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FIFTH BALKAN SCIENTIFIC CONFERENCE ON BIOLOGY – BalkanBio'21
15-16 April 2021, Online Conference

Plovdiv is one of the oldest cities in Europe. It is claimed that the city is a contemporary of Troy and Mycenae, but it is more ancient than Rome, Athens, and Constantinople. Plovdiv is the second-largest and important city in Bulgaria. Plovdiv is strategically important industrial, commercial, scientific, cultural and transportation-communications center on the Balkans region. The city is famous for the international fair, whose spring, autumn and other specialized exhibitions make it a center of economics and business. Plovdiv is a strategic railway junction and the airport "Plovdiv" recently established itself as an alternative to the airport "Sofia".

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- Applied biology and education practices
- Ecology, biodiversity and conservation
- Genetics, cell and molecular biology
- Microbiology, biochemistry and biotechnologies

The program includes several parallel sessions. Each session consists of oral presentations and posters.

Yours sincerely,

Sonya Kostadinova (Dean of the Faculty of Biology)

Gana Gecheva (Chair of the Organizing Committee)

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The Spotted Orb-weaver Neoscona byzanthina (Pavesi, 1876) – An Enigmatic but Common Species on the Balkans (Araneae: Araneidae)

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Abstract. The spotted orb-weaver spider *Neoscona byzanthina* was described from the south-eastern point of the Balkan Peninsula – the town Istanbul (Constantinople) in Turkey by Pavesi (1876) and herein is reported from Albania, Bulgaria, Kosovo and North Macedonia for the first time. Its taxonomy, ecology and general distribution are summarized and discussed.

Key words: Albania, Bulgaria, global distribution, Kosovo, North Macedonia, spider, taxonomic features.

Introduction

The spider *Neoscona byzanthina* was originally placed in a genus *Epeira* Walckenaer, 1805 (now considered a junior synonym of *Araneus* Clerck, 1757) and was described on the base of female specimens from European part of Istanbul (Constantinople) independently by Pavesi (1876) and Simon (1879), respectively as *Epeira byzanthina* and *E. turcica*, after specimens collected by Spagnolini (Spagnolini, 1877). Simon (1884) subsequently synonymized the two and much later (Simon, 1929) pointed out that *N. byzanthina* might be a local variety of *N. adianta* (Walckenaer, 1802). The species was accepted in the catalogue of Bonnet (1955), whereas in the catalogue of Roewer (1955) the author considered that *N. byzanthina* is synonymous with *N. adianta*. Since then, *N. byzanthina* was not cited (except in Ledoux & Canard (1981) under a single male palp drawing), until Ledoux (2008) differentiated it from *N. adianta*. Ledoux (2008) studied in detail and illustrated the species in France and concludes that: 1) the individuals of *N. byzanthina* are larger than those of *N. adianta* and the genitalia size follows that difference in size between the two species; 2) opisthosoma design is more variable in *N. byzanthina* than in *N. adianta* and the first elongations of the bands are a little bit larger in *N. adianta*. In addition 3) the apical ends of the femurs are usually darker in *N. byzanthina* (after Simon, 1929) and 4) the hook of the epigyne has a triangular form in *N. adianta*, whereas in *N. byzanthina* it is more elongated and rounded. Also, the phenology of both species is different, since adults of *N. adianta* are frequent on June and July and adults of *N. byzanthina* are frequent on August and September.

The aim of this work is to present new faunistic and taxonomic data of the Balkans population of *Neoscona byzanthina*.

Material and Methods

The spider material was collected by hand picking and also includes some observations documented by digital images. Specimens were examined and measured using Wild M5A stereomicroscope. Digital images were taken by Canon EOS1300D digital camera, attached to a Carl Zeiss Stemi 2000-c stereomicroscope and with Canon EOS1100D attached to a Carl Zeiss Amplival microscope. The final processing of the figures was done in Adobe Photoshop CS6. The specimens are preserved in 70-80% ethanol and deposited in the Institute of Biodiversity and Ecosystem Research (IBER) and Laboratory of Zoology, University of Prishtina (UP). The geographical coordinates are given in decimal degrees and the altitudes are given in metres above sea level. Country codes are according to ISO 3166-2 (www.iso.org). Maps visualization: projection coordinate system "WGS 84 UTM 35N". All measurements are in millimetres.

Results

A total of 44 specimens (3 ♂♂, 18 ♀♀, 5 imm♂♂, 13 imm♀♀, 5 jj) of *Neoscona byzanthina* were collected or observed in 15 localities within the Balkans (Fig. 1).

Araneidae Clerck, 1757
Neoscona Simon, 1864
Neoscona byzanthina (Pavesi, 1876)

Epeira byzanthina Pavesi, 1876: 59.
Epeira turcica Simon, 1879: 36.
Epeira byzanthina Simon 1884: 328.
Neoscona adianta forma *byzanthina* Simon (1929): 693.
Neoscona byzanthina Ledoux, 2008: 49.

Material: ALBANIA: 1 ♀ (deposited in IBER), Krongji, near Syri i Kaltër (Blue Eye spring) (Fig. 1: 1), N 39.9180˚, E 20.1855˚, 151 m, 05.10.2019, lgt. M. Naumova; BULGARIA: 1 ♀ (deposited in IBER), Plovdiv, Yagodovo village (Fig. 1: 5), N 42.1284˚, E 24.8556˚, 152 m, 28.09.2018, lgt. V. Genchev, backyard; 1 ♀ (deposited in IBER), the same locality and legator, 03.10.2018; 1 ♂ (deposited in IBER), Plovdiv, Yagodovo village (Fig. 1: 5), N 42.1145˚, E 24.8284˚, 158 m, 14.08.2019, lgt. V. Genchev; 1 ♀, S Black Sea coast, Aheloy (Fig. 1: 2), N 42.6431˚, E 27.6456˚, 8 m, 11.09.2018, 1 ♀, 16.09.2018 (observed and photographed by I. Yanev); 1 ♀, S Black Sea coast, Burgas (Fig. 1: 3), N 42.4804˚, E 27.4150˚, 5 m, 23.09.2018 (observed and photographed by I. Yanev); 1 ♀, N Black Sea coast, Shabla (Fig. 1: 7), N 43.5678˚, E 28.5604˚, 2 m, 03.08.2016 (observed and photographed by Z. Barzov); 1 imm♀, Varna, Beloslav village (Fig. 1: 9), N 43.1931˚, E 27.7214˚, 2 m, 26.07.2018; 1 ♀, Ruse, Batin village (Fig. 1: 6), N 43.6692˚, E 25.6798˚, 18 m, 06.10.2019 (observed and photographed by I. Angelova); 1 imm♀, Svilengrad (Fig. 1: 8), N 41.7704˚, E 26.1948˚, 50 m, (observed and photographed by H. Hristov); 1 ♀, Haskovo (Fig. 1: 4), N 41.9595˚, E 25.5287˚, 205 m, 08.10.2019 (observed and photographed by E. Nankova); 1 ♀, Sashtinska Sredna Gora Mts., Zmeyovo village (Fig. 1: 10), N 42.5075˚, E 25.6069˚, 440 m, 29.09.2020 (observed and photographed by V. Ilieva); KOSOVO: 1 ♀, 5 imm♂♂, 6 imm♀♀, 3 jj (deposited in UP), Prilep village near Deçan (Fig. 1: 11), N 42.4955˚, E 20.3087˚, 547 m, 14.07.2018, lgt. D. Geci; 2 ♂♂, 3 ♀♀ (deposited in UP) the same locality, 30.07.2020, lgt. D. Geci; 3 ♀ (deposit in UP), Vaganicë village (Fig. 1: 14), Mitrovicë Municipality, N 42.8489˚, E 20.8624˚, 621 m, 24.08.2020, lgt. D. Geci; 3 ♀♀ (deposited in UP) the same locality and legator. 17.10.2020; 1 ♀ (deposited in UP), Henc wetland (Fig. 1: 12), N 42.5822˚, E 21.0486˚, 538 m, 02.09.2020, lgt. D. Geci; 2 jj (deposited in UP) Deiq village, Klinë Municipality (Fig. 1: 13), N 42.6121˚ E 20.5592˚, 383m, 26.07.2020, lgt. D. Geci; 1 ♀ (deposited in UP), the same locality and legator 30.08.2020; 3 imm♀ (deposited in UP), Dollc village, Klinë Municipality (Fig. 1: 13), N 42.5947˚ E 20.5923˚, 394m, 26.07.2020, lgt. D. Geci; 1 ♀ (deposited in UP), the same locality and legator, 30.08.2020. 2 imm♀♀ (deposited in UP), Zajm village, Klinë Municipality (Fig. 1: 13), N 42.5930˚ E 20.5552˚, 411m, 26.07.2020, lgt. D. Geci; NORTH MACEDONIA: 1 ♀ (deposited in IBER), Skopie, Stajkovsko Ezero lake (Fig. 1: 15), N 42.0240˚, 21.4942˚, 266 m, 14.09.2019, lgt. G. Dimovski.

Comparative material examined: N. adianta: 3 ♂♂, 1 ♀ (deposited in IBER), N Black Sea coast, Kranevo village, N 43.3470˚, E 28.0627˚, 8 m, 11.08.2015, lgt. M. Naumova; 2 ♀♀ (deposited in IBER), Plovdiv, Yagodovo village, N 42.1284˚, E 24.8556˚, 152 m, 10.07.2018, lgt. V. Genchev.

Description: General appearance: relatively large (7.0-13.0 mm) araneid spiders with ovoid opisthosoma without tubercles and appendages. Carapace: yellowish to pale brown with dark longitudinal median and lateral stripes; regularly covered with short grey hairs. Sternum: dark brown to black. Legs: light to medium yellow-brownish, distally dark on femora, patellae and tibiae. Femora with grey lateral stripes. Opisthosoma: dorsally coloured in yellow with brown reticulate pattern and 6-8 pairs of black dots or short horizontal stripes, the posterior ones converging. White/light median part, formed from ovoid spots, at an angle with the symmetry axis present in most specimens but may be reduced. Continuous wavy black longitudinal bands are never present (Figs 2-3). Ventrally coloured in black with two bright longitudinal stripes between the epigastric furrow and spinnerets, 2 pairs of bright spots and one curved stripe surround the spinnerets (Fig. 3).

Males (n=3): total length 7.0-8.0; prosoma: length 2.5-3.0, width 2.0-3.5; opisthosoma length 4.0-5.5, width 4.3-4.5. Tibia II strongly armed as Fig. 4A. Palpal organ as in Fig. 4B-C.

Females (n=10): total length 10.0-13.2; prosoma length 3.0-5.0, width 2.0-4.0; opisthosoma length 5.5-8.0, width 4.0-7.0. Colouration as in males, slightly lighter. Epigyne as in Fig. 5A-C. Scape with shape of elongated rounded triangle, longer than wide, wider at its base, reaching distinctly beyond epigyne; easily distinguished from N. adianta (Fig. 5D-F) especially in lateral view (Fig. 5B, E).

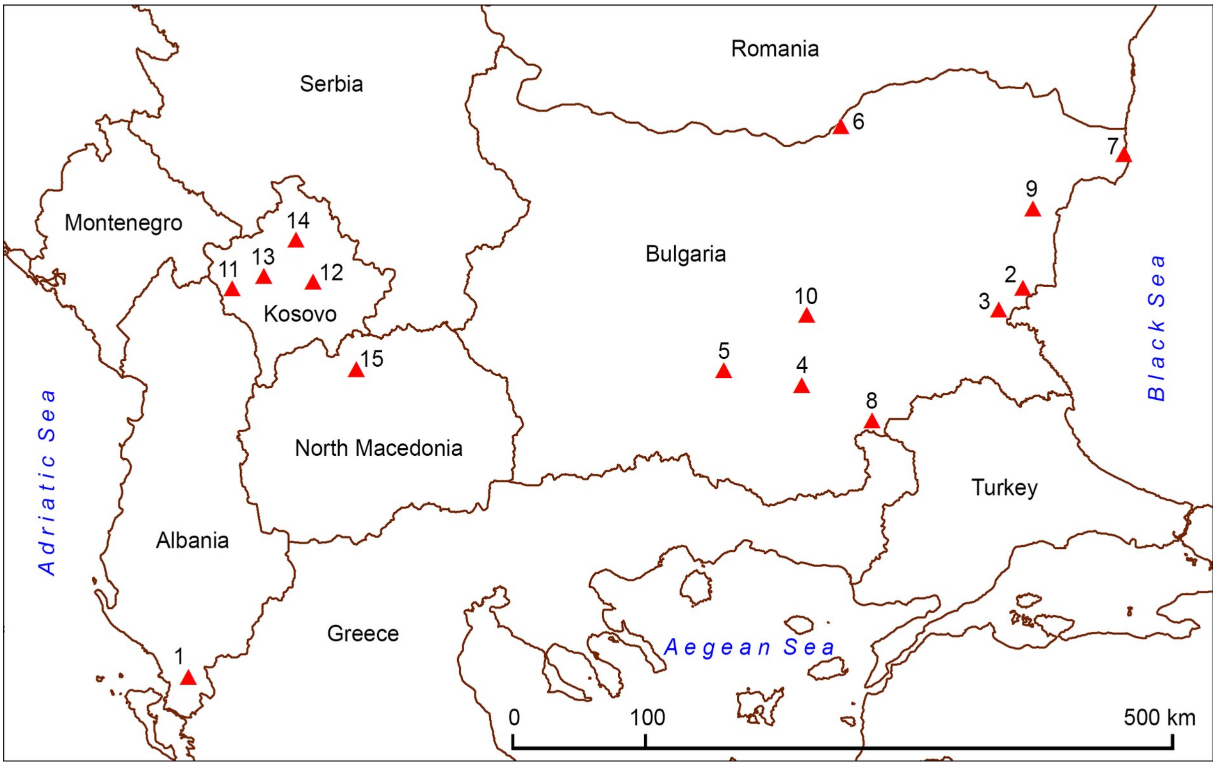

Fig. 1. Map with the new localities of *Neoscona byzanthina* in Albania (1 = Syri i Kaltër), Bulgaria (2 = Aheloy, 3 = Burgas, 4 = Haskovo, 5 = Plovdiv, 6 = Ruse, 7 = Shabla, 8 = Svilengrad, 9 = Varna, 10 = Zmeyovo), Kosovo (11 = Deçan, 12 = Henc wetland, 13 = Klinë, 14 = Vaganicë) and North Macedonia (15 = Skopie).

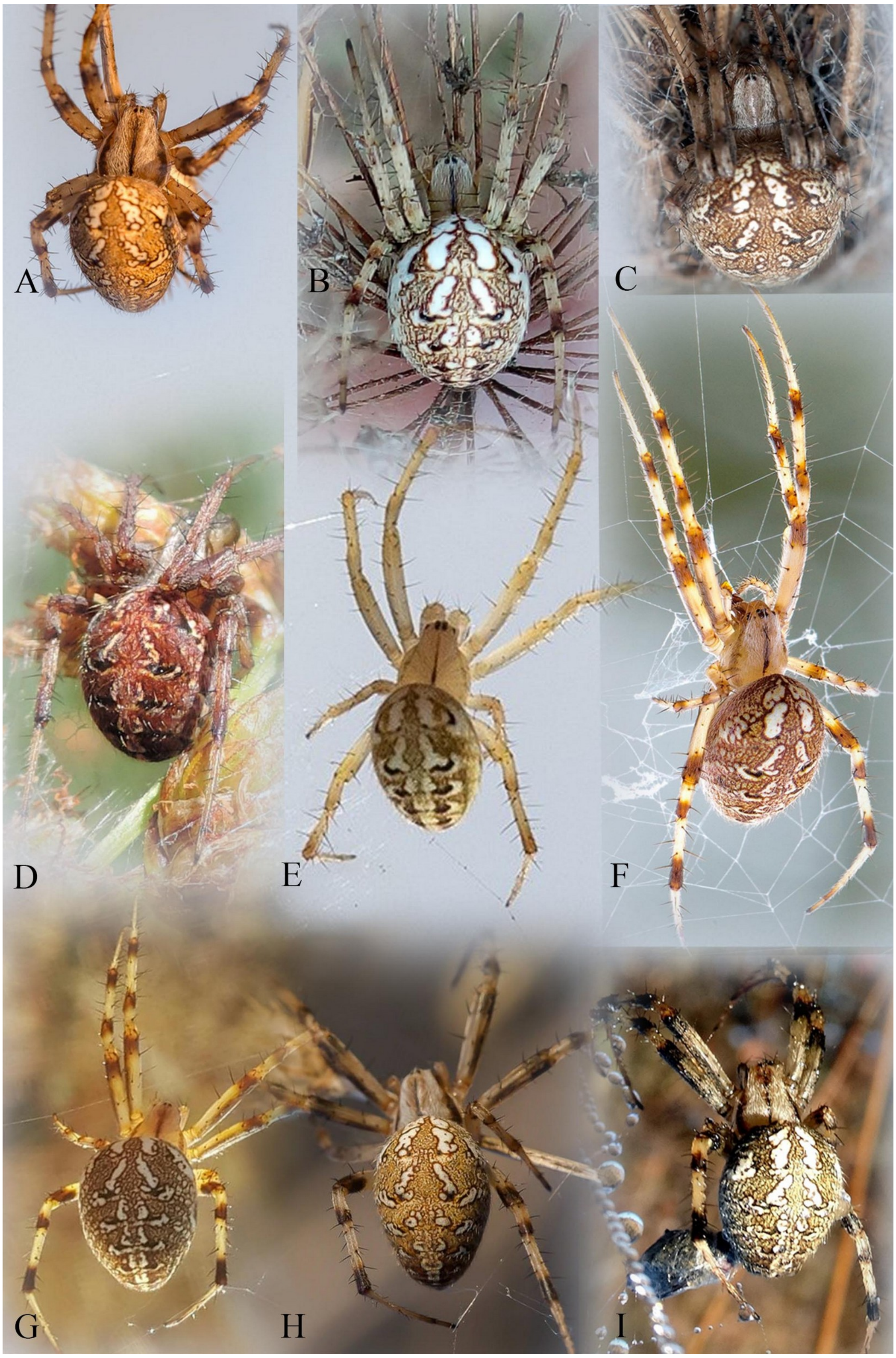

Fig. 2. Variations of colouration and opisthosomal patterns of *Neoscona byzanthina.* Adult females and immature specimens from Bulgaria and Kosovo, dorsal view.

Fig. 3. Variations of opisthosomal patterns of *Neoscona byzanthina*: dorsolateral (A), lateral (B-D), caudal (E-F) and ventral (G-I) views.

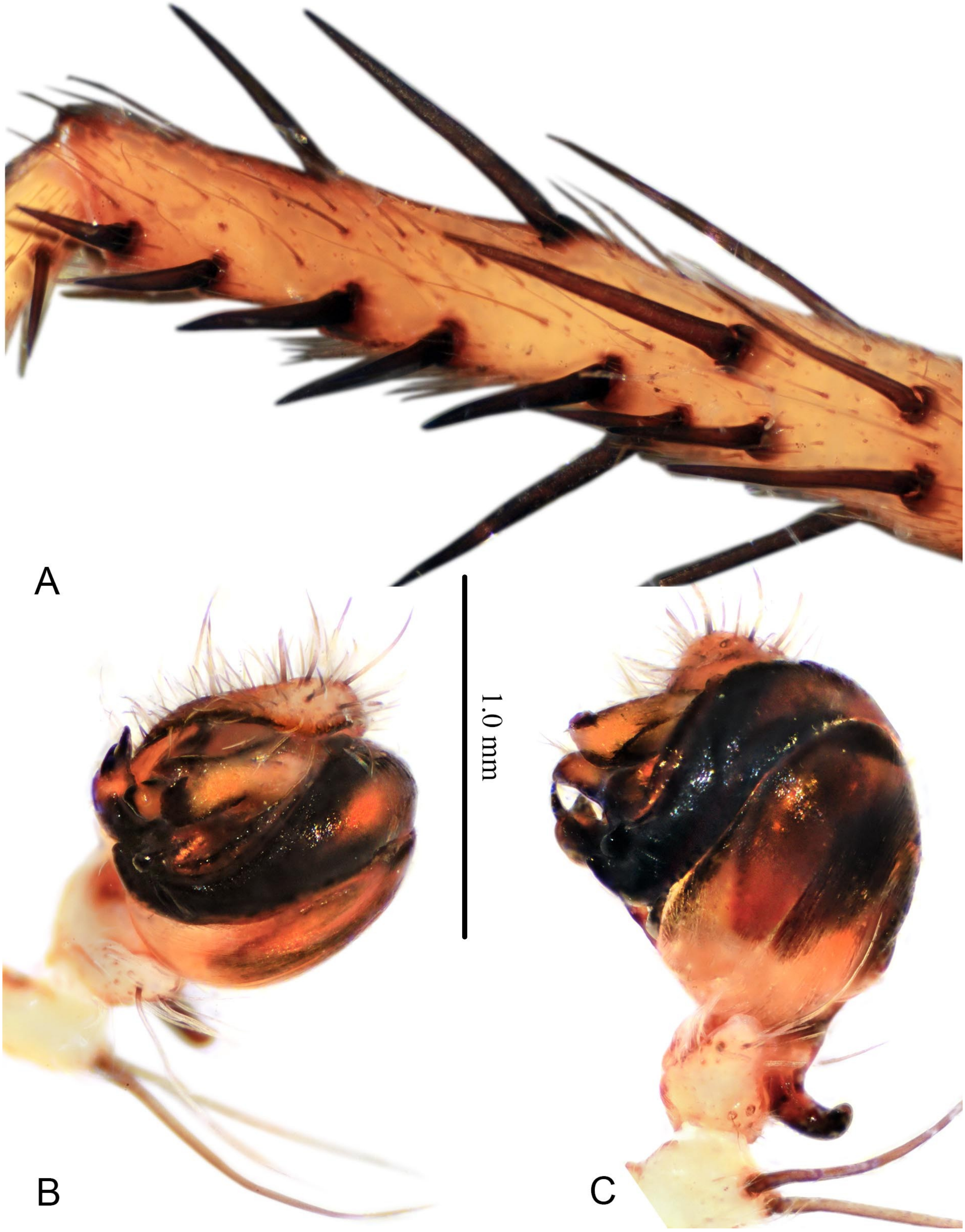

Fig. 4. *Neoscona byzanthina*, male: right tibia II (A) and left pedipalp (B-C), prolateral (A), apical (B) and ventral (C) views.

Fig. 5. *Neoscona* sp. female, scape of epigyne: *N. byzanthina* (A-C) and *N. adianta* (D-F), ventral (A, D), lateral (B, E) and posterior views. Scales: 0.5 mm. Both specimens are almost equal in body size.

Discussion

The large and attractive orb web spider *Neoscona byzanthina* was "invisible" until 12 years ago, because it was erroneously synonymized with the frequent congener *N. adianta*, which is abundant in the Holarctic region. In this study *N. byzanthina* is reported for the first time from Albania, Bulgaria, Kosovo and North Macedonia. The mature specimens were found from mid-July to mid-October. All observations were in humid areas (a variety of wetlands and mesophilous grasslands near lakes, swamps, bogs or river shores) in altitudes between 2 and 621 m. The Balkan population, compared with the relatively well-studied population in France (Ledoux, 2008) shows no differences in individual body measurements, variety of body patterns, distally darker femora, habitat preferences and phenology.

The long period during which *Neoscona byzanthina* was assigned as *N. adianta* requires revision of the historical records, so earlier citations of *N. adianta* should all be reviewed. The males of both species are hard to distinguish based on their copulatory organs but easier to distinguish on the base of the opisthosomal patterns (discussed above) and also on the body size and armament of tibia II, as males *N. byzanthina* are obviously larger and more strongly armed. The females of both species are easier to identify both by the somatic and the genital aspects; the body sizes overlapping insignificantly and *N. byzanthina* being obviously larger. The current range of *N. byzanthina* is shown in the Fig. 6. Until now, the known localities in the literature are in Western Europe (France, Italy and Spain), South-eastern Europe (Greece and Turkey) and Asia Minor (Turkey) (Bolognin et al., 2021, Ledoux, 2008, Mora-Rubio et al., 2019, Pavesi, 1876, Simon, 1879, 1884). The new records partially fill the range gaps and hint at a wider distribution in Europe and the Western Palearctic.

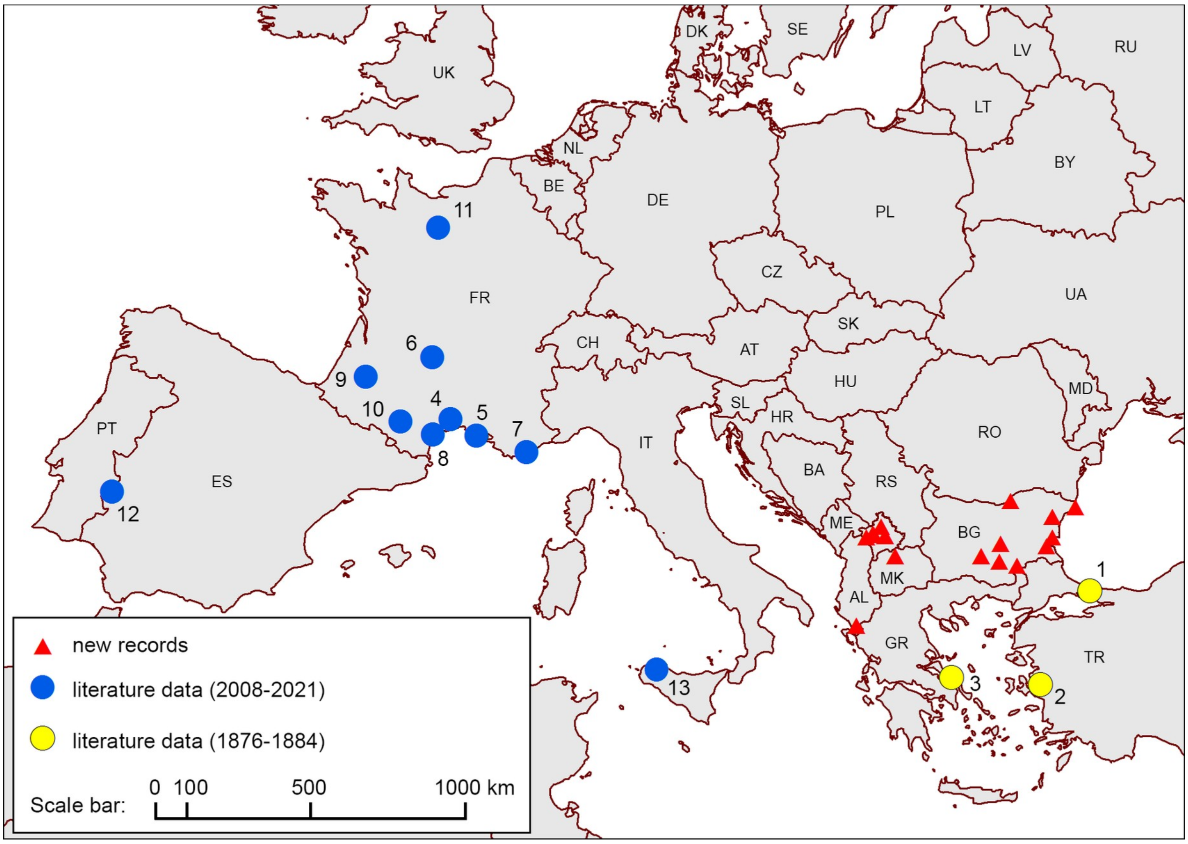

Fig. 6. Currently established global range of *Neoscona byzanthina*. Historical records (circles, numbered chronologically) in France (4 = Aramon, 5 = Bouches-du-Rhône, 6 = Corrèze, 7 = Fréjus, 8 = Galargues, 9 = Lot-et-Garonne, 10 = Monclar de Quercy, 11 = Sainte-Opportune-la-Mare), Greece (3 = Steni Dirfyos, Euboea isle), Italy (13 = Palermo, Sicily isle), Spain (12 = Extremadura: Badajoz) and Turkey (1 = Istanbul, 2 = Izmir) and new records (triangles, for details see Fig. 1).

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A Preliminary Checklist of the Spiders of Kosovo (Arachnida: Araneae)

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Abstract. The Republic of Kosovo is the territory with the least known spider fauna within the Balkans. The present list of spiders is based on all published records available to the authors and also includes original unpublished data. The checklist comprises only 159 species belonging to 29 families and 108 genera. This low number is due to different reasons but mostly because the spiders herein has never been studied in their entirety and because we excluded all the records from the "Balkans", "Yugoslavia", "Serbia", and the border mountain "Kopaonik", for which cannot certainly be argued that relate to Kosovo, so we worked only with reports, containing reliable data on the spider fauna from there. The aim of this study is to presents annotated preliminary checklist of the spiders of Kosovo with additional faunistic data.

Key words: Balkans, catalogue, fauna, former Yugoslavia, Jugoslavia, Serbia.

Introduction

The Republic of Kosovo is a small country in the Western part of the Balkans and has a total area of 10,908 km^2 with an altitude range from 265 m to 2656 m. The mountains of Kosovo belong to the Dinarides range with two major mountain massifs, Sharr (Šar) and Bjeshkët e Nemuna (Prokletije). They round the lowlands Rrafshi i Kosovës (Kosovo Polje plain) and Dukagjin, separated from the chain of mountain Carralevë.

The spider fauna of Kosovo has never been studied in its entirety, and remains one of the least explored in Europe. Our knowledge of this fauna originates from the beginning of 20th century when Bresjančeva (1907) lists 9 species from 6 families for the territory of today's Kosovo (at that time still a part of the Ottoman Empire). Twenty two years later, in his extensive work on Serbian spiders, Stojićević (1929) provides 47 species that correspond to the current territory of Kosovo, 44 of which were first reports. In the following years, the list increased due to the papers of Kratochvíl (1935) with four species; Kolosvary (1938, 1940) with eleven more; Šilhavý (1944) with three; Deeleman-Reinhold (1974, 1986) with two; Wunderlich (1984) with one and Grimm (1985) with two more species. In the only recent work, Vrenozi & Jager (2013) added 19 more species and listed 106 spiders for Kosovo. One more species is under review (Geci &

Naumova, 2021). Addition data can be found in few other papers (Drensky, 1936, Nikolić & Polenec, 1981, Knoflach, 1996, Deltshev *et al.*, 2003, Rezac *et al.*, 2014, Mammola *et al.*, 2018, Naumova *et al.*, 2019a, 2019b) and that seems to be the final list for the moment.

The aim of this study is to summarizes and presents all available data of the spiders of Kosovo, both from the literature and from original records by providing a preliminary checklist and an annotated catalogue of the spiders in Kosovo.

Material and Methods

The presented list of the spiders of Kosovo is based on the critical review of the existing literature records on the distribution of spiders in the studied area. We excluded all the records from \`Serbia\`, \`Yugoslavia\` and \`Balkans\` for which cannot certainly be argued that relate to Kosovo, so we worked only with reports, contained reliable data on the spider fauna from there. We also excluded all the records from mount Kapaonik originated from Stojićević, (1929) and their later citations in Drensky (1936), Nikolić & Polenec (1981), Deltshev *et al*. (2003) and Vrenozi & Jager (2013), because they refer to specific localities in Serbia (Jelak nr. Brus, Jošanička Banja nr. Baljevac, Kriva reka, Srebrnac, Suvo Rudište (Pančićev Vrh)). Only two species (*Erigone atra* Blackwall, 1833 and *Mansuphantes mansuetus* (Thorell, 1875)) have been listed from \`Kopaonik\` without precise locations, but there is no evidence that Stojićević worked with material on the Kosovo\`s part of the mountain, so we exclude them as well. Other excluded records (actually refer to North Macedonia) are: Kaçanik (in Mammola *et al.*, 2018 after Drensky, 1935: Kaçanik, cave in village Blace) and Gnjilane (in Drensky, 1936 after Stojićević, 1929: Končulj, nr. Gnjilane). Four other records (Šilhavý, 1944), may be located in both Kosovo and North Macedonia (\`Sar – Bačila, 1700 m\`, \`Sar – Jezerska, 2000 m\`, \`Sar, Ljubotin\` and \`Sar – pod Ljubotinem u Nikolic\`). They are included herein as very probable and are marked with question mark in parentheses in the list of localities. The species, erroneously reported from Kosovo are presented in Table 1.

The localities were mapped on the basis of exact (taken in with a GPS-receiver) or approximate (on the basis of the location of the settlements/geographic objects) geographic coordinates (decimal), rounded to 4 decimal places (Fig. 1). The list of localities (alphabetically by districts and municipalities) includes the sites with numbers 1 to 50 (mapped), followed by site 51 (Kosovo, without precise locality; not mapped). Mapping and visualization of the map were done by ArcGIS 10.1 (ESRI, Redlands, California, USA).

The nomenclature follows the World Spider Catalog (2021) and the taxa are listed alphabetically. The newly recorded species are marked with an asterisk. The literature sources are listed chronologically (except in cases where the authors have more than one cited publications – then the years are listed after the first mention). The general distribution of the species is provided mainly according to World Spider Catalog (2021) and Nentwig *et al*. (2021). The additional sources were cited. The names of the collectors are abbreviated: AZ=Alexey Zhalov, BP=Boyan Petrov, DG=Donard Geci, MN=Maria Naumova. The material is deposited in the National Museum of Natural History-Sofia (collected from AZ, BP), the University of Prishtina (collected from DG) and in the Institute of Biodiversity and Ecosystem Research (collected from MN). Other abbreviations used: j=juvenile, jj = juveniles.

Results

The preliminary spider checklist presented herein includes 159 species belonging to 108 genera and 29 families (Table 2). From them 63 species (marked with an asterisk), 42 genera and 9 families are newly discovered during this study.

Table 1. List of the localities (alphabetically per DISTRICTS and Municipalities: L1–L51 – numbers used in the Map (Fig. 1) and in the List of species. Arrangement: number; locality (alternative names and transliterations), Geographical object, coordinates (decimal), altitude (m)).

N	Locality	Object/area	Coordinates	Alt.
	FERIZAJ (Uroševac)			
	Ferizaj (Uroševac)			
L1	Nerodime (Nerodimlje)	Sharr (Šar) Mts.	N42.3605°, E21.0940°	690
L2a	Nerodime e Epërme (Gornje Nerodimlje) 1	Carralevë (Crnoljeva) Mts.	N42.3671°, E21.0329°	714
L2b	Nerodime e Epërme (Gornje Nerodimlje) 2	Carralevë (Crnoljeva) Mts.	N42.3637°, E21.0527°	666
L3	Sazli (Saslja, Saslija, Sazlija)	Rrafshi i Kosovës (Kosovo Polje plain)	N42.4060°, E21.1876°	570
	Kaçanik (Kačanik)			
L4	Doganaj village	Rrafshi i Kosovës (Kosovo Polje plain)	N42.2667°, E21.1822°	590
L5a	Kaçanik (Kačanik, Kachanik)	Karadak (Skopska Crna Gora) Mts	N42.2257°, E21.2674°	580
L5b	Kaçanik gorge (Kačaniska Klisura, Gryka e Kaçanikut)	Karadak (Skopska Crna Gora) Mts	N42.2102°, E21.2491°	480
	Shtërpcë (Štrpce)			
L6	Brezovicë	Sharr (Šar) Mts.	N42.2058°, E20.9532°	1090
L7	Firajë	Sharr (Šar) Mts.	N42.2485°, E21.0383°	690
L8	Gotovuse (Gotovuša) village, Ropotski Potok river	Sharr (Šar) Mts.	N42.2344°, E21.0767°	1140
L9	Livadh Lake (Strbacko Jezero, Liqeni i Malit Sharr "Gjoli")	Sharr (Šar) Mts.	N42.1909°, E21.0734°	200
L10a	(?) Luboten1 (`Sar, N Shar Mts, Ljubotin`)	Sharr (Šar) Mts.	N42.2084°, E21.1153°	245
L10b	(?) Luboten2 (`Sar - pod Ljubotinem u Nikolic`)	Sharr (Šar) Mts.	N42.2084°, E21.1153°	245
L11	Shtërpcë (Štrpce)	Carralevë (Crnoljeva) Mts.	N42.2257°, E21.0092°	891
	Shtime (Štimlje)			
L12	Carralevë (Crnoljeva) village	(Crnoljeva) Mts.	N42.4574°, E20.9818°	640
L13	Petrove village, cave Shpella Devetakut	Carralevë (Crnoljeva) Mts.	N42.3973°, E20.9722°	791
	GJILAN (Gnjilane)			
	Gjilan (Gnjilane)			
L14	Gjilan (Gnjilane)	Rrafshi i Kosovës (Kosovo Polje plain)	N42.4994°, E21.4605°	600
	Viti (Vitina)			
L15	Pozharan	Rrafshi i Kosovës (Kosovo Polje plain)	N42.3496°, E21.3496°	715
	MITROVICË (Mitrovica)			
	Mitrovicë (Mitrovica)			
L16a	Mitrovicë (Kosovska Mitrovica) 1	Rrafshi i Kosovës (Kosovo Polje plain)	N42.8913°, E20.8858°	540
L16b	Mitrovicë (Kosovska Mitrovica) 2	Rrafshi i Kosovës (Kosovo Polje plain)	N42.8687°, E20.8593°	548
L16c	Mitrovicë (Kosovska Mitrovica) 3	Rrafshi i Kosovës (Kosovo Polje plain)	N42.8698°, E20.8828°	503
L17	Vaganicë village	Rrafshi i Kosovës (Kosovo Polje plain)	N42.8489°, E20.8624°	621
L18	Zasellë	Kopaonik Mts.	N42.8854°, E20.8988°	671
	Zveçan (Zvečan)			
L19	Bajskë (Banjska, Banska)	Rogozna Mts.	N42.9700°, E20.7849°	590
L20	Zveçan (Zvečan, Zvechan)	Rogozna Mts.	N42.9076°, E20.8307°	535
	PEJË (Peć)			
	Deçan (Dečani)			
L21a	Deçan (Dečani)	Dukagjin	N42.5372°, E20.2849°	620
L21b	Manastiri i Deçanit (Visoki Dečani Monastery)	Bjeshket e Nemuna (Prokletije)	N42.54805°, E20.2663°	650

L22	Prilep village	Dukagjin	N42.4955°, E20.3087°	547
	Istog (Istok)			
L23	Istog (Istok)	Mokna (Mokra Gora) Mts.	N42.7908°, E20.4740°	700
L24	Veriq village	Dukagjin	N42.7511°, E20.5518°	505
	Klinë (Klina)			
L25	Deiq village	Dukagjin	N42.6121°, E20.5592°	383
L26	Dollc village	Dukagjin	N42.5947°, E20.5923°	394
L27	Ujëvarët e Mirushës (Mirusha Waterfalls , Slapovi Miruše)	Dukagjin	N42.5241°, E20.5999°	455
L28	Zajm village	Dukagjin	N42.5930°, E20.5552°	411
	Pejë (Peć)			
L29	Bellopojë	Dukagjin	N42.6409°, E20.2891°	514
L30	Kopranik (Koprivnik) Mts	Bjeshket e Nemuna (Prokletije)	N42.6367°, E20.2552°	1140
L31	Novosellë (Novo selo)	Dukagjin	N42.7311°, E20.3381°	505
L32	Pejë (Peć, Pech, Pedgh, Ipek)	Dukagjin	N42.6551°, E20.2859°	535
L33a	Peje: Rugova Canyon: cave Gryka e Madhe (Great Canyon cave)	Bjeshket e Nemuna (Prokletije)	N42.6647°, E20.2025°	637
L33b	Peje: Rugova Canyon: cave Shpella e Karamakazit (cave Karamakis, Black Scissors cave)	Bjeshket e Nemuna (Prokletije)	N42.6661°, E20.2001°	830
L34a	Shpella e Drinit Bardhë cave(Radaci Cave, Sleeping Beauty cave; Bukuroshja e Fjetur; Shpella Radacit-Cave, Radove (Radovac) cave)	Bjeshket e Nemuna (Prokletije): Zhleb Mt.	N42.7370°, E20.3066°	627
L34b	White Drin falls (Drini i Bardhë)	Bjeshket e Nemuna (Prokletije): Zhleb Mt.	N42.7390°, E20.3072°	585
L35	Zhleb (Zljeb, Zleb, Zljb) Mt	Bjeshket e Nemuna (Prokletije)	N42.7148°, E20.2653°	1480
	PRISHTINË			
	Fushë Kosovë (Kosovo Polje)			
L36	Henc wetland	Rrafshi i Kosovës (Kosovo Polje plain)	N42.5822°, E21.0486°	538
	Lipjan (Lipljan)			
L37	Konjuh	Rrafshi i Kosovës (Kosovo Polje plain)	N42.5388°, E21.1407°	562
L38	Ribar i Vogël village	Rrafshi i Kosovës (Kosovo Polje plain)	N42.5144°, E21.0679°	573
	Prishtinë (Priština)			
L39	Gazimestan	Rrafshi i Kosovës (Kosovo Polje plain)	N42.6906°, E21.1237°	652
L40a	Germia Protected landsacape	Gollak (Goljak) Mts.	N42.6760°, E21.2028°	773
L40b	Gollakë (Gollaku, Malësinë e Gollakut)	Rrafshi i Kosovës (Kosovo Polje plain)	N42.6853°, E21.1809°	617
L41	Lebane (Labjani)	Gollak (Goljak) Mts.	N42.7425°, E21.1523°	630
L42	Prishtinë	Rrafshi i Kosovës (Kosovo Polje plain)	N42.6381°, E21.1334°	561
	PRIZEREN (Prizren)			
	Dragash (Dragaš)			
L43	(?) Bačila (`Sharr-Bačila Staletovičova, most N Shar Mts, 1700 m`)	Sharr (Šar) Mts.	N42.2966°, E21.0408°	1650
L44	(?) Jezerska (`Sar – Jezerska, 2000 m`)	Sharr (Šar) Mts.	N42.0978°, E20.7878°	2000
L45	Plavë (Plave, Plava, Plavenica) village	Sharr (Šar) Mts.	N42.0959°, E20.6477°	950
	Prizeren (Prizren)			
L46	Lubiqevë (Ljubičevo, Ljubichevo)	Sharr (Šar) Mts.	N42.1523°, E20.7386°	730
L47	Prizeren (Prizren)	Dukagjin	N42.2162°, E20.7370°	541
L48	Sredskë (Sredska, Sretska)	Sharr (Šar) Mts.	N42.1721°, E20.8561°	740
	Suharekë (Suva reka)			

L49	Carralevë (Mali i Carralevës) 1	Carralevë (Crnoljeva) Mts	N42.4275°, E20.9036°	795
L50a	Carralevë (Mali i Carralevës) 2	Carralevë (Crnoljeva) Mts	N42.4446°, E20.9157°	759
L50b	Carralevë (Mali i Carralevës) 3	Carralevë (Crnoljeva) Mts	N42.4416°, E20.9165°	720
	Without precise locality			
L51	Kosovo (Kossovo, Kossowo)			

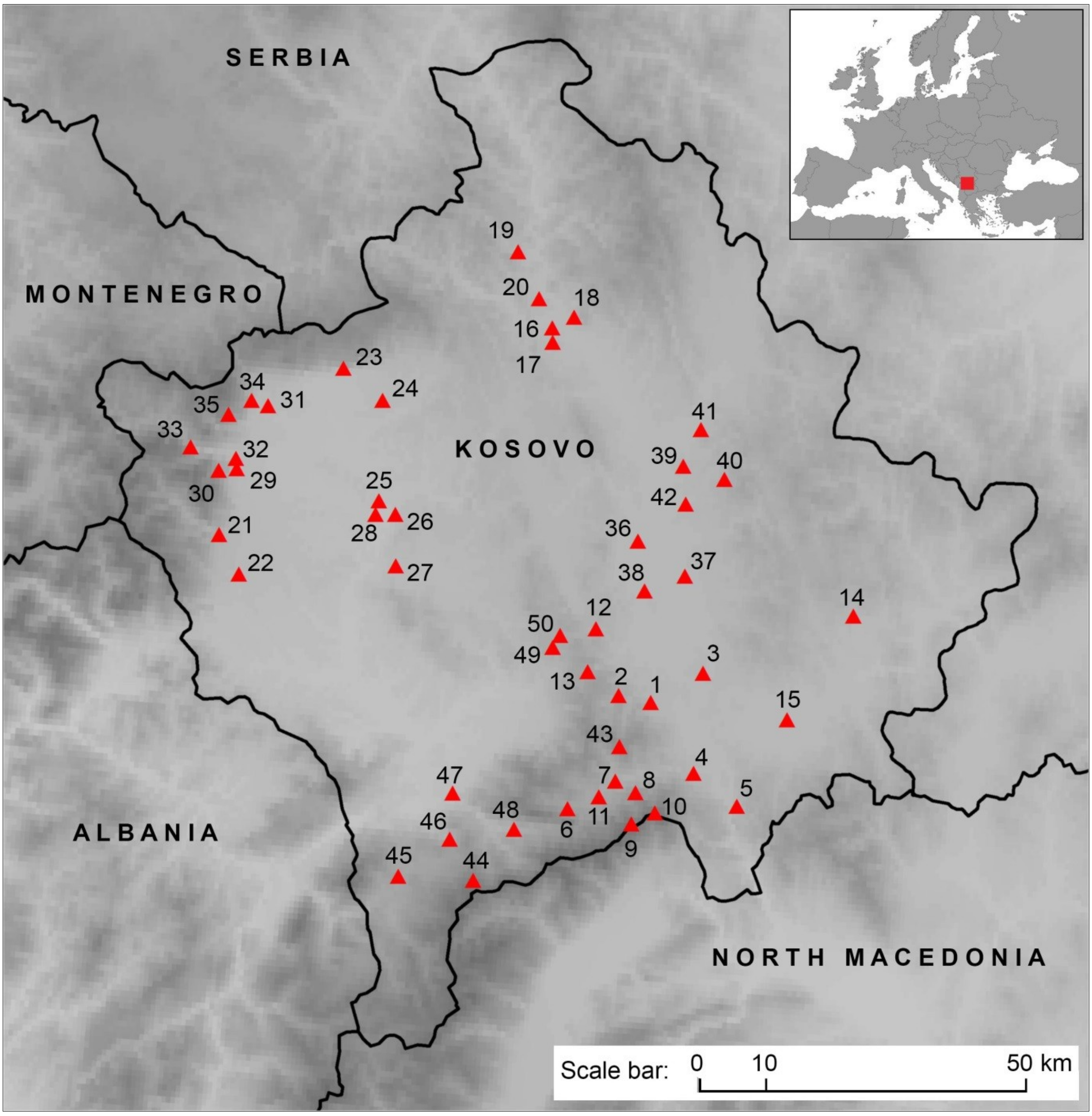

Fig. 1. Map with localities of the spiders in Kosovo. For details, see List of localities.

Annotated list & catalogue of the spiders in Kosovo:

AGELENIDAE
**Eratigena agrestis* (Walckenaer, 1802)
New data: L16 (1 ♂, 29.VII.2020, DG).
Global distribution: Palearctic, introduced to USA and Canada.

**Histopona torpida* (C. L. Koch, 1837)
New data: L37 (2♂, 24.V.2019, DG).
Global distribution: West Palearctic.

Inermocoelotes falciger (Kulczyński, 1897)
Literary data: L31 (sub *Coelotes f.*: Kolosvary 1940), L32 (sub *Coelotes f.*: Kolosvary 1938, Deltshev *et al.*, 2003, sub *Inermocoelotes f.*: Vrenozi & Jäger, 2013).
New data: L40a (5♂, 1♀, 27.IV.2019, DG).
Global distribution: Balkans, Hungary, Romania, Ukraine.

Inermocoelotes inermis (L. Koch, 1855)
Literary data: L30 (sub *Coelotes i.*: Kolosvary, 1938, Deltshev *et al.*, 2003, sub *Inermocoelotes i.*: Vrenozi & Jäger, 2013), L32 (sub *Coelotes i.*: Kolosvary, 1940, Deltshev *et al.*, 2003, sub *Inermocoelotes i.*: Vrenozi & Jäger, 2013).
Global distribution: Europe.

**Inermocoelotes kulczynskii* (Drensky, 1915)
New data: L2b (2♂, 1♀, 15.V.2019, DG).
Global distribution: Balkan endemic known from Bulgaria and North Macedonia.

Lycosoides coarctata (Dufour, 1831)
Literary data: L35 (Vrenozi & Jäger, 2013).
Global distribution: Mediterranean.

**Tegenaria bosnica* Kratochvíl & Miller, 1940
New data: L33a (2♀, 27-29.I.2008, BP).
Global distribution: Balkan endemic, known from Albania, Bosnia and Herzegovina, Croatia, Montenegro and North Macedonia.

**Tegenaria campestris* (C. L. Koch, 1834)
New data: L15 (7♂, 3♀, 06.VI.2019, DG).
Global distribution: Western Palearctic.

**Tegenaria domestica* (Clerck, 1757)
New data: L16b (1♀, 29.VII.2020, DG; 1♀, 18.X.2020, DG), L24 (1♂, 10.X.2020, DG), L27(1♂, 1♀, 25.VII.2020, DG), L34a (1♂, 2♀, 27.VIII.2020, DG).
Global distribution: Palearctic, introduced to Australia, New Zealand, North and South America.

AMAUROBIIDAE
**Amaurobius phaeacus* Thaler & Knoflach, 1998
New data: L40a (2♂, 27.IV.2019, DG).
Global distribution: Balkan endemic, known from Albania, Greece and North Macedonia.

ANYPHAENIDAE
**Anyphaena accentuata* (Walckenaer, 1802)
New data: L40a (2♀, 18.V.2019, DG).
Global distribution: Palearctic.

ARANEIDAE
Aculepeira ceropegia (Walckenaer, 1802)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Agalenatea redii (Scopoli, 1763)
Literary data: L16a (sub *Araneus r.*: Kolosvary, 1938, 1940, sub *Agalenatea r.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L7 (1♂, 7♀, 27.V.2019, DG), L15 (4♀, 27.V.2019, DG), L17 (9♂, 35♀, 04.X-06.XI.2020, DG), L23 (1♀, 04.XI.2018, MN), L40a, (3♀, 09.V.2018, DG).
Global distribution: Palearctic.

Araneus angulatus Clerck, 1757
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Araneus circe (Audouin, 1826)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Araneus quadratus Clerck, 1757
New data: L17 (4♀, 17.X.2020, DG), L47 (1♀, 04.X.2019, MN).
Global distribution: Palearctic.

Araniella cucurbitina (Clerck, 1757)
Literary data: L1 (sub *Aranea c.*: Stojićević, 1929), L5a (Stojićević, 1929), L32 (Vrenozi & Jäger, 2013), L46 (sub *Epeira c.*: Bresjančeva, 1907).
Global distribution: Palearctic.

Araniella opisthographa (Kulczyński, 1905)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Cyclosa conica (Pallas, 1772)

Literary data: L5a (Stojićević, 1929, Vrenozi & Jäger, 2013), L20 (Stojićević, 1929).
New data: L40a (1♀, 14.IV.2019, DG).
Global distribution: Holarctic.

**Gibbaranea bituberculata* (Walckenaer, 1802)
New data: L40a (1♀, 18.V.2019, DG).
Global distribution: Palearctic.

Hypsosinga albovittata (Westring, 1851)
Literary data: L3 (sub *Singa a.*: Stojićević, 1929, sub *Hypsosinga a.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Hypsosinga pygmaea (Sundevall, 1831)
Literary data: L14 (sub *Singa p.*: Stojićević, 1929, sub *Hypsosinga p.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Holarctic.

Hypsosinga sanguinea (C. L. Koch, 1844)
Literary data: L3 (sub *Singa s.*: Stojićević, 1929, sub *Hypsosinga s.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L51 (sub *Singa s.*: Drensky, 1936).
Global distribution: Palearctic.

**Larinioides patagiatus* (Clerck, 1757)
New data: L11 (1♂, 2♀, 27.V.2019, DG).
Global distribution: Holarctic.

**Larinioides suspicax* (O. P.-Cambridge, 1876)
New data: L49 (1♂, 3♀, 06.VI.2019, DG).
Global distribution: Palearctic.

**Leviellus thorelli* (Ausserer, 1871)
New data: L16b (1♀, 18.IX.2020, DG).
Global distribution: Europe.

Mangora acalypha (Walckenaer, 1802)
Literary data: L1 (Stojićević, 1929, Vrenozi & Jäger, 2013), L14 (Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L2a (2♂, 5♀, 06.VI.2019, DG), L40a (4♀, 18.V.2019, DG).
Global distribution: Palearctic.

Neoscona byzanthina (Pavesi, 1876)
Literary data: L17, L22, L25, L26, L28, L36 (Geci & Naumova, 2021).
Global distribution: Western Palearctic.

**Nuctenea umbratica* (Clerck, 1757)
New data: L6 (2♀, 27.V.2019, DG), L7 (1♀, 27.V.2019, DG), L16b (2♂, 1♀, 29.VII.2020, DG), L18 (1j, 02.VIII.2020, DG), L40a (1♀, 27.IV.2019, DG).
Global distribution: Palearctic.

Singa hamata (Clerck, 1757)
Literary data: L1 (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L46 (Bresjančeva, 1907, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L2a (1♀, 06.VI.2019, DG), L49 (2♀, 06.VI.2019, DG).
Global distribution: Palearctic.

**Zilla diodia* (Walckenaer, 1802)
New data: L4 (2♀, 27.V.2019, DG), L7 (1♂, 16.VI.2019, DG), L40a (1♂, 18.V.2019, DG).
Global distribution: Palearctic.

Zygiella keyserlingi (Ausserer, 1871)
Literary data: L32 (Kolosvary, 1938, 1940, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

ATYPIDAE
Atypus piceus (Sulzer, 1776)
Literary data: L47 (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic and Iran.

CHEIRACANTHIIDAE
Cheiracanthium elegans Thorell, 1875
Literary data: L47 (sub *Chiracanthium e.*: Stojićević, 1929, sub *Cheiracanthium e.*: Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

CLUBIONIDAE
Clubiona stagnatilis Kulczyński, 1897
Literary data: L32 (Vrenozi & Jäger, 2013).

Global distribution: Palearctic.

**Clubiona terrestris* Westring, 1851
New data: L50b (2♂, 1♀, 16.VI.2019, DG).
Global distribution: Western Palearctic.

**Porrhoclubiona genevensis* (L. Koch, 1866)
New data: L11 (1♂, 2♀, 06.VI.2019, DG).
Global distribution: Palearctic.

DICTYNIDAE
**Brigittea latens* (Fabricius, 1775)
New data: L2a (6♂, 7♀, 24.VI.2019, DG), L15 (1♂, 3♀, 24.VI.2019, DG).
Global distribution: Palearctic.

Dictyna uncinata Thorell, 1856
Literary data: L5a (Stojićević, 1929).
Global distribution: Palearctic.

**Nigma flavescens* (Walckenaer, 1830)
New data: L32 (1♂, 01.X.2019, MN).
Global distribution: Palearctic.

DYSDERIDAE
**Dysdera crocata* C. L. Koch, 1838
New data: L2a (2♂, 24.VI.2019, DG), LL49 (3♂, 27.V.2019, DG), L50a (1♂, 27.V.2019, DG), L50b (2♂, 27.V.2019, DG).
Global distribution: Western Palearctic, introduced to North America, Chile, Brazil, South Africa, Australia, New Zealand and Hawaii.

Dysdera longirostris Doblika, 1853
Literary data: L35 (Vrenozi & Jäger, 2013).
Global distribution: Central to Eastern Europe, Turkey, Caucasus.

Dysderocrates storkani (Kratochvíl, 1935)
Literary data: L9 (sub *Harpactocrates s.*: Kratochvíl, 1935).
Global distribution: Balkan endemic, known from Albania, Croatia, Montenegro, North Macedonia and Serbia.

**Harpactea hombergi* (Scopoli, 1763)
New data: L15 (4♂, 27.V.2019, DG), L40a (5♂, 2♀, 14.IV.2019, DG), L49 (2♂, 27.V.2019, DG), L50a (3♂, 27.V.2019, DG), L50b (1♂, 06.VI.2019, DG).
Global distribution: Europe.

**Harpactea nausicaae* Brignoli, 1976
New data: L34b (1♀, 01.X.2019, MN).
Global distribution: Balkan endemic, known from Albania, Greece and North Macedonia.

**Harpactea saeva* (Herman, 1879)
New data: L40a (4♂, 3♀, 27.IV.2019, DG), L50a (4♂, 18.VI.2019, DG).
Global distribution: Eastern Europe.

GNAPHOSIDAE
Berlandina plumalis (O. P.-Cambridge, 1872)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: West Africa, Mediterranean to Central Asia and Iran.

**Callilepis cretica* (Roewer, 1928)
New data: L40a (1♂, 13.V.2018, DG).
Global distribution: North-eastern Mediterranean and Azerbaijan.

**Drassodes cupreus* (Blackwall, 1834)
New data: L40a (1♀, 07.VII.2017, DG).
Global distribution: Palearctic.

Drassodes lapidosus (Walckenaer, 1802)
Literary data: L5a (Stojićević, 1929).
New data: L40a (2♂, 14.IV.2019, DG).
Global distribution: Palearctic.

**Drassyllus villicus* (Thorell, 1875)
New data: L15 (1♂, 3♀, 18.VI.2019, DG), L40a (3♂, 1♀, 24.V.2019, DG).
Global distribution: Western Palearctic.

Micaria pulicaria (Sundevall, 1831)
Literary data: L21a (Kolosvary, 1938, 1940), L21b (Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Holarctic.

Nomisia aussereri (L. Koch, 1872)
Literary data: L35 (Grimm, 1985).
Global distribution: Palearctic.

Scotophaeus blackwalli (Thorell, 1871)

Literary data: L39 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L51 (Drensky, 1936).
Global distribution: Palearctic, introduced to North America, Peru and Hawaii.

**Zelotes apricorum* (L. Koch, 1876)
New data: L15 (3♀, 27.V.2019, DG), L40a (1♀, 27.IV.2019, DG).
Global distribution: Palearctic.

Zelotes longipes (L. Koch, 1866)
Literary data: L20 (sub *Z. serotinus*: Stojićević, 1929, sub *Z. longipes*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Zelotes oblongus (C. L. Koch, 1833)
Literary data: L5a (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003), L39 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

Zelotes similis (Kulczyński, 1887)
Literary data: L32 (Grimm, 1985, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

HAHNIIDAE
**Hahnia pusilla* C. L. Koch, 1841
New data: L49 (1♂, 27.V.2019, DG).
Global distribution: Palearctic.

LINYPHIIDAE
Agyneta fuscipalpa (C.L. Koch, 1836)
Literary data: L3 (sub *Micryphantes fuscipalpus*: Stojićević, 1929, Drensky, 1936, sub *Meioneta f.*: Deltshev *et al.*, 2003, sub *Agyneta f.*: Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Erigone dentipalpis (Wider, 1834)
Literary data: L3 (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic, introduced to North America (Buckle *et al.*, 2001, Jennings & Graham, 2007).

Fageiella ensigera Deeleman-Reinhold, 1974
Literary data: L34a (Deeleman-Reinhold, 1974, Vrenozi & Jäger, 2013), L51 (Naumova *et al.*, 2019b).
New data: L34a (1♀, 30.I.2008, BP).
Global distribution: Balkan endemic, known from Kosovo, Montenegro and Serbia.

**Frontinellina frutetorum* (C. L. Koch, 1835)
New data: L40a (1♀, 24.V.2019, DG).
Global distribution: Palearctic.

**Linyphia triangularis* (Clerck, 1757)
New data: L16b (1♀, 08.XI.2020, DG), L18 (1♀, 02.II.2020, DG), L34b (3♀, 01.X.2019, MN).
Global distribution: Palearctic, introduced to Canada and USA.

**Neriene montana* (Clerck, 1757)
New data: L7 (2♂, 3♀, 06.06.2019, DG).
Global distribution: Palearctic, introduced to North America (Paquin & Dupérré, 2003).

Oedothorax gibbosus (Blackwall, 1841)
Literary data: L14 (sub *Stylothorax tuberosa*, Stojićević, 1929, sub *O. tuberosus*: Drensky, 1936, sub *Oedothorax g.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Palliduphantes trnovensis (Drensky, 1931)
Literary data: L33b (Deeleman-Reinhold, 1986, Deltshev *et al.*, 1996, Vrenozi & Jäger, 2013).
Global distribution: Balkan endemic, known from Albania, Bulgaria, Kosovo, Montenegro, North Macedonia and Serbia.

Porrhomma pygmaeum (Blackwall, 1834)
Literary data: L3 (Stojićević 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Styloctetor compar (Westring, 1861)
Literary data: L3 (sub *Lophomma stativum*, Stojićević, 1929, sub *Anacotyle stativa*, Nikolić & Polenec, 1981, sub *S. stativus*, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).

Global distribution: Holarctic.

**Tenuiphantes tenebricola* (Wider, 1834).
New data: L32 (4♀, 01.X.2019, MN).
Global distribution: Palearctic.

Trichoncus affinis Kulczyński, 1894
Literary data: L3 (Stojićević 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L51 (Nikolić & Polenec, 1981, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

LIOCRANIDAE
**Apostenus fuscus* Westring, 1851
New data: L15 (3♂, 06.VI.2019, DG), L40a (1♂, 27.IV.2019, DG).
Global distribution: Europe.

Sagana rutilans Thorell, 1875
Literary data: L30, (sub *Liocranum r.*: Kolosvary, 1940, Deltshev *et al.*, 2003, sub *Sagana r.*: Vrenozi & Jäger, 2013), L32, (sub *Liocranum r.*: Kolosvary, 1938, Deltshev *et al.*, 2003, sub *Sagana r.*: Vrenozi & Jäger, 2013).
New data: L40a (1♂, 24.V.2019, DG).
Global distribution: Western Palearctic.

LYCOSIDAE
Alopecosa aculeata (Clerck, 1757)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Holarctic.

Alopecosa albofasciata (Brullé, 1832)
Literary data: L5b (Stojićević, 1929).
Global distribution: Mediterranean to Central Asia.

**Alopecosa cuneata* (Clerck, 1757)
New data: L49 (2♂, 06.VI.2019, DG).
Global distribution: Palearctic.

Alopecosa trabalis (Clerck, 1757)
Literary data: L41 (sub *Tarentula t.*: Stojićević, 1929, sub *Alopecosa t.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L42, L47 (sub *Tarentula t.*: Drensky, 1936), L48 (sub *Tarentula t.*: Stojićević, 1929, sub *Alopecosa t.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Arctosa cinerea (Fabricius, 1777)
Literary data: L14 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L42 (Drensky, 1936).
Global distribution: Palearctic, Kongo.

Arctosa leopardus (Sundevall, 1833)
Literary data: L3 (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Hogna radiata (Latreille, 1817)
Literary data: L31 (sub *Tarentula r.*: Kolosvary, 1940), L32 (sub *Tarentula r.*: Kolosvary, 1938, sub *Hogna r.*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L16b (1♀, 29.VII.2020, DG, 1♀, 08.XI.2020, DG), L18 (1♀, 02.VII.2020, DG).
Global distribution: Palearctic.

Pardosa agrestis (Westring, 1861)
Literary data: L3 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L14 (Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa alacris (C. L. Koch, 1833)
Literary data: L32 (sub *P. pseudolugubris*: Wunderlich, 1984, Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

Pardosa amentata (Clerck, 1757)
Literary data: L30 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa fulvipes (Collett, 1876)
Literary data: L8 (Kratochvíl, 1935).
Global distribution: Palearctic.

Pardosa hortensis (Thorell, 1872)
Literary data: L1 (sub *Lycosa h.* & *L. saccata*: Stojićević, 1929), L1 (Deltshev, 2003), L5a, (sub *Lycosa h.* & *L. saccata*: Stojićević, 1929), L14 (Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).

Global distribution: Palearctic.

Pardosa lugubris (Walckenaer, 1802)
Literary data: L5a (sub *Lycosa chelata*: Stojićević, 1929), L32 (Wunderlich, 1984).
New data: L7 (4♂, 2♀, 24.V.2019, DG), L15 (3♀, 27.V.2019, DG), L40a (8♂, 27.IV.2019, DG).
Global distribution: Palearctic.

Pardosa mixta (Kulczyński, 1887)
Literary data: L9, L10a, L43, L44 (sub *Lycosa m.*: Kratochvíl, 1935).
Global distribution: Western Palearctic.

Pardosa morosa (L. Koch, 1870)
Literary data: L21a (sub *Lycosa furva*: Kolosvary, 1938, 1940), L21b (Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa paludicola (Clerck, 1757)
Literary data: L3 (Stojićević, 1929, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa prativaga (L. Koch, 1870)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa pullata (Clerck, 1757)
Literary data: L1, L14 (sub *Lycosa p.*: Stojićević, 1929), L14 (Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Pardosa riparia (C. L. Koch, 1833)
Literary data: L5a (sub *Lycosa r.*: Stojićević, 1929)
Global distribution: Palearctic.

Pardosa saltuaria (L. Koch, 1870)
Literary data: L9, L10a (sub *Lycosa s.*: Kratochvíl, 1935).
Global distribution: Western Palearctic.

Pirata piscatorius (Clerck, 1757)
Literary data: L14 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

**Trochosa robusta* (Simon, 1876)
New data: L2a (4♀, 18.VI.2019, DG).
Global distribution: Palearctic.

**Trochosa ruricola* (De Geer, 1778)
New data: L15 (2♀, 18.VI.2019, DG).
Global distribution: Palearctic, introduced to North America, Cuba, Puerto Rico and Bermuda.

Xerolycosa miniata (C. L. Koch, 1834)
Literary data: L39 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L50 (Drensky, 1936).
Global distribution: Palearctic.

**Xerolycosa nemoralis* (Westring, 1861)
New data: L12 (2♂, 2♀, 18.VI.2019, DG), L50a (3♀, 18.VI.2019, DG).
Global distribution: Palearctic.

*MITURGIDAE
**Zora manicata* Simon, 1878
New data: L2b (3♂, 18.VI.2019, DG), L12 (4♂, 2♀, 18.VI.2019, DG), L15 (2♀, 18.VI.2019, DG), L40a (1♀, 14.IV.2019, DG), L50b (1♂, 18.VI.2019, DG).
Global distribution: Western Palearctic.

**Zora silvestris* Kulczyński, 1897
New data: L15 (4♂, 3♀, 06.VI.2019, DG).
Global distribution: Western Palearctic.

*NESTICIDAE
**Nesticus cellulanus* (Clerck, 1757)
New data: L34a (1♀, 30.I.2008, BP).
Global distribution: Western Palearctic, introduced to North America.

OXYOPIDAE
Oxyopes ramosus (Martini & Goeze, 1778)
Literary data: L46 (Bresjančeva, 1907, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

*PHILODROMIDAE
**Rhysodromus histrio* (Latreille, 1819)

New data: L17 (2♀, 17.X.2020, DG).
Global distribution: Holarctic.

*PHOLCIDAE
Holocnemus pluchei (Scopoli, 1763)
New data: L32 (1♂, 1♀, 2jj, 01.X.2019, MN).
Global distribution: Western Palearctic, introduced to USA, Argentina and Australia.

Pholcus opilionoides (Schrank, 1781)
New data: L40a (1♂, 18.V.2019, DG).
Global distribution: Palearctic.

Pholcus phalangioides (Fuesslin, 1775)
New data: L16b (1♂, 2♀, 29.VII.2020, DG), L24 (1♂, 2♀, 11.VII.2020, DG), L38 (1♂, 1♀, 02.VII.2020, DG).
Global distribution: Western Asia, but almost cosmopolitan due to human activity.

*PHRUROLITHIDAE
Phrurolithus szilyi Herman, 1879
New data: L15 (1♂, 3♀, 27.V.2019, DG).
Global distribution: Europe.

PISAURIDAE
Pisaura mirabilis (Clerck, 1757)
Literary data: L1, L3 (sub *P. listeri* Stojićević, 1929, sub *P. mirabilis*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L5a (sub *P. listeri* Stojićević, 1929, sub *P. mirabilis*: Deltshev *et al*., 2003), L21a (sub *P. listeri* Kolosvary, 1938, 1940), L21b (Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L46 (Bresjančeva, 1907, Drensky, 1936, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L51 (Drensky, 1936).
New data: L4 (3♀, 27.V.2019, DG), L6 (2♂, 2♀, 27.V.2019, DG), L15 (2♂, 27.V.2019, DG), L40a (3♀, 18.V.2019, DG), L49 (7♀, 27.V.2019, DG), L50a (3♀, 27.V.2019, DG).
Global distribution: Palearctic.

SALTICIDAE
Attulus floricola (C. L. Koch, 1837)
New data: L49 (1♂, 19.VI.2019, DG).
Global distribution: Holarctic.

Attulus pubescens (Fabricius, 1775)
Literary data: L1 (sub *Sitticus p*.: Stojićević 1929, Deltshev *et al.*, 2003, sub *Attulus p.:* Vrenozi & Jäger, 2013), L5a (sub *Sitticus p*.: Stojićević 1929), L29, L32 (sub *Sitticus p*.: Vrenozi & Jäger, 2013), L51 (Drensky, 1936).
Global distribution: Western Palearctic, introduced to USA and Canada.

Carrhotus xanthogramma (Latreille, 1819)
New data: L15 (7♂, 2♀, 27.V.2019, DG).
Global distribution: Palearctic.

Dendryphantes rudis (Sundevall, 1833)
Literary data: L41 (Stojićević, 1929; Deltshev *et al.*, 2003; Vrenozi & Jäger, 2013), L42 (Drensky, 1936).
Global distribution: Palearctic.

Evarcha falcata (Clerck, 1757)
Literary data: L47 (sub *E. blancardi*: Stojićević, 1929, sub *E. falcata:* Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Heliophanus auratus C.L. Koch, 1835
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Heliophanus cupreus (Walckenaer, 1802)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Heliophanus dampfi Schenkel, 1923
New data: L15 (2♂, 06.VI.2019, DG).
Global distribution: Palearctic.

Heliophanus flavipes (Hahn, 1832)
Literary data: L3, L5a (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L32 (Vrenozi & Jäger, 2013), L51 (Drensky, 1936).
Global distribution: Palearctic.

Marpissa muscosa (Clerck, 1757)
New data: L7 (3♀, 27.V.2019, DG).
Global distribution: Palearctic.

Neon levis (Simon, 1871)
New data: L40a (1♀, 24.V.2019, DG).
Global distribution: Palearctic.

Pellenes nigrociliatus (Simon, 1875)

Literary data: L47 (Stojićević 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L40a (5♂, 14.IV.2019, DG).
Global distribution: Palearctic.

**Pellenes seriatus* (Thorell, 1875)
New data: L6 (3♂, 1♀, 06.VI.2019, DG).
Global distribution: Palearctic.

Philaeus chrysops (Poda, 1761)
Literary data: L5a (Stojićević, 1929).
Global distribution: Palearctic.

Phlegra fasciata (Hahn, 1826)
Literary data: L29 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Salticus cingulatus (Panzer, 1797)
Literary data: L5a (Stojićević, 1929).
Global distribution: Palearctic.

**Sibianor aurocinctus* (Ohlert, 1865)
New data: L50a (2♂, 24.V.2019, DG).
Global distribution: Palearctic.

Synageles dalmaticus (Keyserling, 1863)
Literary data: L29 (Vrenozi & Jäger, 2013).
Global distribution: Western Palearctic.

*Scytodidae
**Scytodes thoracica* (Latreille, 1802)
New data: (1♀, 29.VII.2020, DG).
Global distribution: Palearctic, introduced to North America, Argentina, South Africa, India, Australia and New Zealand.

*Segestriidae
**Segestria senoculata* (Linnaeus, 1758)
New data: L6 (2♀, 24.V.2019, DG).
Global distribution: Western Palearctic.

SPARASSIDAE
Micrommata virescens (Clerck, 1757)
Literary data: L32 (sub *M. viridissima*: Kolosvary 1938, 1940, sub *M. virescens*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L46 (Bresjančeva 1907, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L15 (1♀, 06.VI.2019, DG), L50a (1♂, 2♀, 18.VI.2019, DG).
Global distribution: Palearctic.

TETRAGNATHIDAE
**Meta menardi* (Latreille, 1804)
New data: L13 (2jj, 18.IV.2018, AZ), L34a (1♀, 1j, 30.I.2008, BP).
Global distribution: Western Palearctic.

Metellina merianae (Scopoli, 1763)
Literary data: L45 (Vrenozi & Jäger, 2013).
New data: L40a (1♀, 14.IV.2019, DG).
Global distribution: Palearctic.

Metellina segmentata (Clerck, 1757)
Literary data: L19 (sub *Meta s.*: Kolosvary, 1940, Deltshev *et al.*, 2003, sub *Metellina s.*: Vrenozi & Jäger, 2013).
Global distribution: Palearctic, introduced to Canada.

Pachygnatha degeeri Sundevall, 1830
Literary data: L5a (Stojićević, 1929).
Global distribution: Palearctic.

Tetragnatha extensa (Linnaeus, 1758)
Literary data: L14 (Stojićević, 1929, Vrenozi & Jäger, 2013).
Global distribution: Holarctic.

THERIDIIDAE
**Asagena meridionalis* Kulczyński, 1894
New data: L40a (2♂, 27.IV.2019, DG).
Global distribution: Western Palearctic.

Asagena phalerata (Panzer, 1801)
Literary data: L3 (sub *Asagena p.*: Stojićević, 1929, Drensky, 1936, sub *Steatoda p.*: Deltshev *et al.*, 2003), L35 (Knoflach, 1996).
New data: L40a (1♂, 27.IV.2019, DG).
Global distribution: Palearctic.

Crustulina guttata (Wider, 1834)
Literary data: L3 (Stojićević, 1929, Deltshev *et al.*, 2003), L51 (Drensky, 1936).
New data: L7 (1♀, 18.VI.2019, DG), L40a (1♂, 3♀, 18.VI.2019, DG), L49 (1♀, 14.IV.2019, DG).
Global distribution: Palearctic.

Enoplognatha ovata (Clerck, 1757)

Literary data: L46 (sub *Phyllonethis lineata*: Bresjančeva, 1907, Drensky, 1936).
Global distribution: Palearctic, introduced to North America.

Phylloneta sisyphia (Clerck, 1757)
Literary data: L46 (sub *Theridium sisiphum*: Bresjančeva, 1907, Drensky, 1936).
Global distribution: Palearctic.

**Platnickina nigropunctata* (Lucas, 1846)
New data: L40a (2♀, 16.VI.2019, DG).
Global distribution: Mediterranean.

Robertus arundineti (O. P.-Cambridge, 1871)
Literary data: L1 (Stojićević, 1929, Deltshev *et al.*, 2003), L51 (Drensky, 1936, Nikolić & Polenec, 1981).
Global distribution: Palearctic.

**Steatoda triangulosa* (Walckenaer, 1802)
New data: L16b (3♀, 29.VII.2020, DG), L37 (1♂, 2♀, 12.VII.2020, DG).
Global distribution: Palearctic, introduced to Canada, USA and Canary Is.

Theridion pictum (Walckenaer, 1802)
Literary data: L32 (Vrenozi & Jäger, 2013).
Global distribution: Holarctic.

THOMISIDAE
Diaea livens Simon, 1876
New data: L40a (1♂, 24.V.2019, DG), L40b (1♂, 03.X.2019, MN).
Global distribution: Western Palearctic, introduced to USA.

Ebrechtella tricuspidata (Fabricius, 1775)
Literary data: L1 (sub *Misumena t.*: Stojićević 1929, sub *Misumenops t.*: Deltshev *et al.*, 2003, sub *Ebrechtella t.*: Vrenozi & Jäger, 2013), L32 (sub *Misumena t.*: Kolosvary 1938, 1940, sub *Misumenops t.*: Deltshev *et al.*, 2003, sub *Ebrechtella t.*: Vrenozi & Jäger, 2013), L51 (sub *Misumena t.*: Drensky, 1936).
New data: L15 (1♀, 06.VI.2019, DG), L16c (1♀, 02.VIII.2020, DG).
Global distribution: Palearctic.

Misumena vatia (Clerck, 1757)
New data: L47 (1♀, 04.X.2019, MN).
Global distribution: Holarctic.

Ozyptila praticola (C. L. Koch, 1837)
Literary data: L21b (Kolosvary 1938, 1940, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic, introduced to Canada, USA and Argentina.

Psammitis sabulosus (Hahn, 1832)
Literary data: L10a (sub *Xysticus s.*: Šilhavý, 1944).
Global distribution: Palearctic.

Runcinia grammica (C. L. Koch, 1837)
Literary data: L46 (sub *R. lateralis*: Bresjančeva 1907).
New data: L17 (1♀, 06.XI.2020, DG).
Global distribution: Palearctic, introduced to South Africa and St. Helena.

Synema globosum (Fabricius, 1775)
Literary data: L32 (Vrenozi & Jäger, 2013)
New data: L2a (3♀, 06.VI.2019, DG), L12 (2♀, 27.V.2019, DG), L16b (1♀, 06.XI.2020, DG), L40a (1♀, 14.IV.2019, DG).
Global distribution: Palearctic.

Thomisus onustus Walckenaer, 1805
Literary data: L32 (Vrenozi & Jäger, 2013), L46 (sub *T. albus*: Bresjančeva, 1907, Drensky, 1936, sub *T. onustus*: Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
New data: L16b (2♀, 18.X.2020, DG), L40a (1♀, 21.IV.2018, DG).
Global distribution: Palearctic.

Tmarus piger (Walckenaer, 1802)
Literary data: L32 (Vrenozi & Jäger, 2013).
New data: L2a (2♂, 06.VI.2019, DG).
Global distribution: Palearctic.

**Xysticus acerbus* Thorell, 1872
New data: L12 (1♂, 27.V.2019, DG).
Global distribution: Palearctic.

Xysticus audax (Schrank, 1803)
Literary data: L32 (Vrenozi & Jäger, 2013)
Global distribution: Palearctic.

Xysticus cristatus (Clerck, 1757)
Literary data: L8, L9 (Šilhavý, 1944), L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic, introduced to Canada and USA.

Xysticus ferrugineus Menge, 1876
Literary data: L10 (Šilhavý, 1944), L31 (Kolosvary 1940), L32 (Kolosvary, 1938, Nikolić & Polenec, 1981, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Xysticus kochi Thorell, 1872
Literary data: L3, L20 (Stojićević, 1929, Deltshev *et al.*, 2003, Vrenozi & Jäger, 2013), L10a (Šilhavý, 1944), L32 (Vrenozi & Jäger, 2013).
Global distribution: Palearctic.

Xysticus lanio C. L. Koch, 1835
Literary data: L10b (Šilhavý, 1944).
Global distribution: Palearctic.

*TRACHELIDAE
**Paratrachelas maculatus* (Thorell, 1875)
New data: L16b (1♀, 18.X.2020, DG).
Global distribution: Western Palearctic.

Table 2. A taxonomic count of the spiders in Kosovo.

Family	Genus	Species	Family	Genus	Species
Agelenidae	5	9	Nesticidae	1	1
Amaurobiidae	1	1	Oxyopidae	1	1
Anyphaenidae	1	1	Philodromidae	1	1
Araneidae	15	21	Pholcidae	2	3
Atypidae	1	1	Phrurolithidae	1	1
Cheiracanthiidae	1	1	Pisauridae	1	1
Clubionidae	2	3	Salticidae	13	18
Dictynidae	3	3	Scytodidae	1	1
Dysderidae	3	6	Segestriidae	1	1
Gnaphosidae	8	12	Sparassidae	1	1
Hahniidae	1	1	Tetragnathidae	4	5
Linyphiidae	12	12	Theridiidae	8	9
Liocranidae	2	2	Thomisidae	10	15
Lycosidae	7	25	Trachelidae	1	1
Miturgidae	1	2	**29**	**108**	**159**

Table 3. Species, excluded from the checklist due to the erroneous interpretation of their localities.

Family/Species: reference	Actually refers to	Reference for designation: page
Araneidae		
Araneus diadematus Clerck, 1757: Drensky, 1936	Serbia	Stojićević (1929): 19, present paper
Dysderidae		
Dasumia kusceri (Kratochvíl, 1935): Nikolić & Polenec, 1981	North Macedonia	Naumova *et al.*, (2019a): 471
Dysdera ninnii Canestrini, 1868: Nikolić & Polenec, 1981	*rejected	Rezac *et al.* (2014): 464
Linyphiidae		

Bathyphantes approximatus (O. P.-Cambridge, 1871): Nikolić & Polenec 1981	Serbia	Stojićević (1929): 22, present paper
Erigone atra Blackwall, 1833: Stojićević, 1929	Serbia	present paper
Macrargus rufus (Wider, 1834): Nikolić & Polenec, 1981	Serbia	Stojićević (1929): 26, present paper
Mansuphantes mansuetus (Thorell, 1875): Stojićević, 1929	Serbia	present paper
Oedothorax gibbosus (Blackwall, 1841): Drensky, 1936	Serbia	present paper
Troglohyphantes kratochvili Drensky, 1935: Mammola *et al.*, 2018 (supl.)	North Macedonia	Drensky (1935): 101, present paper
Lycosidae		
Xerolycosa nemoralis (Westring, 1861): Drensky, 1936	North Macedonia	Stojićević (1929): 50, present paper
Theridiidae		
Steatoda bipunctata (Linnaeus, 1758): Drensky, 1936	Serbia	Stojićević (1929): 14, present paper
Steatoda castanea (Clerck, 1757): Drensky, 1936	Serbia	Stojićević (1929): 14, present paper
Zodariidae		
Zodarion aculeatum Chyzer, 1897: Drensky, 1936	Serbia	Stojićević (1929): 41, Bosmans (2009): 226

* Distribution range of *Dysdera ninnii* is restricted to north-eastern Italy, Slovenia, western Croatia and Switzerland, according to Rezac *et al.* (2014).

Discussion

According to the results of the present study, the spider fauna of Kosovo is the least investigated in the Balkans. An original faunistic data can be found in barely eleven literary sources and the total numbers of published species is only 96. Even in Bosnia & Herzegovina (179 species), Montenegro (289 species), European Turkey (313 species) and Albania (571 species), which are also still in their early stage of researches, spider faunas are more species-rich (Helsdingen, 2013, 2020, Kůrka *et al.*, 2020, Naumova *et al,*, 2016, 2019b, Nentwig *et al.*, 2021, Stanković & Ćurĉić, 2020). Most of the species in the current checklist of Kosovo have Palearctic distribution. Only seven species (*Amaurobius phaeacus*, *Dysderocrates storkani*, *Fageiella ensigera*, *Harpactea nausicaae*, *Inermocoelotes kulczynskii*, *Palliduphantes trnovensis* and *Tegenaria bosnica*) can be defined as endemics for the Balkans. As a landlocked territory with a complex historical and recent political environment, Kosovo was not the most appetizing destination for either vacation or work trips, and until recently there was no arachnologist born and working there, but that is slowly changing. The provided comprehensive national checklist is the first purposeful study of the spiders in Kosovo. The established number of 159 spider species is just the beginning, considering the geographical position in the Balkans \`biodiversity hotspot\` (Griffits *et al.*, 2004, Cuttelod *et al.*, 2008), the diverse relief and climate and especially given the complete lack of study of the cave systems.

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Ecological Structure of the Carabidae complex (Coleoptera) from the Sarnena Sredna Gora Mts., Bulgaria

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Abstract. On the basis of material collected during field trips in the Sarnena Sredna Gora Mts. in 2018–2020, and the available bibliographic data, we completed a list of 175 species of ground beetles (Coleoptera: Carabidae), belonging to 59 genera and 21 tribes. During the field work we collected 7961 specimens from 164 species. This study aimed at analyzing the ecological structure of the carabid fauna. The dominant structure was characteristic with the presence of 1 eudominant numbering 11% of all specimens (*Laemostenus cimmerius*), 5 dominants (32%), 6 subdominants (22%), 12 recedents (23%) and 82 subrecedents (12%). Analysis of the life forms showed a predominance of the zoophages (107 species, 61%) over the mixophytophages (68 species, 39%). Similar ratio (65: 35%) is mostly approaching to the typical for the forest-steppe zones of Eurasia. Humidity preferences analysis showed the larger share of the mesophilous and mesoxerophilous carabids. The macropterous carabids were 57% of all species. All dominant mountain forest species are wingless. The results suggest a well-differentiated and preserved forest carabid fauna and carabid coenosis, with a typical mountain zoning, consisting in distinguishing between typical montane forest carabids and species characteristic of all types of deciduous forests. Established forest species have a relatively high density and a high level of evenness, characteristics of the climax or near-climax forest communities.

Key words: carabids, ecological structure, Sarnena Gora, life forms, wing morphology.

Introduction

The Sarnena Sredna Gora Mts. (Sarnena Gora) is the easternmost part of the Sredna Gora Mts. It falls on the border of two biogeographical regions and three subregions (Gruev, 1988; Teofilova & Kodzhabashev, 2020a). Geographical location, relief, edaphic conditions and specific climatic factors in the Sarnena Sredna Gora Mts. suggest an exceptional variety of habitats (oak forests, beech forests, coniferous plantations, broadleaf plantations, bushes, riparian woods and bushes, dry, mesophilous and hygrophilous grasslands, pastures, inland standing and running surface waters, as well as some artificial landscapes – villages, chalets, agrocoenoses, etc. All this suggests a diverse fauna with variety of forms and complexes.

During the research of the animal diversity of the Sarnena Gora, two very rare species of Coleopterans characteristic of old climax forests from the beech mountain belt

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were found (Teofilova et al., 2021a, 2021b). A similar pattern has also been established about earthworm (Lumbricidae) species characteristic of territories with typical mountain climate (Zdravkova et al., 2020). Despite the relatively small altitude of the Sarnena Gora, the established fauna indicates the presence of a well-structured and differentiated vertical distribution, characteristic of the middle and low mountain belts of other Bulgarian mountains.

This paper represents the second part of the first purposive study on the ground beetles (Coleoptera: Carabidae) from Sarnena Gora Mts. So far, 175 species (23% of all Bulgarian Carabidae species) are to be found there (Teofilova & Kodzhabashev, 2020a). Ground beetles represent one of the largest beetle families with cosmopolitan distribution and with decisive importance for the functioning of ecosystems. Their high taxonomic richness, the large numbers and the diverse life specializations are the reasons they cover the entire environmental spectrum of fundamental natural gradients.

Ecological classifications of carabids are numerous and various, but for their practical application in assessing the condition of the faunas and coenoses and the degree of anthropogenic influence, as main factors determining their presence and distribution the feeding, the diet and the mode of movement (respectively life forms), as well as the hydrothermal regime and the state of the soil, the type of plant cover, climatic and geographical features, are used (Kotze et al., 2011; Kryzhanovskij, 1983; Sharova, 1981).

The present study aimed at analyzing the ecological structure of the carabid complex in Sarnena Gora in relation to main ecological parameters, e.g. dominance structure, life form categorization, wing development, humidity and habitat preferences, with a subsequent assessment of the environmental trends and anthropogenic impact in the studied area.

Material and Methods

The species list is completed on the basis of the available bibliographic data and material collected during field trips carried out in 2017–2020. Ground beetles were collected with pitfall traps, hand picking and light attraction, and different types of habitats were sampled (see Teofilova & Kodzhabashev, 2020a). Main sampling sites were: 1) Svezhen Region – I. Pasture with single bushes and trees; II. Coniferous pine-spruce plantation; III. Mesophilous ridge beech forest; IV. Old beech forest; V. Mesoxerothermic oak forest; VI. Ridge beech forest with many old trees; VII. Ridge coniferous pine-spruce-Douglas fir plantation; VIII. Ridge pasture, surrounded by forests; IX. Mixed oak-beech-hornbeam forest; X. River bank with oak, cornel and white willow; XI. Oak forest with *Ruscus aculeatus*; 2) Chirpan Heights Region – XII. Black locust plantation; XIII. Pasture with *Paliurus spina-christi* and *Opuntia* sp.; XIV. Mixed riverine forest; XV. Linden forest with *Ruscus aculeatus;* XVI. Mixed oak-linden-maple forest with *Ruscus*; XVII. Dry oak-Oriental hornbeam forest on shallow and stony soil; XVIII. Abandoned pasture with single bushes and trees; XIX. Edge of alfalfa field; XX. Wheat field and small river ecotone with walnuts; 3) Bratan Region – XXI. Scots pine plantation near walnut and linden plantations; XXII. Riverine forest with beech, alder, hazel and hornbeam. They are presented on Fig. 1.

The analysis of the specific community ecology included only the material from the 141 pitfall traps set in the main 22 sampling sites in the period 22 March 2019 – 9 May 2020, when 5948 specimens and 106 species were collected. In order to determine the dominance structure, the relative abundance (or degree of dominance) was used: $D = (n_i/N).100$, where n_i is the number of individual representatives of each species, and N – their total number. The classical four-level classification of Tischler (1949) for invertebrates, modified by Sharova (1981), was adopted: eudominants (> 10% of all individuals); dominants (5 to 10%); subdominants (3 to 5%); recedents (1 to 3%); subrecedents (< 1%). Frequency of occurrence was calculated and species were divided to constant (F > 50%), auxiliary (F = 25–50%), and accompanying (F < 25%).

Main ecological analysis included all collected data about the species composition (both from the literature and the field work). The total number of all collected beetles was 7961, belonging to 164 species, and 11 species were known from the literature.

Categorization of the species in respect of their life forms followed the classification of Sharova (1981). Species were also classified into three groups according to their hind wing development: macropterous (always possessing wings), wing dimorphic/polymorphic (only part of the population being fully winged), and brachypterous (wingless), according to Den Boer et al. (1980). According to their ecological requirements in terms of humidity, the established carabid species were divided into six categories (Teofilova, 2018): hygrophilous, mesohygrophilous, mesophilous, mesoxerophilous, xerobionts, and eurybionts.

Captured animals are deposited in the first author's collection in the Institute of Biodiversity and Ecosystem Research (Bulgarian Academy of Sciences, Sofia).

Results

The results from the study revealed that in the Sarnena Sredna Gora Mts. 175 species of ground beetles occur, belonging to 59 genera and 21 tribes. For details about the sampling sites and collecting methods for each species, see Teofilova & Kodzhabashev (2020a). The complete check-list of the established species with their full name, author and year of description, information about their wing morphology, humidity and habitat preferences, life form, dominance degree and occurrence (the last two parameters calculated only on the basis of the data from the pitfall traps sampling in 2019–2020) is given in the Appendix 1.

Dominance structure

The dominance structure of the entire carabid complex was characterised by the presence of only one eudominant (with a total number of 11% of all caught specimens), five dominants (32%), six subdominants (22%), 12 recedents (23%) and 82 subrecedents (12%). The eudominant species was *Laemostenus cimmerius*, dominants were *Aptinus bombarda*, *Carabus montivagus*, *C. convexus*, *Molops piceus*, *Pterostichus oblongopunctatus*, and the subdominant species were *Abax carinatus*, *Calathus distinguendus*, *C. fuscipes*, *Carabus hortensis*, *Harpalus tardus*, *Myas chalybaeus* (Table 1). *Carabus intricatus* , *Limodromus assimilis*, and *Xenion ignitum* had degree of dominance close to 3% (see Appendix 1), but they were considered recedent.

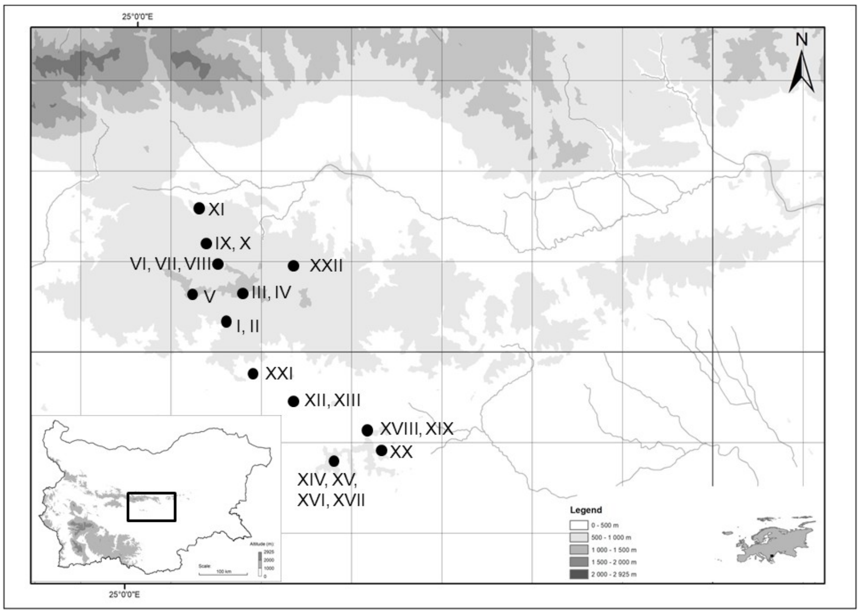

Fig. 1. Indicative map of the study area.

Table 1. Dominance structure of the carabid complex in Sarnena Gora during the whole-year research in 2019–2020**.**

Category	Species	No
Eudominant	*Laemostenus cimmerius*	1
Dominant	*Aptinus bombarda*, *Carabus montivagus*, *C. convexus*, *Molops piceus*, *Pterostichus oblongopunctatus*	5
Subdominant	*Abax carinatus*, *Calathus distinguendus*, *C. fuscipes*, *Carabus hortensis*, [*C. intricatus*], *Harpalus tardus*, [*Limodromus assimilis*], *Myas chalybaeus*, [*Xenion ignitum*]	6
Recedent	*Abax ovalis*, *A. parallelus*, *Brachinus crepitans*, *Br. explodens*, *Calosoma inquisitor*, *Carabus intricatus*, *C. coriaceus*, *Limodromus assimilis*, *Notiophilus rufipes*, *Pterostichus niger*, *Trechus quadristriatus*, *Xenion ignitum*	12
Subrecedent	all the rest	82

Frequency of occurrence

The occurrence reflects the uniformity or the evenness of the distribution of species in space. In whole-year catches in Sarnena Gora we found the highest share of auxiliary species occurring in less than 25% of the sampling sites – 81 species (76.4% of all). Only in one sampling site (F = 5%) occurred 44 (41%) of the species. The accompanying species occurring in 25–50% of the sampling sites were 14 (13.2%). As constant (F > 50%) we found 11 species (10.4%), and euconstant species (F = 100%) were missing (Table 2). *Laemostenus cimmerius* was not found only in sites III and XIX.

Richest in rare species occurring in only one of the studied habitats were sampling sites XIX (alfalfa field edge) (10 species), followed by sites X (river bank) and XX (river-field ecotone) with 6 rare species each, and site XXII (riverine forest) with 5 species. This fact points the peculiarity of these habitats. In site XIII we found 4 species, which were not found in the remaining sites; in sites I, VIII, XII and XIV – 2 such species; in III, V, XVI and XVIII – only 1 species. No "unique" species were found in sites II, IV, VI, VII, IX, XI, XV, XVII and XXI).

Life forms

The 175 ground beetle species belonged to two classes and 22 life form groups proposed by Sharova (1981) – 16 zoophagous and 6 mixophytophagous. The life forms of each species were given in the Appendix 1. The analysis of the life forms showed a predominance of the zoophages (107 species, 61%) over mixophytophages (68 species, 39%) (Table 3). The most numerous life form groups were the harpaloid geohortobionts from class Mixophytophaga (33 species), and the litter & soil-dwelling digging stratobionts (21 species) and the surface & litter-dwelling stratobionts (19 species) from class Zoophaga (Table 3). The significant percentage of the mixophytophagous harpaloid geohortobionts was mainly resulting from the increased presence of species from the genus *Harpalus*.

Wing morphology

The degree of hind wing development allowed distinguishing of three groups of carabids: brachypterous (hind wings shorter than elytra or missing), macropterous (winged), and dimorphic (some individuals have fully developed wings, others have only vestigial ones). Macropterous beetles represented 57% (101 species) of all collected carabid species. Pteridimorphic species were 22% of all (39 species), and brachypterous were 16% (30 species). For five species in our study (5%) there were no data about their wing morphology (Fig. 1).

Humidity and habitat preferences

The analysis of the humidity preferences (Fig. 2) of the ground beetles from Sarnena Gora showed the prevalence of the mesoxerophilous (59 species, 34% of all established species) and mesophilous carabids (50 species, 27%). Mesohygrophilous were 29 species (17%). Less represented were strictly hygrophilous (14 species, 9%) and xerophilous (11 species, 6%) carabids, as well as eurybionts (12 species, 7%). The habitat-preferential structure showed a strong dispersion in the distribution of the carabid complex in the region of Sredna Gora. The inhabitants of the dry oak forests are less than 5%. The majority of the xerophiles (about 30% of all species) were open-habitat forms, and many of them are broad-spectrum thermophiles able to inhabit both natural or semi-natural habitats and agrocoenoses. The mesophilic complex included mainly forest mesophiles (about 15% of all) found in the beech (*Fagus sylvatica* L.) mountain belt, with lesser share in the north-facing pre-mountain mixed forests with linden as co-edificator. One third of the hygrophilic carabid fauna were coastal extra- or intrazotal species, and the rest of it inhabited biotopes with a high degree of moisture regardless the vegetation type. Relatively small shares had eurybionts, which are usually very common among the main entomofauna of agrocoenoses and are also among the dominant component of the whole carabid complex, as well as the specific groups of stenobionts, such as halophiles and bothrobionts (about 1% each). Specific group were the inhabitants of the ecotones, which in Sarnena Gora were about 10% of all species.

Table 2. Frequency of occurrence in the carabid complex from the Sarnena Gora.

F	*Species*	*No*
> 50%	91%: *Laemostenus cimmerius*; 82%: *Carabus convexus*, *Myas chalybaeus*; 68%: *Abax carinatus*, *Carabus montivagus*; 64%: *Carabus coriaceus*, *Trechus quadristriatus*; 55%: *Calathus fuscipes*, *Calosoma inquisitor*, *Harpalus tardus*, *Pterostichus oblongopunctatus*	11
25–50%	45%: *Carabus intricatus*, *Molops piceus*, *Notiophilus rufipes*; 36%: *Calathus distinguendus*, *Carabus hortensis*, *Xenion ignitum*; 32%: *Cychrus semigranosus*, *Pterostichus niger*; 27%: *Abax ovalis*, *Aptinus bombarda*, *Brachinus explodens*, *Carabus scabrosus*, *Laemostenus venustus*, *Molops alpestris*	14
< 25%	all other	81

Table 3. Life forms of the ground beetles from the Sarnena Sredna Gora Mts. The first figure in the index shows the class of life form, the second shows the subclass, and the third indicates the life form group; the figure in brackets after the subclass shows the series, if any.

	Life forms	**No sp.**	%
	Class: Zoophagous		
	Life form subclass: 1.1 – Phytobios		
1.1.2	Stem-dwelling hortobionts	1	0.6
1.1.3	Leaf-dwelling dendrohortobionts	3	1.7
	Life form subclass: 1.2 – Epigeobios		
1.2.2	Large walking epigeobionts	12	6.9
1.2.2(1)	Dendroepigeobionts	1	0.6
1.2.3	Running epigeobionts	3	1.7
1.2.4	Flying epigeobionts	3	1.7
	Life form subclass: 1.3 – Stratobios		
	Series: 1.3(1) – crevice-dwelling stratobionts		
1.3(1).1	Surface & litter-dwelling	19	10.6
1.3(1).2	Litter-dwelling	16	9.1

1.3(1).3	Litter & crevice-dwelling	18	10.3
1.3(1).4	Endogeobionts	2	1.1
1.3(1).5	Litter & bark-dwelling	2	1.1
1.3(1).6	Bothrobionts	3	1.7
	Series: 1.3(2) – digging stratobionts		
1.3(2).1	Litter & soil-dwelling	21	12.0
1.3(2).2	Litter & crevice-dwelling	1	0.6
1.3(2).3	Bothrobionts	1	0.6
	Life form subclass: 1.4 – Geobionts		
1.4.1(1)	Narrow-headed running & digging geobionts	1	0.6
	Zoophagous total:	***107***	***61.0***
	Class Mixophytophagous		
	Life form subclass: 2.1 – Stratobios		
2.1.1	Crevice-dwelling stratobionts	9	5.1
	Life form subclass: 2.2 – Stratohortobios		
2.2.1	Stratohortobionts	16	9.1
	Life form subclass: 2.3 – Geohortobios		
2.3.1	Harpaloid geohortobionts	33	18.9
2.3.1(1)	Crevice-dwelling harpaloid geohortobionts	2	1.1
2.3.2	Zabroid geohortobionts	5	2.6
2.3.3	Dytomeoid geohortobionts	3	1.7
	Mixophytophagous total:	***68***	***39.0***

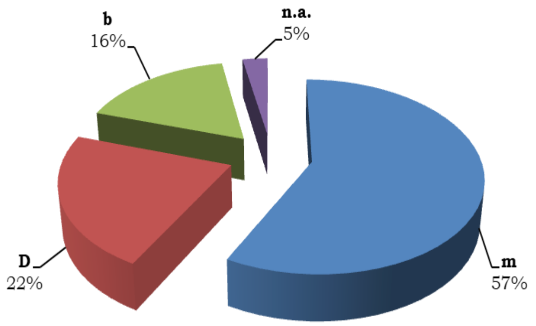

Fig. 1. Wing morphology of carabid species in Sarnena Gora: m – macropterous, D – wing di(poly)morphic, b – brachypterous, n.a. – no data.

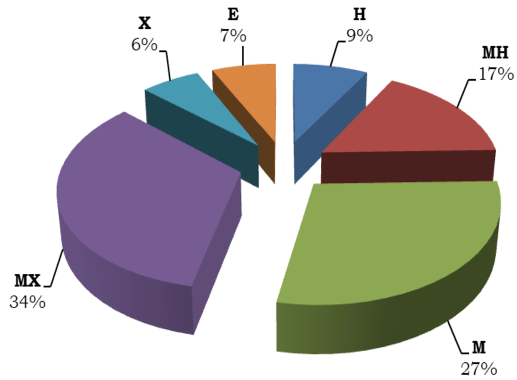

Fig. 2. Humidity preferences (number of species) of the carabids: H – hygrophilous, MH – mesohygrophilous, M – mesophilous, MX – mesoxerophilous, X – xerobiont, E – eurybiont.

Discussion

The results of the present study show that the region of the Sarnena Sredna Gora Mts. keeps a very rich, diverse and heterogeneous ground beetle fauna.

The dominance structure demonstrates the quantitative significance of the species and provides information on their quantitative share, which is a way of establishing the ecological situation and condition in the particular habitat or region. In Sarnena Gora it has a less concentrated dominance with more salient evenness between the dominant and recedent categories, unlikely the more anthropogenically loaded regions near Sofia (Kodzhabashev & Mollov, 2000), on Cape Emine (Teofilova, 2015), and in Zlatiya Plateau (Teofilova & Kodzhabashev, 2020c). Foreign authors studying the anthropogenic impacts on carabids are many, and works dealing with agrocoenoses (e.g. Batáry et al., 2012; Mast et al., 2012; Meissle et al., 2012; Pizzolotto et al., 2018; Porhajašová et al., 2008) and forests (e.g. Cobb et al., 2007; de Warnaffe & Leburn, 2004; Koivula & Niemelä, 2002; Lange et al., 2014; Magura et al., 2002; Skłodowski, 2014) are especially numerous. In Bulgaria, however these aspects are relatively rarely concerned (Popov & Krusteva, 1999; Teofilova et al., 2012; Teofilova, 2016, 2017). Main anthropogenic impacts in the studied region are agriculture, livestock farming and logging.

We found 12 species in the dominant component, and 8 of them are typical forest species. Characteristic of mountain mesophilic forests are *Molops piceus*, *Carabus hortensis*, *C. intricatus* and *Xenion ignitum*, and typical for all old deciduous forests are *Laemostenus cimmerius*, *Aptinus bombarda*, *C. convexus*, *C. montivagus*, *Abax carinatus*, *Harpalus tardus*, *Pterostichus oblongopunctatus* and *Myas chalybaeus*. The recedent component of the dominant structure without exceptions includes mesophiles and mesohygrophiles, which clearly confirms the mountainous mesophilic appearance of the region as a whole. The established subrecedent component includes 77% of the species and 23% of the specimens caught, which is closer to the normal distribution for natural ecosystems with a low degree of anthropogenic influence. All xerophilous open-habitat species are in the subrecedent category, which confirms the presence of mesophilic montane appearance of the area as a whole. In particular, many xerophilous species, all of which have very low abundance, are established in the Chirpan Heights area, where many of the open habitats have been dried and steppificated as a result of forest felling and cultivation of the soil for agricultural purposes and pastures (habitats XII, XIII, XVII, XVIII, XIX, XX).

In relation to the frequency of occurrence, it is notable the absence of euconstant species (*F* = 100%). It seems that such common species are more often in areas with higher anthropogenic load, as the region of Cape Emine (Teofilova, 2015), where five euconstant species were found in various types of ecosystems, and the Zlatiya Plateau, where three species of *Harpalus* were found in all sampling sites (Teofilova & Kodzhabashev, unpublished results).

All species having a frequency of occurrence > 25% are forest mesophiles (20 species) or eurybionts (5 species), which confirms the middle-montane character and mesophilic forest appearance of the area, despite large anthropogenic transformations occurred in the last 80–100 years. This group of species, in addition to relatively high occurrence, also has a relatively high degree of dominance, and most species are represented in the dominant component of the dominance structure. Of all 25 species, 13 are representatives of the nemoral European carabid fauna, and another 8 are forest species characteristic of the southeastern parts of Europe.

Relatively low frequency of occurrence have all intra- and extrazonal species, such as coastal hygrophiles (*Elaphrus aureus*, *Perileptus areolatus*, *Pterostichus vernalis*, *Pt. anthracinus*, *Tachyura haemoroidalis*), certain halobionts (*Apotomus clypionitens*, *Bembidion subfasciatum*, *Carterus dama*, *Harpalus hospes*, *Microlestes corticalis*, *M. fulvibasis*) and synanthropic xerobionts (*Brachinus alexadri*, *Dixus obscurus*, *Licinus cassideus*, *Microlestes lactuosis*, *Harpalus albanicus*, *H. angulatus*, *H. smaragdinus*, H. *subcylindricus*). Their specific ecological requirements are the cause of their local habitation, low frequency of occurrence and, usually, density.

The morphological structure of the life forms of carabids shows the overall adaptability of the group to the complex of all environmental factors, i.e. the "life form" can be accepted as a specific ecological adaptation (specialization) or as a measurement of a specific "ecological niche". The life forms' spectra of a region or habitat may provide information on the ecological structure, environmental conditions and regularities in the distribution along ecological gradients (Sharova, 1981). The share of the two main classes of Zoophages: Mixophytophages established in Sarnena Gora (61%: 39%), is characteristic of wooded areas with wide open spaces among or around them. Zoophagous life form groups are normally more numerous, especially in stable ecosystems (Sharova, 1981) and in forest regions, as it was found in 'Leshnitsa' Reserve (Teofilova, 2016), Vrachanska Planina Mts. (Teofilova, 2019b), and in the Western Rhodope Mts. (Teofilova, 2018). According to Sharova (1981), the normal ratio between the two classes is 60%: 40% for the forest-steppe areas, and 70%: 30% for the nemoral forest zone. Close to the normal ratio between the classes values were established in the Srebarna Reserve – 60%: 40% (Kodzhabashev, 2016, PhD thesis, unpublished results), the region of Cape Emine and Eastern Rhodope Mts. – 57%: 43% (Teofilova et al., 2015; Teofilova & Kodzhabashev, 2020b), and the Lower Tundzha Valley – 62.5%: 37.5% (Teofilova, 2017). Close to the normal for the nemoral zone was the ratio in the Western Rhodopes Mts. – 67%: 33% (Teofilova, 2018). The proximity of the Upper Thracian Lowland and the substantial transformation of much of the territory of Sarnena Gora into agricultural land and pastures are the reason for the significant preponderance of life forms specializing in living in open, secondary stepped spaces. In forest habitats, the percentage of the two classes is 70%: 30%, which is due to the high density of zoophages of the subclasses of Stratobios (46%) and Epigeobios (11%).

The increased share of mixophytophages indicates that the area is anthropogenically influenced, which is characteristic of the vast open territories occupied mainly by arable agricultural lands, pastures and sparsely vegetated old clearings on shallow soils. In arable lands, mainly representatives of the geohortobionts occur, which use the crumbly surface layer as shelter, but feed upon herbaceous plants. This group is very often ephemeral in nature, predetermined by the technical activities of the management of agricultural lands. Many of the species are steppic or thermophilic xerobionts adapted to live in conditions of prolonged droughts. Stratohorobionts and stratobionts include species that mainly inhabit pastures and degraded open habitats, grassed and shrubbed with xero- and mesoxerophytic vegetation. Increased number of mixophytophages is been found in xerophytic pseudomaquis communities in SW Bulgaria (Teofilova, 2020). It seems that fossorial mixophytophagous harpaloid geohortobionts are the dominating life form not only in this study but also in many other regions of Bulgaria (e.g. Kodzhabashev & Penev, 2006; Teofilova, 2017, 2018, 2019a, 2019b; Teofilova & Kodzhabashev 2020b, 2020c).

The analysis of the established results about the development of the carabids' wings shows a ratio between the three main types characteristic of areas with wide forest massifs located among plain open areas. All dominant mountain forest species are wingless, and some of the recedent species are di(poly)morphic. Similar results found Brandmyr (1983) in a study of the mountain carabid fauna of the Alps. According to Darlington (1943), Brandmayr (1983) and Desender (1989, 2000), wingless carabid assemblages are characteristic of ecologically homogeneous and stable environments, where resources are sufficient for beetles' entire life cycle. These theories explain the presence of the specific wingless carabid fauna in mountain forest habitats. According to Desender (2000), Kotze et al. (2011) and Venn (2007, 2016), the proportions in the ecological groups can be successfully used to register changes in environmental conditions, i.e. for bioindication and monitoring purposes. The increase in winged forms among dimorphic species and the appearance of winged males in species for which it is characteristic to have mainly winged females, is a signal of changes requiring migration or resettlement for colonization. Unfortunately, despite all summaries on this ecological aspect (e.g. Den Boer et al., 1980; Desender, 1989, 2000; Venn, 2016), many of the regularities are still at the level of a scientific hypothesis, or apply to a particular geographic region, a particular species or taxonomic group, making it difficult to fully discuss the topic. In Bulgaria, such researches are still beginning to develop (Teofilova & Kodzhabashev, 2020c; Teofilova, 2021). Here, we have a ratio between the winged, dimorphic and wingless species of 57%, 22% and 16%, respectively. As a comparison, that ratio is, respectively, 69%, 22% and 8% in Zlatiya Plateau (Teofilova & Kodzhabashev, 2020c), and 73%, 17% and 10% in Bulgarian rapeseed (*Brassica napus* L.) fields (Teofilova, 2021). An increased presence of winged forms is recorded in all arable agricultural and degraded lands in our study, as well as in many agrocoenoses in Europe (e.g. Pizzolotto et al., 2018; Teofilova, 2021). If we follow Gray's hypothesis, that the proportion of flight capable pioneer species should increase with increasing disturbance, and the proportion of flightless species should decrease (Gray, 1989), we can conclude that the environment in Sarnena Gora is more stable and determines carabids' wing morphology structure with a lesser share of winged beetles, in comparison with other regions in Bulgaria.

According to a number of carabidologists (Eyre et al., 2005; Kryzhanovskij, 1983; Lindroth, 1992; Thiele, 1977), the main factors of the environment of particular importance for the distribution of ground beetles are the type and hydrothermal regime of the soils. They, in turn, determine the plant cover and many other dependent factors important for the carabids. Changes in these conditions are usually the result of human activities related to destruction or transformation of the vegetation and, respectively, of all conditions determining the normal gradient distribution of ground beetles.

Our results about the humidity preferences of beetles show that mesoxerophiles predominate in Sarnena Gora. They are mostly inhabitants of open areas, such as arable lands, pastures, hay meadows and clearings, and of natural origin are probably the dry grassland communities in the Chirpan Heights and the easternmost regions of the mountain. Carabid fauna of open territories is a mix of naturally occurring and some ecologically plastic species, some of which in process of initial invasive expansion. This effect is particularly pronounced in intensive agricultural lands. A number of xerobionts specific to Eurasian steppes and Mediterranean succulent communities are also found among this fauna (*Acinopus megacephalus*, *Carterus dama*, *Dixus obscurus*, *Harpalus angulatus*, *H. smaragdinus*). Xerophilous and mesoxerophilous carabid fauna (about 40% of the species) is disjunct, with low occurrence and low relative density, but with an extreme variety of species. Perhaps the proximity of the Upper Thracian Lowland to the south and the Sub-Balkan Valleys to the north, which have been converted into large agricultural areas for intensive farming, have had a strong impact on the modern state of the carabid coenoses found near them. Most of the xerobionts are registered in pastures, alfalfa fields and degraded due soil erosion sloping pre-mountainous terrains. Increased number of xerophiles and mesoxerophiles (over 60%) is found in xerophytic pseudomaquis communities in SW Bulgaria (Teofilova, 2020). Arable lands and pastures serve as peculiar corridors and refugia for xerophilous carabid fauna; annual agricultural and livestock activities, combined with prolonged periods of drought and geographical location, favour its invasive expansion.

In synanthropic habitats, there is also an increased percentage of eurybionts, which are mostly ecologically plastic species, tolerant to a wide range of environmental conditions and their frequent changes, with extensive Palaearctic or Eurasian ranges (Kryzhanovskij, 1983). The share of eurytopic species in the region of Cape Emine is 9% (Teofilova et al., 2015), and in Zlatiya Plateau it is 11% (Teofilova & Kodzhabashev, 2020c), pointing the more stable conditions in Sarnena Gora, where only 7% of all species are eurybionts.

A significant share of the species composition of the carabids from the Sarnena Gora is occupied by the mesophilous and mesohygrophilous carabid fauna – 79 species, representing 44% of all established species. This fauna mainly includes forest species characteristic of the middle beech mountain belt of the Sarnena Gora. Characteristic species for montane forest carabid fauna are *Carabus hortensis*, *Cychrus semigranosus*, *Molops* spp., *Pterostichus merklii*, *Pt. vecors*, *Tapinopterus cognatus*, *Xenion ignitum*. The distribution of these species coincides with the distribution limits of the beech. Widespread mesophilous forest species in all height belts are *Abax carinatus*, *A. ovalis*, *A. parallelus*, *Aptinus bombarda*, *Calosoma inquisitor*, *Carabus scabrosus*, *Myas chalybaeus*, *Pterostichus oblongopunctatus*, etc. As a comparison, the share of mesophilous and mesohygrophilous carabids was 38% in the Eastern Rhodope Mts. (Teofilova & Kodzhabashev, 2020b), 32% in Zlatiya Plateau (Teofilova & Kodzhabashev, 2020c), and under 25% in pseudomaquises in SW Bulgaria (Teofilova, 2020).

Hygrophilous carabid fauna is mainly concentrated around montane rivers and includes 15 species (9%), some of which are found in only one of the four riparian habitats. Such stenotopic riparian species are *Bembidion dalmatinum, B. deletum*, *Chlaenius nitidulus, Elaphrus aureus, Drypta dentata, Perileptus areolatus*, *Pterostichus vernalis.*

The results of this work demonstrate the predominantly mesophilic nature of Sarnena Gora as a whole, similar to that in the western part of the Rhodope Mts. (Teofilova, 2018) and Vrachanska Planina Mts. (Teofilova, 2019b), and contrasting with the predominantly mesoxerophilic conditions in the Eastern Rhodope Mts. (Teofilova & Kodzhabashev, 2020b) and pseudomaquises in SW Bulgaria (Teofilova, 2020).

Conclusions

The present study proves that the region of the Sarnena Sredna Gora Mts. keeps a very rich, diverse and heterogeneous ground beetle fauna, and has a significant conservation value. The insufficient research in the area and the large carabid species richness suggest that future targeted studies would contribute to the enrichment of the species list presented here.

The results of our research and analysis suggest a well-differentiated and preserved forest carabid fauna and carabid coenose, with a typical mountain zoning, consisting in distinguishing between species characteristic of all types of old mesophilic deciduous forests, and montane forest carabids typical for the beech mountain belt. Established forest coenoses have a relatively small number of species with high density and high level of evenness, characteristics of the climax or close to climax state forest ecosystems. All established forest species are wingless, mesophilous or mesohygrophilous stratobionts and epigeobionts, which confirms the naturalness and autochtonity of the ground beetle communities and their habitats. The established in the dominant component of the dominant structure species can be used as indicators of mountain forest mesophilic fauna. Such are also some typical forest species from the recedent category, which are being represented by a relatively large number of specimens only in mountain forest ecosystems.

Despite the large and drastic changes in the environment, there is an exceptional variety of preserved habitats of carabids in the Sarnena Gora, which is confirmed by the great species richness, the high density of species specific to natural habitats and their stable carabid coenoses.

A significant problem for natural ground beetle communities is the rapid human intervention associated with the felling of old, diverse in age forests and their transformation into even-age forests, as well as the conversion of natural forests into coniferous or exotic plantations. Another significant problem is the intensive agriculture, which destroys soil structure annually, and pesticides and mineral fertilizers destroy soil organisms and lead to irreversible degradation.

In order to assure the preservation of the natural habitats and significant species, a proclamation of some protected areas and/or zones is recommendable.

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Appendix 1. Species list and ecological characteristics of carabids from Sarnena Sredna Gora Mts. WM – Wing morphology (m – macropterous, D – wing di(poly)morphic, b – brachypterous, n.a. – no data); HP - Humidity preferences (H – hygrophilous, MH – mesohygrophilous; M – mesophilous, MX – mesoxerophilous, X – xerophilous, E – eurybiont); HT - Habitat type (1 – inhabitants of dry open habitats; 2 – inhabitants of humid open habitats; 3 – inhabitants of dry forest habitats; 4 – inhabitants of humid forest habitats; 5 – inhabitants of open, sunny coasts; 6 – inhabitants of canopy, shady shores; 7 – halobionts; 8 – bothrobionts; 9 – eurybionts, 10 – species of ecotones); LF – Life forms (descriptions of life forms codes are given in Table 4); *D* – Dominance degree; *F* – Occurrence. *only data from the pitfall traps sampling in 2019 – 2020.

Species	WM	HP	HT	LF	*D*%*	Sampling site [*F*%]*
Abax (Abax) carinatus carinatus (Duftschmid, 1812)	b	M	4	1.3(2).1	3.04	all without IV, VI, XII, XIII, XVIII, XIX, XX [*68*]
Abax (Abax) ovalis (Duftschmid, 1812)	b	MH	4	1.3(2).1	1.34	III, IV, VI, VII, IX, XXII [*27*]
Abax (Abax) parallelus parallelus (Duftschmid, 1812)	b	M	4	1.3(2).1	2.00	XXI, XXII [*9*]
Acinopus (Acinopus) picipes (Olivier, 1795)	D	MX	1	2.3.2.	0.02	III [*5*]
Acinopus (Oedematicus) megacephalus (P. Rossi, 1794)	m	MX	1	2.3.2.		
Acupalpus (Acupalpus) dubius Schilsky, 1888	m	H	2,6	2.1.1.		
Acupalpus (Acupalpus) meridianus (Linnaeus, 1760)	m	MH	2,5	2.1.1.		
Amara (Amara) aenea (De Geer, 1774)	m	E	9	2.3.1.	0.08	X, XVIII, XIX, XX [*18*]
Amara (Amara) anthobia A. Villa et G. B. Villa, 1833	m	MX	1	2.1.1.	0.18	X, XII, XVIII [*14*]
Amara (Amara) communis (Panzer, 1797)	m	M	2,3,10	2.3.1.		
Amara (Amara) convexior Stephens, 1828	m	MX	1	2.3.1.	0.62	XX, XXI, XXII [*14*]
Amara (Amara) eurynota (Panzer, 1796)	m	M	1,2	2.3.1.	0.03	VIII [*5*]
Amara (Amara) familiaris (Duftschmid, 1812)	m	MX	1,2	2.1.1.		
Amara (Amara) lucida (Duftschmid, 1812)	m	M	1,2	2.3.1.	0.02	XVIII [*5*]
Amara (Amara) montivaga Sturm, 1825	m	M	2	2.3.1.		
Amara (Amara) ovata (Fabricius, 1792)	m	E	1,2	2.3.1.	0.02	XI [*5*]
Amara (Amara) saphyrea Dejean, 1828	m	M	3,4	2.3.1.	0.59	X, XI, XII, XX, XXI [*23*]
Amara (Amara) similata (Gyllenhal, 1810)	m	MX	1,2	2.3.1.		
Amara (Bradytus) consularis (Duftschmid, 1812)	m	MX	1,10	2.3.1(1)		
Amara (Percosia) equestris equestris (Duftschmid, 1812)	m	MX	1	2.3.2.	0.02	XX [*5*]
Amara (Xenocelia) municipalis (Duftschmid, 1812)	m	MX	1,10	2.3.1.		
Amara (Zezea) chaudoiri incognita Fassati,1946	m	M	2	2.2.1.		
Amara (Zezea) fulvipes (Audinet-Serville, 1821)	m	MX	1	2.2.1.		
Amblystomus metallescens (Dejean, 1829)	m	MH	2,5	2.1.1.		
Amblystomus rectangulus Reitter, 1883	m	MH	2	2.1.1.		
Anchomenus (Anchomenus) dorsalis dorsalis (Pontoppidan, 1763)	m	MX	1,2,10	1.3(1).1	0.29	XVIII, XIX, XX, XXI [*18*]
Anisodactylus (Anisodactylus) binotatus (Fabricius, 1787)	m	MH	2,10	2.3.1.	0.02	I [*5*]
Apotomus clypeonitens adanensis Jedlička, 1961	m	MH	5,7	1.4.1(1)		
Aptinus (Aptinus) bombarda (Illiger, 1800)	b	M	4	1.3(1).3	7.8	III, IV, V, VII, IX, XXII [*27*]
Asaphidion flavicorne (Solsky, 1874)	m	MH	6	1.2.3.	0.02	X [*5*]
Asaphidion flavipes (Linnaeus, 1760)	m	MH	2,5,10	1.2.3.	0.02	XX [*5*]
Bembidion (Metallina) lampros (Herbst, 1784)	D	M	1,2,10	1.3(1).2	0.08	X, XX [*9*]
Bembidion (Metallina) properans (Stephens, 1828)	D	MH	2,5	1.3(1).2	0.02	XX [*5*]
Bembidion (Ocyturanes) balcanicum Apfelbeck, 1899	n.a.	MH	2	1.3(1).1		
Bembidion (Peryphanes) castaneipenne Jacquelin du Val, 1852	m	H	6	1.3(1).1		

Bembidion (Peryphanes) dalmatinum dalmatinum Dejean, 1831	m	H	2,6	1.3(1).1	0.02	XIV [*5*]
Bembidion (Peryphanes) deletum deletum Audinet-Serville, 1821	m	H	6	1.3(1).1		
Bembidion (Peryphus) femoratum Sturm, 1825	m	MH	2,5	1.3(1).1		
Bembidion (Talanes) subfasciatum Chaudoir, 1850	m	H	7	1.3(1).1		
Brachinus (Brachinus) alexandri F. Battoni, 1984	m	X	1	1.3(1).3		
Brachinus (Brachinus) crepitans (Linnaeus, 1758)	D	MX	1,10	1.3(1).3	2.10	XII, XVII, XIX, XX, XXI [*23*]
Brachinus (Brachinus) psophia Audinet-Serville, 1821	m	MX	1	1.3(1).3		
Brachinus (Brachynidius) brevicollis Motschulsky, 1844	n.a.	MX	3	1.3(1).3	0.02	XX [*5*]
Brachinus (Brachynidius) explodens Duftschmid, 1812	m	MX	1	1.3(1).3	1.63	XII, XVIII, XIX, XX, XXI, XXII [*27*]
Calathus (Calathus) distinguendus Chaudoir, 1846	D	MX	1	1.3(1).2	4.00	IV, V, VIII, XI, XVII, XVIII, XIX, XX [*36*]
Calathus (Calathus) fuscipes fuscipes Goeze, 1777	D	E	9	1.3(1).2	4.00	I, II, IV, V, VIII, IX, X, XI, XII, XVI, XIX, XX [*55*]
Calathus (Calathus) longicollis Motschulsky, 1865	D	MX	1	1.3(1).2	0.03	XII [*5*]
Calathus (Neocalathus) cinctus Motschulsky, 1850	D	MX	1	1.3(1).2	0.05	XIII [*5*]
Calathus (Neocalathus) melanocephalus (Linnaeus, 1758)	D	M	1,2,10	1.3(1).2	0.07	I, VIII, X, XX [*18*]
Calosoma (Calosoma) inquisitor inquisitor (Linnaeus, 1758)	m	MX	3	1.2.2(1)	1.51	II, IV, V, VII, IX, XI, XII, XVI, XVII, XVIII, XXI, XXII [*55*]
Calosoma (Campatita) auropunctatum auropunctatum (Herbst, 1784)	m	MX	1	1.2.2.		
Carabus (Archicarabus) montivagus montivagus Palliardi, 1825	b	M	3,10	1.2.2.	6.20	I, II, V, VII, VIII, IX, X, XI, XII, XIII, XV, XVII, XIX, XXI, XXII [*68*]
Carabus (Carabus) granulatus granulatus Linnaeus, 1758	D	MH	4,6	1.2.2.		
Carabus (Chaetocarabus) intricatus intricatus Linnaeus, 1760	b	MH	4,6	1.2.2.	2.76	III, IV, VI, VII, VIII, IX, XIV, XV, XVI, XXII [*45*]
Carabus (Eucarabus) ulrichii rhilensis Kraatz, 1876	b	M	2,4	1.2.2.		
Carabus (Megodontus) violaceus azuresens Dejean, 1826	b	M	2,4	1.2.2.	0.54	III, IV, VI, VIII, XXII [*23*]
Carabus (Pachystus) hortensis hortensis Linnaeus, 1758	b	M	3,4,10	1.2.2.	3.26	II, III, IV, VI, VII, VIII, IX, XXII [*36*]
Carabus (Procerus) scabrosus scabrosus Olivier, 1790	b	M	4	1.2.2.	0.29	XII, XIV, XV, XVI, XX, XXI [*27*]
Carabus (Procrustes) coriaceus cerisyi Dejean, 1826	b	E	9	1.2.2.	1.66	I, VII, VIII, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX, XXI [*64*]
Carabus (Tachypus) cancellatus Illiger, 1798	b	MH	2,4,6	1.2.2.		
Carabus (Tomocarabus) convexus dilatatus Dejean, 1826	b	MX	1,3,10	1.2.2.	5.63	all without III, VI, XIV, XX [*82*]
Carterus (Carterus) dama (P. Rossi, 1792)	n.a.	X	1,7	2.3.3.		
Chlaenius (Chlaeniellus) nitidulus (Schrank, 1781)	m	H	2,6,10	1.3(1).1	0.03	X [*5*]
Chlaenius (Chlaeniellus) vestitus (Paykull, 1790)	m	H	2,5	1.3(1).1		
*Chlaenius (Dinodes) decipiens (*L. Dufour, 1820)	m	MX	1,7	1.3(1).1	0.13	XIX [*5*]
Chlaenius (Trichochlaenius) aeneocephalus aeneocephalus Dejean, 1826	m	M	1,5	1.3(1).1	0.02	XIX [*5*]
Cicindela (Cicindela) campestris campestris Linnaeus, 1758	m	MX	1	1.2.4.		
Cicindela (Cicindela) sylvicola Dejean, 1822	m	MX	1	1.2.4.		
Cychrus semigranosus balcanicus Hopffgarten, 1881	b	M	4	1.2.2.	0.50	II, III, IV, VI, VII, XXI, XXII [*32*]
Cylindera (Cylindera) germanica germanica (Linnaeus, 1758)	m	M	2,5,7	1.2.4.		
Cymindis (Cymindis) axillaris axillaris (Fabricius, 1794)	D	MX	1,2	1.3(1).3	0.02	XIII [*5*]
Diachromus germanus (Linnaeus, 1758)	m	MH	2	2.2.1.		
Dicheirotrichus (*Trichocellus) discicollis* (Dejean, 1829)	m	MH	6	2.1.1.	0.02	XII [*5*]
Ditomus calydonius calydonius (P. Rossi, 1790)	m	MX	1	2.3.3.		
Dixus obscurus (Dejean, 1825)	m	X	1	2.3.3.	0.02	XIII [*5*]
Dromius (Dromius) quadrimaculatus (Linnaeus, 1758)	m	M	4	1.3(1).5		
Drypta (Drypta) dentata (P. Rossi, 1790)	m	H	2,6	1.1.2.		
Elaphrus (Elaphroterus) aureus aureus P. W. J. Müller, 1821	m	H	6	1.2.3.	0.03	XXII [*5*]
Gynandromorphus etruscus (Quensel en Schönherr, 1806)	m	MX	1	2.2.1.		

Harpalus (Harpalus) affinis (Schrank, 1781)	m	MX	1,9	2.3.1.		
Harpalus (Harpalus) albanicus Reitter, 1900	m	X	1	2.3.1.	0.17	XVIII, XIX, XX [*14*]
Harpalus (Harpalus) angulatus scytha Tschitschérine, 1899	n.a.	X	1	2.3.1.		
Harpalus (Harpalus) atratus Latreille, 1804	D	MX	1,3	2.3.1.	0.10	X, XV, XVII, XXI [*18*]
Harpalus (Harpalus) attenuatus Stephens, 1828	m	MX	1,3	2.3.1.	0.05	XVIII, XIX [*9*]
Harpalus (Harpalus) caspius (Steven, 1806)	m	X	1	2.3.1.	0.32	XVII, XVIII, XIX, XX [*18*]
Harpalus (Harpalus) cupreus fastuosus Faldermann, 1836	m	MX	1,10	2.3.1.		
Harpalus (Harpalus) dimidiatus (P. Rossi, 1790)	m	MX	1,3	2.3.1.		
Harpalus (Harpalus) distinguendus (Duftschmid, 1812)	m	E	9	2.3.1.	0.15	IV, V, X, XIX, XX [*23*]
Harpalus (Harpalus) flavescens (Piller et Mitterpacher, 1783)	m	MX	5	2.3.1(1)		
Harpalus (Harpalus) flavicornis flavicornis Dejean, 1829	D	MX	1,3	2.3.1.	0.47	XIII, XVIII, XIX, XXI [*18*]
Harpalus (Harpalus) honestus (Duftschmid, 1812)	D	MX	1	2.3.1.	0.05	VII, XIX [*9*]
Harpalus (Harpalus) hospes hospes Sturm, 1818	m	X	1,7	2.3.1.	0.74	XIX [*5*]
Harpalus (Harpalus) picipennis (Duftschmid, 1812)	D	MX	1	2.3.1.	0.02	XIX [*5*]
Harpalus (Harpalus) pumilus Sturm, 1818	D	MX	1	2.3.1.		
Harpalus (Harpalus) pygmaeus Dejean, 1829	m	MX	1,3	2.3.1.		
Harpalus (Harpalus) rubripes (Duftschmid, 1812)	m	E	1,9	2.3.1.	0.20	V, X, XII, XVIII, XIX [*23*]
Harpalus (Harpalus) rufipalpis rufipalpis Sturm, 1818	m	MX	1	2.3.1.	0.03	VIII [*5*]
Harpalus (Harpalus) saxicola Dejean, 1829	m	MX	1	2.3.1.		
Harpalus (Harpalus) serripes serripes (Quensel, 1806)	m	MX	1,3	2.3.1.	0.1	XII, XIII [*9*]
Harpalus (Harpalus) smaragdinus (Duftschmid, 1812)	m	X	1,3	2.3.1.		
Harpalus (Harpalus) subcylindricus Dejean, 1829	m	X	9	2.3.1.	0.05	XVIII, XX [*9*]
Harpalus (Harpalus) tardus (Panzer, 1796)	m	E	9	2.3.1.	3.08	V, X, XI, XII, XIII, XIV, XV, XVI, XVIII, XIX, XX, XXI [*55*]
Harpalus (Pseudoophonus) griseus (Panzer, 1796)	m	MX	1	2.2.1.		
Harpalus (Pseudophonus) rufipes (De Geer, 1774)	m	E	9	2.2.1.	0.18	XIX, XX [*9*]
Harpalus (Semiophonus) signaticornis (Duftschmid, 1812)	m	MX	1	2.2.1.	0.07	XIII, XVIII, XIX [*14*]
Laemostenus (Laemostenus) venustus (Dejean, 1828)	m	M	4	1.3(1).6	0.22	IV, VI, IX, XV, XVI, XXI [*27*]
Laemostenus (Pristonychus) cimmerius weiratheri J. Müller, 1932	b	M	8	1.3(1).6	11.25	all without III, XIX [*91*]
Laemostenus (Pristonychus) terricola punctatus (Dejean, 1828)	D	M	8	1.3(1).6	0.12	V, XVI, XVII, XXII [*18*]
Lebia (Lebia) cruxminor cruxminor (Linnaeus, 1758)	m	M	2,10	1.1.3.		
Lebia (Lebia) humeralis Dejean, 1825	m	M	1,10	1.1.3.		
Lebia (Lebia) scapularis scapularis (Geoffroy, 1785)	m	MX	3	1.1.3.		
Leistus (Pogonophorus) rufomarginatus (Duftschmid, 1812)	D	M	4	1.3(1).1	0.32	II, V, VIII, X, XI [*23*]
Licinus (Licinus) cassideus cassideus (Fabricius, 1792)	b	X	1,3	1.3(1).1	0.03	XIX [*5*]
Licinus (Licinus) depressus (Paykull, 1790)	D	M	2,4,10	1.3(1).1		
Limodromus assimilis (Paykull, 1790)	m	MH	4,6,10	1.3(1).2	2.96	IX, X, XI, XVIII, XXII [*23*]
Microlestes corticalis (L. Dufour, 1820)	m	M	1,7	1.3(1).3		
Microlestes fissuralis (Reitter, 1901)	D	M	1,3	1.3(1).3	0.05	XIX [*5*]
Microlestes fulvibasis (Reitter, 1901)	b	M	1,7	1.3(1).3		
Microlestes luctuosus luctuosus Holdhaus, 1904	m	X	1	1.3(1).3	0.03	XIX [*5*]
Microlestes maurus maurus (Sturm, 1827)	D	MX	1,3	1.3(1).3	0.17	XIX [*5*]
Microlestes minutulus (Goeze, 1777)	D	MX	1,3	1.3(1).3	0.17	I, XVIII, XIX [*14*]
Microlestes negrita negrita (Wollaston, 1854)	D	MX	1	1.3(1).3		
Molops (Molops) alpestris kalofericus Mlynář, 1977	b	M	4	1.3(2).1	0.64	III, V, VI, IX, XXI, XXII [*27*]
Molops (Molops) dilatatus angulicollis J. Müller, 1936	b	M	4	1.3(2).1	0.40	III, V, X, XI, XXII [*23*]
Molops (Molops) piceus bulgaricus Mařan, 1938	b	M	4	1.3(2).1	6.42	II, III, IV, V, VI, VII, VIII, IX,

						XIX, XXII [*45*]
Myas (Myas) chalybaeus (Palliardi, 1825)	b	M	3,4	1.3(2).1	4.14	all without I, XII, XIII, XIV [*82*]
Nebria (Nebria) brevicollis brevicollis (Fabricius, 1792)	D	MH	2,4	1.3(1).1	0.10	X [*5*]
Notiophilus aestuans Dejean, 1826	D	M	1,4	1.3(1).1		
Notiophilus biguttatus (Fabricius, 1779)	D	MH	2,4	1.3(1).1	0.17	VII, XI [*9*]
Notiophilus rufipes Curtis, 1829	m	M	3,4	1.3(1).1	1.03	II, III, V, VII, VIII, IX, X, XI, XX, XXI [*45*]
Ophonus (Hesperophonus) azureus (Fabricius, 1775)	D	MX	1	2.2.1.	0.12	XVIII, XIX [*9*]
Ophonus (Hesperophonus) cribricollis (Dejean, 1829)	m	MX	1	2.2.1.		
Ophonus (Metophonus) brevicollis (Audinet-Serville, 1821)	m	MX	1	2.2.1.	0.03	XIX [*5*]
Ophonus (Metophonus) laticollis Mannerheim, 1825	D	MX	1,3,10	2.2.1.	0.59	XX, XXI, XXII [*14*]
Ophonus (Metophonus) parallelus (Dejean, 1829)	m	MX	1,10	2.2.1.	0.02	XIX [*5*]
Ophonus (Ophonus) sabulicola (Panzer, 1796)	m	MX	1	2.2.1.	0.34	XIX, XX [*9*]
Parophonus (Parophonus) laeviceps (Ménétriés, 1832)	m	M	1	2.2.1.		
Parophonus (Parophonus) maculicornis (Duftschmid, 1812)	m	M	2	2.2.1.	0.03	X, XII [*9*]
Parophonus (Parophonus) mendax (P. Rossi, 1790)	m	MH	2,4	2.2.1.	0.02	XIII [*5*]
Pedius inquinatus (Sturm, 1824)	D	MX	1,3	1.3(2).1		
Perileptus (Perileptus) areolatus (Creutzer, 1799)	m	H	5	1.3(1).2		
Philorhizus notatus (Stephens, 1827)	D	MX	1,10	1.3(1).3	0.07	I, X, XIII [*14*]
Platyderus (Platyderus) rufus rufus (Duftschmid, 1812)	b	M	4	1.3(1).2	0.02	V [*5*]
Poecilus (Poecilus) cupreus cupreus (Linnaeus, 1758)	m	E	9	1.3(2).1	0.05	XX [*5*]
Poecilus (Poecilus) cursorius cursorius (Dejean, 1828)	m	MH	2,6	1.3(2).1		
Poecilus (Poecilus) versicolor (Sturm, 1824)	m	M	1,2	1.3(2).1	0.03	I [*5*]
Polystichus connexus (Geoffroy in Fourcroy, 1785)	m	MH	2	1.3(1).3		
Pterostichus (Argutor) vernalis (Panzer, 1796)	D	H	2,6	1.3(1).2	0.02	X [*5*]
Pterostichus (Bothriopterus) oblongopunctatus oblongopunctatus (Fabricius, 1787)	D	MH	4,10	1.3(2).1	6.24	I, II, III, IV, V, VI, VII, IX, X, XI, XXI, XXII [*55*]
Pterostichus (Bothriopterus) quadrifoveolatus Letzner, 1852	m	MH	4,6	1.3(2).1	0.02	XVI [*5*]
Pterostichus (Feronidius) incommodus Schaum, 1858	b	MX	3	1.3(2).1	0.02	XVII [*5*]
Pterostichus (Feronidius) melas depressus (Dejean, 1828)	b	E	9	1.3(2).1	0.76	VIII, XIV, XV, XVI, XVII [*23*]
Pterostichus (Parahaptoderus) vecors (Tschitschérine, 1897)	b	M	4	1.3(2).1	0.03	XXII [*5*]
Pterostichus (Petrophilus) melanarius (Illiger, 1798)	D	E	9	1.3(2).1		
Pterostichus (Phonias) strenuus (Panzer, 1796)	D	MH	2,4,6	1.3(1).2	0.03	X [*5*]
Pterostichus (Platysma) niger niger (Schaller, 1783)	D	MH	2,4,10	1.3(2).1	1.01	III, IV, VII, VIII, X, XIV, XXII [*32*]
Pterostichus (Pseudomaseus) anthracinus anthracinus (Illiger, 1798)	D	H	2,4,6	1.3(2).1		
Pterostichus (Pseudomaseus) nigrita nigrita (Paykull, 1790)	D	MH	2,4,6	1.3(2).1	0.03	XXII [*5*]
Pterostichus (Pterostichus) merklii J. Frivaldszky, 1879	b	M	4	1.3(2).1	0.12	XXII [*5*]
Sphodrus leucophthalmus (Linnaeus,1758)	m	M	8	1.3(2).3		
Stenolophus (Stenolophus) abdominalis persicus Mannerheim, 1844	m	H	5,6	2.1.1.		
Stenolophus (Stenolophus) teutonus (Schrank, 1781)	m	MH	2,5,10	2.1.1.		
Syntomus obscuroguttatus (Duftschmid, 1812)	m	M	1	1.3(1).3	0.03	XX [*5*]
Syntomus pallipes (Dejean, 1825)	D	MX	1	1.3(1).3	0.05	XII, XXI [*9*]
Synuchus (Synuchus) vivalis vivalis (Illiger, 1798)	D	M	2,4	1.3(1).2	0.03	X [*5*]
Tachys (Paratachys) bistriatus bistriatus (Duftschmid, 1812)	m	MH	2,6	1.3(1).4		
Tachyta (Tachyta) nana (Gyllenhall, 1810)	m	M	4	1.3(1).5		
Tachyura (Sphaerotachys) hoemorroidalis (Ponza, 1805)	m	H	2,5	1.3(1).1		
Tapinopterus (Tapinopterus) cognatus kalofirensis Mařan, 1933	b	M	4	1.3(2).2	0.49	III, VI, VII, VIII, XXII [*23*]
Trechus (Trechus) crucifer Piochard de la Brûlerie, 1876	m	M	4	1.3(1).2	0.07	II, VII [*9*]

Trechus (Trechus) irenis Csiki, 1912	n.a.	M	4	1.3(1).2	0.02	XXII [5]
Trechus (Trechus) quadristriatus (Schrank, 1781)	m	E	9	1.3(1).2	2.13	I, III, VI, VII, VIII, X, XI, XIV, XVII, XVIII, XIX, XX, XXI, XXII [*64*]
Xenion ignitum (Kraatz, 1875)	b	M	4	1.3(1).4	2.84	II, III, IV, V, VI, VII, IX, XXII [*36*]
Zabrus (Zabrus) tenebrioides (Goeze, 1777)	m	MX	1	2.3.2.		
Zabrus (Pelor) spinipes spinipes (Fabricius, 1798)	b	MX	1	2.3.2.		

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Macroinvertebrate Communities of sub-Mediterranean Intermittent Rivers in Bulgaria: Association with Environmental Parameters and Ecological Status

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Abstract. Intermittent rivers and ephemeral streams drain more than 50% of the land surface on Earth. Yet, their ecology remains insufficiently understood. In Bulgaria, temporary rivers are typically small- or medium-sized rivers (national type R14), flowing in areas with sub-Mediterranean climate. We present the first data focused explicitly on macrozoobenthos from intermittent rivers in four Bulgarian river basins within the drainage of the Aegean Sea. We identified 114 taxa from nine rivers (5÷33 taxa/site), with abundance varying between 61 and 994 ind.m^{-2}. The most common were taxa of Ephemeroptera and Chironomidae, followed by the crab *Potamon ibericum.* There were considerable differences among macroinvertebrates at different sites at taxon level, with similarities among samples increasing when using lower taxonomic resolution. The distinctness of communities was likely a reflection of the high variability in environmental conditions and local human impacts. Redundancy analysis identified key groups for the sites with fast flow (e.g. Ephemeroptera, Trichoptera, Plecoptera, Coleoptera and Diptera Varia, taxa associated with altitude and the higher share of stone substrata). Most of the river sites were classified as having high ecological status according to the Bulgarian legislation. Only the sites in the Vrabcha and Dereorman Rivers were with moderate status; these were the sites with the lowest taxon richness. We could speculate that the structuring of the benthic community was affected by other factors that have not been accounted for in the present study, i.e. great annual fluctuations in river flow, characteristic for R14, or by loading with nutrients or other pollutants.

Key words: benthic invertebrates, temporary rivers, environmental factors, ecological status, Southern Bulgaria.

Introduction

Intermittent or temporary rivers and streams have highly variable flow that periodically ceases along parts of their courses (Matthews, 1988; Uys & O'Keeffe, 1997). Worldwide, together with ephemeral streams, intermittent rivers drain more than half of the land surface on our planet (Datry et al., 2014). Nonetheless, their ecology is not sufficiently studied.

In Bulgaria, intermittent rivers are common in areas with sub-Mediterranean climate (Isheva & Ivanov, 2016; Wolfram et al., 2016). These are small- or medium-sized rivers, classified as R14 under the national river typology (Cheshmedjiev et al., 2013). Bulgarian intermittent rivers belong to the four Bulgarian river basins (of Struma, Arda, Maritsa and Tundzha Rivers) within the drainage of the Aegean Sea.

Wolfram et al. (2016) identified two subtypes within R14 national river type: R14a with low slope and altitude, with low to medium flow velocity and R14b with high slope and maximum altitude in the catchment area, with medium high flow velocity. The authors verified and refined the Bulgarian river typology, as well as the class boundaries for the ecological status of R14 based on an adapted version of the Biotic index (as described in Cheshmedjiev & Varadinova, 2013), other biological quality elements and environmental factors. Wolfram et al. (2016) did not provide any details on the benthic invertebrate communities inhabiting rivers of R14 national river type.

This is the first faunistic study to focus explicitly on macrozoobenthos of intermittent rivers in Bulgaria. Our aims were to: (i) analyse the basic environmental parameters (i.e. altitude, water temperature, conductivity, pH, oxygen content and saturation, substrata), (ii) study the benthic invertebrate communities (their abundance, diversity and dominant taxa at both lowest possible taxon level (TL) and at major-groups level (GL), (iii) test for associations between environmental factors and macroinvertebrate communities (at GL) and (iv) to assess their ecological status based on benthic macroinvertebrates (at TL).

Material and Methods

Study area

Nine sites representative of intermittent rivers in Bulgaria were selected and sampled once in June 2020 (Fig. 1, Table 1). The selection criteria were defined for the purposes of a bigger project (see Acknowledgements), aiming at developing a new classification system for assessment of the ecological status, based on the biological quality element macrozoobenthos. Thus, the selected river sections represented various ecological situations: from relatively unaffected to anthropogenically impacted (as identified by East Aegean RBMP, 2016-2021; West Aegean RBMP, 2016-2021). All sampled rivers were situated in Southern Bulgaria and belonged to the basins of the Struma (Ludata and Vrabcha Rivers), Tundzha (Fishera and Dereorman Rivers), Arda (Kodzha Dere, Chataldzhevitsa, Kulidzhinska and Aterinska Rivers) and Maritsa (Luda River) Rivers (Fig. 1).

Sample collection and processing

Bottom habitat structure was described on site: the presence of stone, gravel, sand and silt was recorded. Physical and chemical water parameters, i.e. water temperature (WT), oxygen concentration (DO [$mg.dm^{-3}$]) and saturation (OSat [%]), conductivity (Cond [$\mu S.cm^{-1}$]) and pH, were measured in situ using portable Windaus Labortechnik Package and HANNA multi-parameter instruments. Water samples were processed following ISO 5667-3: 2018.

Macroinvertebrates were collected by wading in the river for ca. 100 m, following an adapted version of the pro-rata multi-habitat procedure described in Cheshmedjiev et al. (2011) and in accordance with BDS EN ISO 10870:2012 and EN 16150:2012 standards. The method was regulated by the national water legislation trough Regulation No H-4 (2012). Invertebrate samples were cleaned from coarse substrata, washed through a sieve (500 μm) on site, transferred into containers with 70% alcohol and transported to the laboratory. Benthic specimens were sorted by systematic groups, enumerated and then identified to the lowest possible level.

Analyses

Pearson correlations and their level of significance among water parameters were identified using R 4.0.3. (R Core Team, 2020) and the PerformanceAnalytics package (Peterson et al., 2014). Principal component analysis (PCA), based on the correlation matrix by centring and standardisation, was used to summarise the major patterns of variation of environmental variables (i.e. altitude, physical and chemical water parameters and substrata).

Log-transformed absolute abundances were used in analyses on macroinvertebrate communities. Cluster analyses were performed using Bray-Curtis similarities for taxa (TL) and major groups (GL) with PRIMER-E Vers. 6. Redundancy analysis (RDA) explored associations among the correlation matrices of 16 macroinvertebrate groups and seven environmental parameters. Parameters identified by RDA to have the lowest contribution on explaining associations among environmental factors and communities were excluded from further statistical analyses. Ordinations (PCA and RDA) were performed using CANOCO Vers. 5 statistical package for Windows.

The ecological status of the studied river sections was assessed using the Biotic index (BI) and the Total number of taxa (TNT) according to a river-type specific scale developed in the national legislation (Regulation No H-4, 2012).

Results

Environmental factors

The study sites were situated between 50 and 582 m a.s.l. (Table 1). The lowest temperature was recorded at the site in the Ludata River (13.7°C at 582 m a.s.l.). The highest value of conductivity was measured at the site in the Dereorman River (1332 $\mu S.cm^{-1}$), followed by the Fishera River (669 $\mu S.cm^{-1}$). Pearson correlations identified a significant association ($p=0.032$) between pH and conductivity and an association bordering on significance between pH and altitude ($p=0.077$; Fig. 2).

According to the results of the PCA, the first three principal components (PCs) explained 81.91% of the total variance in environmental data (eigenvalues $\lambda1 = 0.362$; $\lambda2 = 0.321$; $\lambda3 = 0.137$). Axis 1 represented two environmental gradients: the first was related to conductivity and silt; this gradient separated the Dereorman River with the highest values of these parameters. The second gradient of PC1 was associated with water temperature and the presence of sand and separated the Vrabcha and Luda Rivers from the rest of the river sites (Fig. 3). Axis 2 was also related to two gradients: the first was associated with altitude and stone substratum that separated the Ludata, Kodzha Dere and Aterinska Rivers. The second gradient of PC2 was related to oxygen concentration (used also as a proxy for oxygen saturation as identified by the PCA). The highest values of dissolved oxygen were measured in Fishera (its position on the biplot was associated also with the higher conductivity) and Luda Rivers. Chataldzhevitsa River was separated as the site with the highest pH and an altitude of 406 m a.s.l.

Biota: diversity and dominant taxa

We identified 114 taxa of 53 families and 16 systematic groups of bottom invertebrates in the studied R14 rivers (Table 2).

The number of taxa varied between five (Dereorman River) and 33 (Kulidzhinska River) taxa per site (Table 2), while the absolute abundance varied between 61 (Vrabcha River) and 994 (Kulidzhinska River) $ind.m^{-2}$ (Fig. 4). The most common species were the mayflies *B. rhodani* (recorded at six of the nine sites), *C. macrura*, *E. ignita*, *E.* (*E.*) *dispar* and the chironomid *Cr. sylvestris*-gr. (all recorded at five sites), followed by the crab *P. ibericum* and the chironomid *Conchapelopia* sp. (recorded at four of the sites). The most abundant was *Gammarus* sp. with absolute abundance of 550 $ind.m^{-2}$ (or relative abundance of 96.32%), recorded from the Dereorman River. The family Simuliidae (Insecta: Diptera) was recorded at six of the sites.

Table 1. Rivers with sampling sites, codes and environmental characteristics.

N	River	Site	ID	**Altitude** Alt [m a.s.l.]	**Tempe-rature** WT [°C]	**Conduc-tivity** Cond [µS.cm^{-1}]	**pH**	**Dissolved oxygen** DO [mg.dm^{-3}]	**Oxygen saturation** OSat [%]	**Substrata presence** Stone	Gravel	Sand	Silt
1	Ludata	Yanovski bridge after Senokos site	Luda_YS	582	13.7	443	8.15	7.3	81	x	x	x	
2	Vrabcha	Upstream of delta, E79 road before Strumyani Town	Vrab_D	129	18.9	442	8.06	7.3	85		x	x	
3	Fishera	500 m upstream the border with Turkey	Fish_B	104	15.6	669	7.66	8.6	89	x	x	x	x
4	Dere-orman	Delta, bridge on Yambol - Elhovo road	Der_D	109	15.4	1332	7.71	7.4	74	x	x		x
5	Luda	Before confluence in Byala Rver	Luda_C	50	18.3	170	8.01	8.3	74		x	x	
6	Kodzha Dere	Malko Kamenyane Village	Kod_MK	284	17.2	358	8.07	7.2	87	x		x	
7	Chatal-dzhevitsa	Delta under dam of Borovitsa Reservoir	Chat_D	406	16.9	94	8.67	7.3	81	x	x	x	
8	Kuli-dzhinska	Bryagovets Village, 800 m from Ivailovgrad Reservoir	Kuli_D	139	18.1	476	8.02	7.8	86	x	x	x	
9	Aterinska	Bridge between Ivailovgrad Town and Svirachi Village	Ater_IS	180	14.2	406	7.96	7.3	71	x	x	x	x

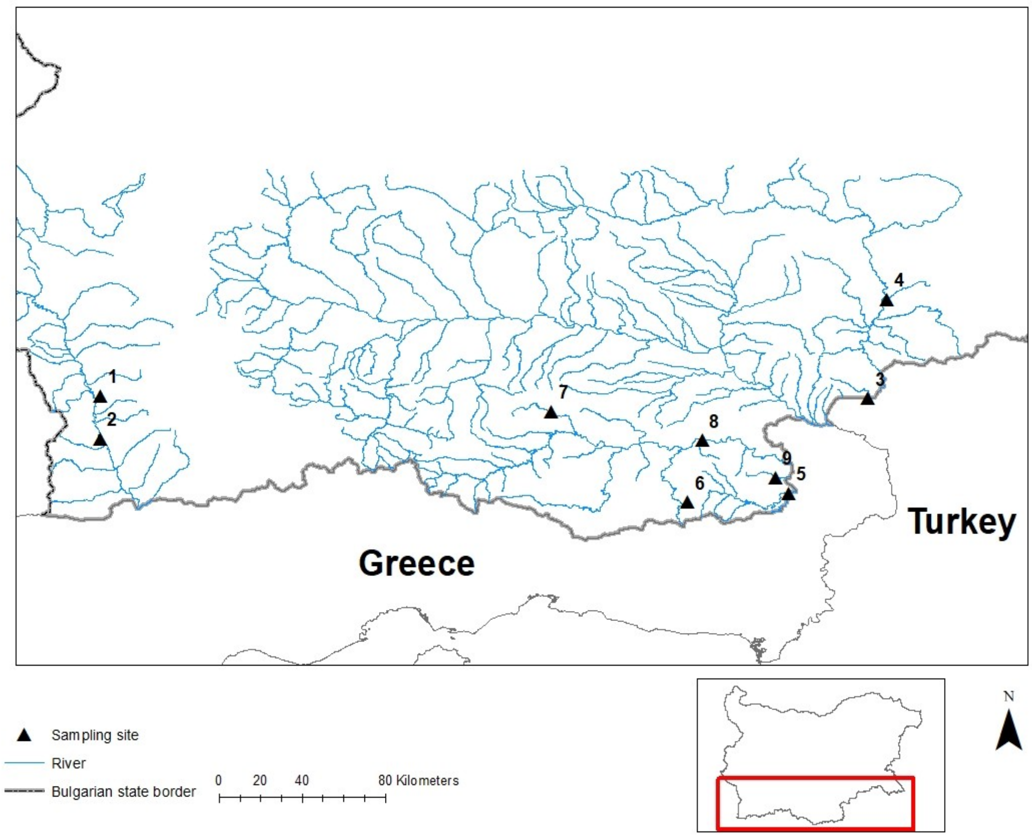

Fig. 1. Map of Bulgaria with sampling area and sites. See Table 1 for site numbers.

Table 2. List of taxa recorded at each of the sites. Site numbers are as in Table 1.

Group	Taxa	Sites								
		1	2	3	4	5	6	7	8	9
Oligochaeta	Oligochaeta indet.									x
	Lumbricidae Gen. sp.		x			x			x	
Hirudinea	*Erpobdella octoculata* (Linnaeus, 1758)	x	x							
Hydracarina	*Lebertia inaequalis* (Koch, 1837)						x	x		x
Gastropoda	*Belgrandiella* sp.								x	
	Radix labiata (Rossmässler, 1835)			x		x				x
	Radix sp.	x								
	Physella acuta (Draparnaud, 1805)		x							
	Planorbis planorbis (Linnaeus, 1758)			x						
	Ancylus fluviatilis O. F. Müller, 1774		x						x	x
Amphipoda	*Gammarus* sp.				x		x		x	x
Isopoda	*Asellus aquaticus* (Linnaeus, 1758)			x						
Decapoda	*Potamon ibericum* (Bieberstein, 1809)			x			x		x	x
Ephemeroptera	*Acentrella sinaica* Bogoescu, 1931					x				
	Alainites muticus (Linnaeus, 1758)								x	
	Baetis rhodani (Pictet, 1843)	x	x				x	x	x	x
	B. fuscatus (Linnaeus, 1761)					x			x	
	B. vernus Curtis, 1834	x								
	B. melanonyx (Pictet, 1843)	x								
	B. scambus Eaton, 1870							x		
	B. subalpinus Bengtsson, 1917		x							
	B. vardarensis Ikonomov, 1962								x	
	Procloeon bifidum (Bengtsson, 1912)					x				
	Cloeon simile Eaton, 1870			x						
	Caenis macrura Stephens, 1835	x	x	x			x	x		
	Oligoneuriella rhenana (Imhoff, 1852)					x	x		x	
	Ephemera danica Müller, 1764	x								
	Ephemerella ignita (Poda, 1761)					x	x	x	x	x
	Ecdyonurus (*Ecdyonurus*) *dispar* (Curtis, 1834)					x	x	x	x	x
	Ecdyonurus (*E.*) *insignis* (Eaton, 1870)							x		
	Ecdyonurus (*Helvetoraeticus*) sp.	x								x
	Electrogena affinis (Eaton, 1887)							x		x
	E. quadrilineata (Landa, 1969)						x			
	Rhithrogena bulgarica Braasch, Soldan & Sowa, 1985	x						x	x	
	Siphlonurus aestivalis (Eaton, 1903)									x
	Habrophlebia eldae Jacob & Sartori, 1984								x	

	Habrophlebia sp.						x			
	Habroleptoides sp.							x		
Plecoptera	*Leuctra* sp.	x							x	
	Nemoura sp.	x								
	Isoperla grammatica (Poda, 1761)	x							x	x
	Perla sp.							x		
Odonata	*Anax imperator* Leach, 1815	x						x		
	Calopteryx splendens (Harris, 1782)			x						
	Calopteryx sp.			x						
	Cordulegaster sp.			x						
	Gomphus sp.	x								
	Ophiogomphus sp.		x							
	Onychogomphus sp.							x		
Trichoptera	*Agapetus orchipes* Curtis, 1834									x
	Agapetus sp.								x	
	Cyrnus trimaculatus (Curtis, 1834)								x	
	Plectrocnemia conspersa conspersa (Curtis, 1834)							x		
	Polycentropus ierapetra Malicky, 1972						x			
	Hydropsyche angustipennis (Curtis, 1834)									x
	H. bulbifera McLachlan, 1878	x							x	
	H. incognita Pitsch, 1993								x	
	H. gr. *instabilis*							x		
	H. gr. *incognita*							x		
	Hydropsyche sp.	x							x	
	Hydropsychidae Gen. sp.									x
	Sericostoma sp.	x								x
	Rhyacophila sp.	x				x			x	
	Potamophylax sp.	x								
	Halesus digitatus (Schrank, 1781)	x						x		
	H. tesselatus (Rambur, 1842)						x			
	Limnephilidae Gen. sp.	x								
	Wormaldia sp.							x		
Megaloptera	*Sialis lutaria* (Linnaeus, 1758)			x						
Coleoptera	Dytiscidae Gen. sp.	x		x		x				
	Platambus maculatus (Linnaeus, 1758)								x	
	Elmidae Gen. sp.	x					x			
	Limnius volckmari (Panzer, 1793)					x			x	
	Hydraena sp.								x	
	Limnichidae Gen. sp.					x			x	
Hemiptera	Gerridae Gen. sp.			x	x					x

	Microvelia sp.	x								
	Veliidae Gen. sp.				x					x
	Mesovelia sp.									x
	Nepa cinerea Linnaeus, 1758					x				
	Ablabesmyia sp.			x			x			
Diptera (Chironomidae)	*Aspectrotanypus* sp.		x							
	Cladotanytarsus sp.			x						
	Chironomus sp.				x					
	Conchapelopia sp.		x	x					x	x
	Cricotopus sylvestris-Gr.			x		x	x		x	x
	Cricotopus (*Isocladius*) sp.								x	
	Cricotopus sp.						x			
	Dicrotendipes sp.			x						
	Kiefferulus tendipediformis (Goetghebuer, 1921)			x						
	Macropelopia sp.	x								
	Microtendipes sp.			x		x	x			
	Micropsectra sp.	x								
	Orthocladius sp.			x						
	Paramerina sp.			x						
	Paratanytarsus sp.			x						
	Paratrissocladius sp.							x		
	Procladius sp.			x						
	Polypedilum convictum (Walker, 1856)		x						x	x
	Polypedilum sp.		x	x			x			
	Prodiamesa olivacea (Meigen, 1818)		x		x					
	Psectrocladius sp.			x						
	Tanytarsus sp.			x						
	Brillia sp.	x	x							x
	Simuliidae Gen. sp.	x				x	x	x	x	x
Diptera varia	*Atherix* sp.						x		x	x
	Tipula sp.	x	x							
	Dicranota sp.	x								x
	Limoniidae Gen. sp.							x	x	x
	Hexatoma sp.					x				
	Tabanus sp.	x						x		
	Empididae Gen. sp.			x			x			x
	Psychodidae Gen. sp.									x
	Ceratopogonidae Gen. sp.		x				x			

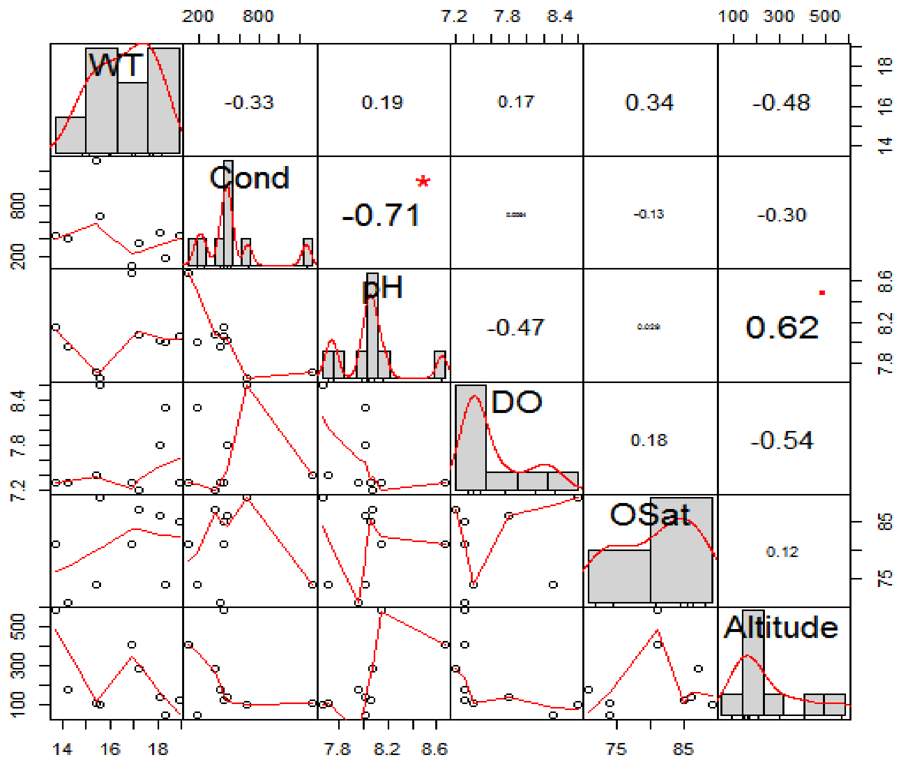

Fig. 2. Pearson correlations among environmental parameters. Asterisk (*) denotes statistically significant correlations. Parameter abbreviations are according to Table 1.

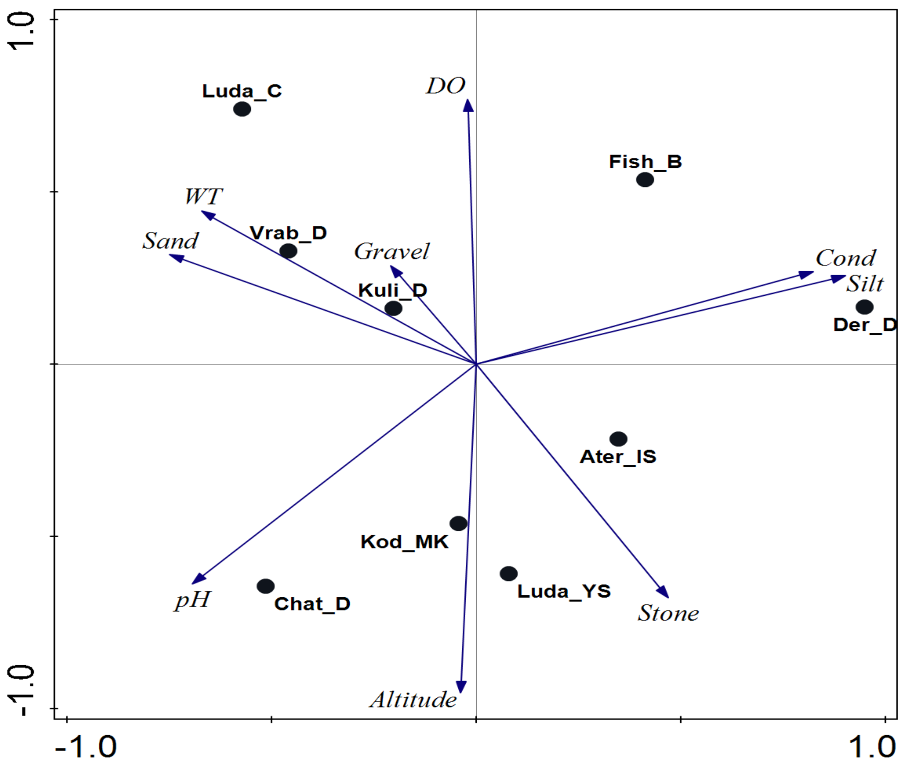

Fig. 3. Ordination biplot diagram based on principal component analysis of altitude, water parameters and four substratum types in the nine river sites (for codes see Table 1).

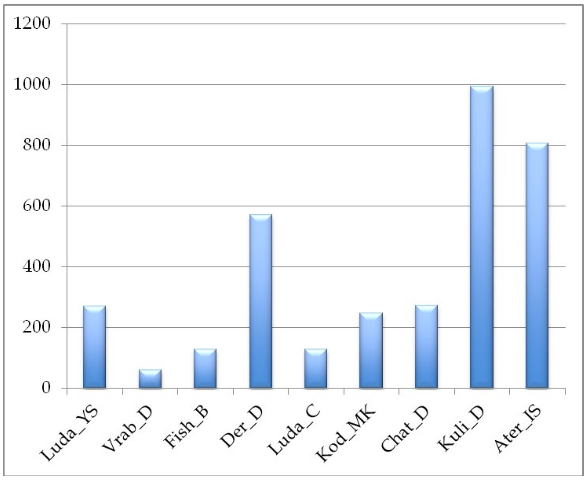

Fig. 4. Absolute abundance of macro-invertebrates at each site (for codes see Table 1).

The major groups included Oligochaeta, Hirudinea, Hydracarina, Gastropoda, Amphipoda, Isopoda, Decapoda, Ephemeroptera, Plecoptera, Odonata, Trichoptera, Megaloptera, Coleoptera, Hemiptera, Chironomidae, Diptera Varia (excluding Chironomidae). The most diverse were the groups of Ephemeroptera (25 taxa), Chironomidae (24 taxa) and Trichoptera (19 taxa). The most frequent were taxa of Chironomidae (recorded at all sites), followed by Ephemeroptera and Diptera varia (at eight sites) and by Trichoptera and Gastropoda (at six sites; Table 2).

Mayflies were dominant or subdominant (based on their abundance) at all but two sites (Fig. 5). Most numerous were *B. rhodani*, *E. ignita* and *E. (E.) dispar*, but due to a variety of microhabitats we also found some rare species, e.g. *A. sinaica*, *Pr. bifidum*, *Electrogena* spp., *H. eldae*, usually occurring in small populations. We recorded six families of caddisflies, of which the most common were Hydropsychidae (*Hydropsyche* spp.) at five sites, Polycentropodidae and Limnephilidae at three. Representatives of four subfamilies of Chironomidae were recorded: Tanypodinae, Orthocladiinae, Prodiamesinae and Chironominae (tribes Tanytarsini and Chironomini). From the sample in the Fishera River, we recorded the highest number of taxa of the family (14), while at the other sites the number of taxa varied between one and six. Snails of four families were recorded at six of the sites (Table 2). The most common were the taxa belonging to Planorbidae (recorded at four sites).

Taxon-level cluster analyses indicated that benthic assemblages were rather variable at the different sites and the similarities among sites were low (max= 44.60%; mean=17.42%; Fig. 6A). The most distinct from the others were the sites in the Dereorman, Fishera and Vrabcha Rivers, while the least different were the Kodzha Dere, Kulidzhinska, Aterinska and Chataldzhevitsa Rivers. Group-level cluster analyses suggested higher similarities among sites (max=75.48%; mean=46.43%; Fig. 6B) and this motivated us to explore the associations between biota and their environment at GL.

Biota: association with environment and ecological status

Three of the parameters (WT, DO and OSat) had similar values among sites and little contribution to explaining the associations among environmental factors and communities (as demonstrated by RDA), therefore they were excluded from further statistical analyses.

According to the RDA ordination of biota and the seven environmental variables retained in the analyses (Fig. 7), 77.3% of the total variance in benthic invertebrate communities was explained by the first three axes (eigenvalues $\lambda1$ = 0.399; $\lambda2$ = 0.273; $\lambda3$ = 0.101). Axis 1 was positively related to conductivity (9.6% explained variation) and silt substratum (29.2% explained variation) and separated the Dereorman River, where most abundant were gammarids of order Amphipoda (96% of the total abundance, Fig. 4), followed by order Hemiptera. Subdominant at the site in the Fishera River was order Hemiptera; that was the only site where the orders Megaloptera and Isopoda were recorded (Figs. 5 and 7). Altitude (9.8% explained variation) and stone substratum (11.3% explained variation) were associated with taxa of orders Ephemeroptera, Trichoptera, Plecoptera, Coleoptera and Diptera Varia (i.e. elmids, simuliids), taxa that were mostly recorded in the Ludata, Kulidzhinska, Aterinska and Kodzha Dere Rivers. The highest abundance of class Gastropoda was found in the Vrabcha River. Taxa of order Odonata were more frequent on gravel (4.9% explained variation) and sand substrata (14.3% explained variation). Shorter gradients related to water mites (Hydracarina) and order Decapoda were evident along PC2 and correlated with the site in the Kodzha Dere River. Altitude and pH explained 11.6% of the total variation in communities and determined the position in the ordination of Chataldzhevitsa River; the lowest absolute and relative abundance of Chironomidae were recorded at the same site.

Our results regarding the ecological assessment based on BI and TNT indicated that all river sites but two were classified as having high ecological status. Both metrics and TNT suggested moderate ecological status at the sites in the Dereorman and Vrabcha Rivers, correspondingly (Fig. 8).

Discussion

Intermittent rivers provide diverse and dynamic conditions that are associated with their intermittence, which in turn shape distinctive invertebrate communities (Datry et al., 2014). Additionally, man-driven climate change or anthropogenic factors such as pollution or modification of riverbed morphology and river flow trigger alterations in the structure and diversity of macrozoobenthos. Our results suggest diverse communities inhabit R14 in Southern Bulgaria, with mayflies predominating in most of them. Cluster analyses indicated considerable differences among macroinvertebrates at different sites at taxon level, with similarities among samples increasing when using group-level resolution. To some extent, important for the grouping of rivers was their affiliation to river basins. In addition, seven of the measured environmental parameters explained more than 3/4 (grouped in gradients along the first three PCs) of the total variation in macrozoobenthic communities.

The most taxon-rich was the site in the Kulidzhinska River, characterised as undisturbed according to the East Aegean RBMP (2016-2021). This was confirmed also by the recorded moderate to low conductivity, high oxygen saturation and its high ecological status (based on both BI and TNT). Relatively taxon-rich were also the Ludata and Aterinska Rivers. Aterinska River was assessed as having high ecological status according to the East Aegean RBMP (2016-2021) and in the present study. In 2009–2011, Stoyanova et al. (2014) studied taxa of Ephemeroptera, Plecoptera and Trichoptera (EPT taxa) at six sites along the Ludata River. The authors also recorded high diversity of these sensitive taxa at all sites but one that was impacted by the toxic compounds in the seepage waters from a mine upstream the site.

The macroinvertebrate communities in the Dereorman, Fishera and Vrabcha Rivers, were the least similar to the rest of the studied rivers. The lowest taxon richness was recorded for the Dereorman River, due to organic pollution from agriculture as recorded also by Borisova et al. (2013). The lowest macroinvertebrate abundance and the second lowest richness at the site in the Vrabcha River we attribute to the higher share of sand substratum, which is associated with lower organic matter and food availability and the resulting low biotic diversity (Leitao et al., 2014; Tolonen et al., 2001).

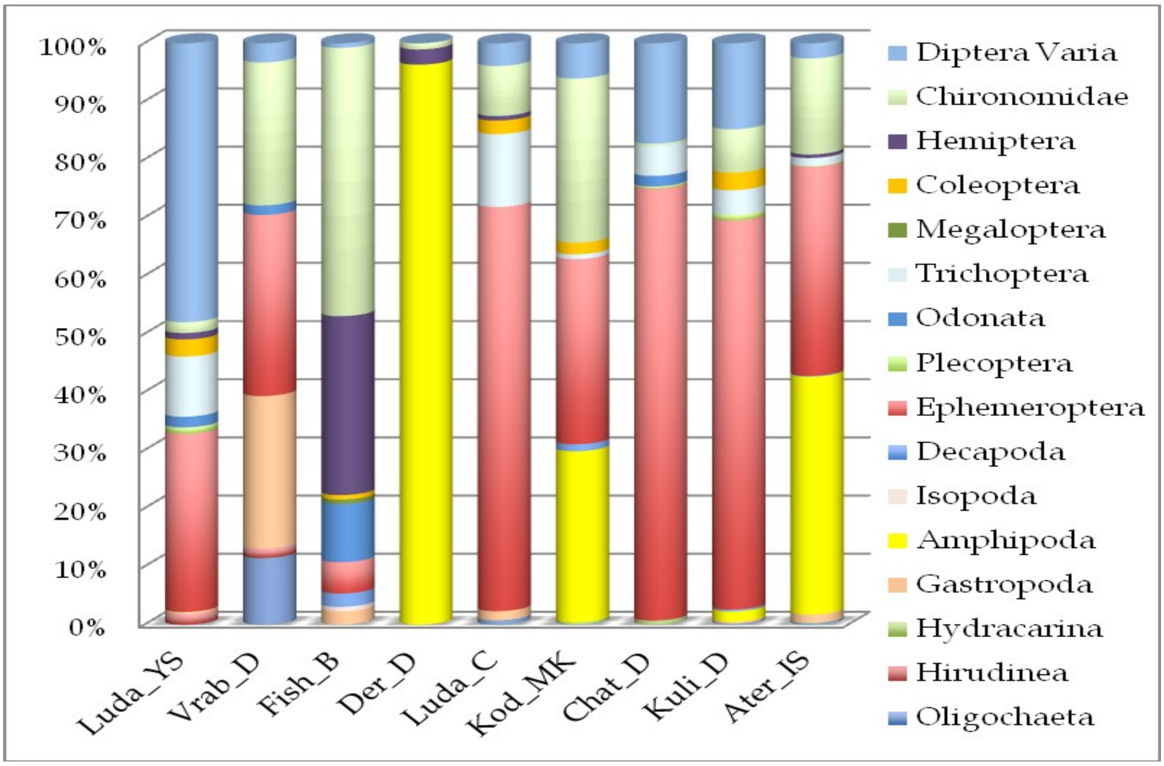

Fig. 5. Relative abundance (%) of the benthic groups at each of the nine sites (for codes see Table 1).

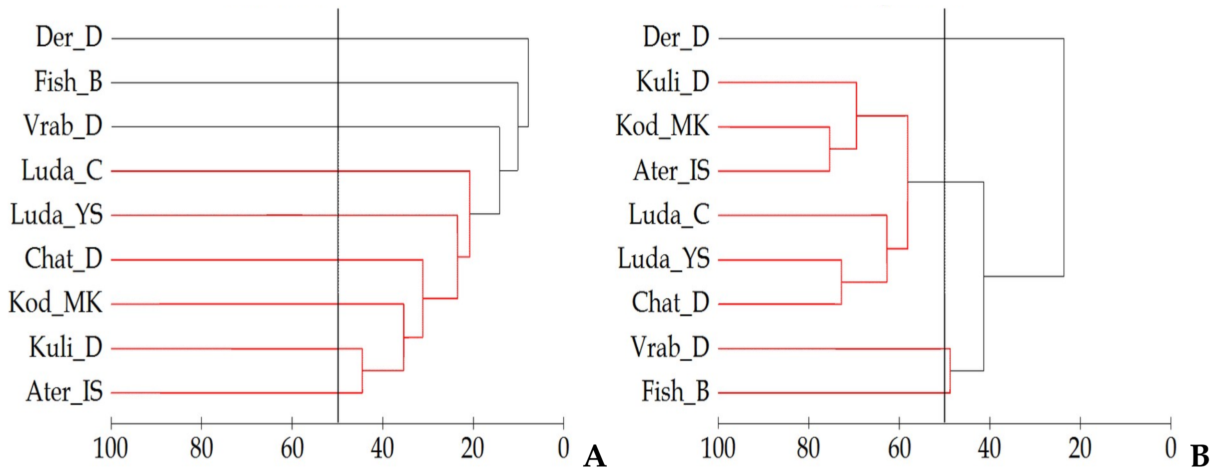

Fig. 6. Bray-Curtis similarity among samples based on macroinvertebrate communities at taxon level (A) and at group level (B). Vertical lines denote 50% of similarity between couples of samples (for codes see Table 1).

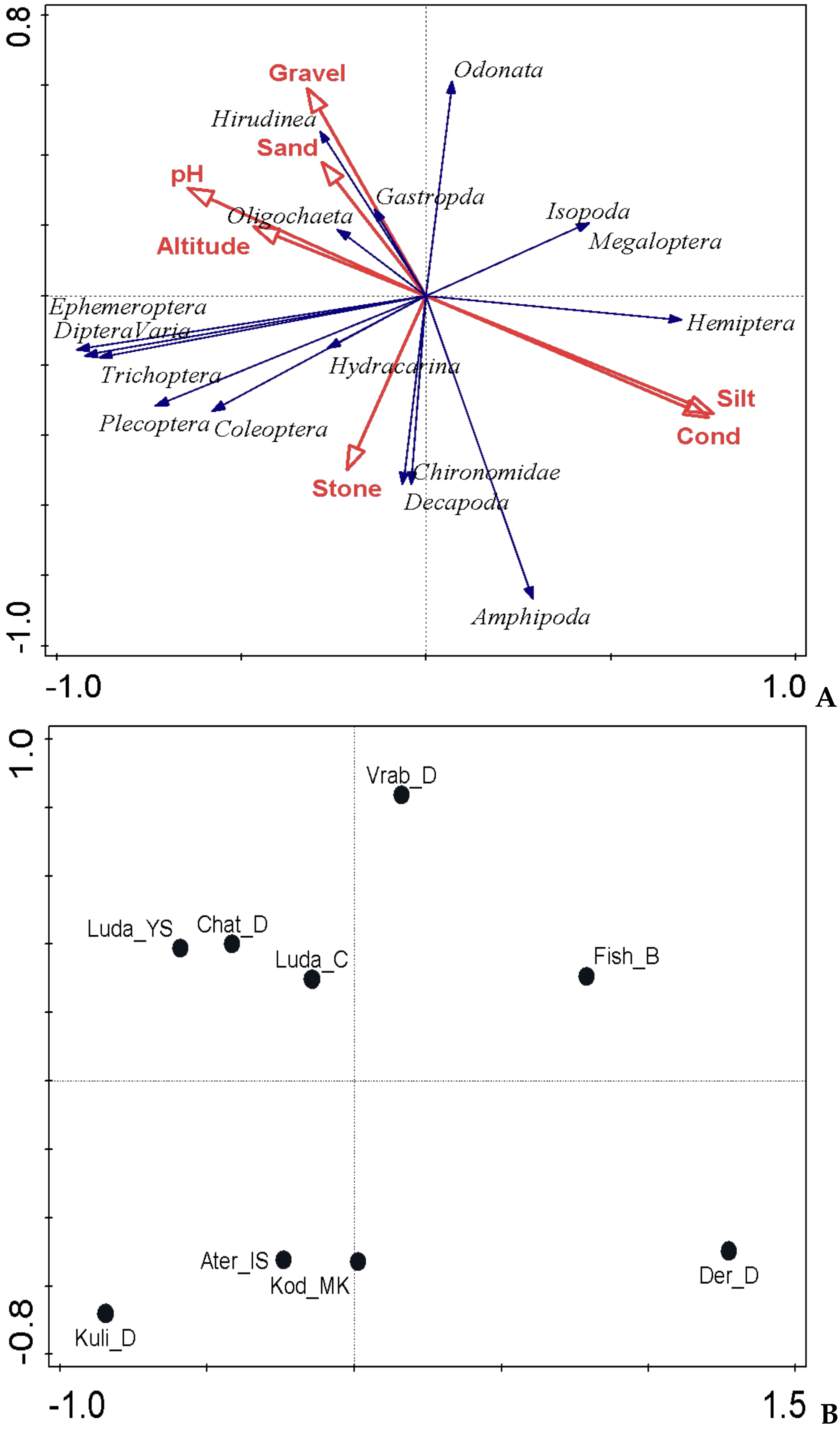

Fig. 7. Redundancy analysis ordinations of environmental variables and macroinvertebrate groups at the studied river sites. (A) associations among the seven environmental variables and the distribution patterns of benthic groups and (B) ordination of river sites (for codes see Table 1).

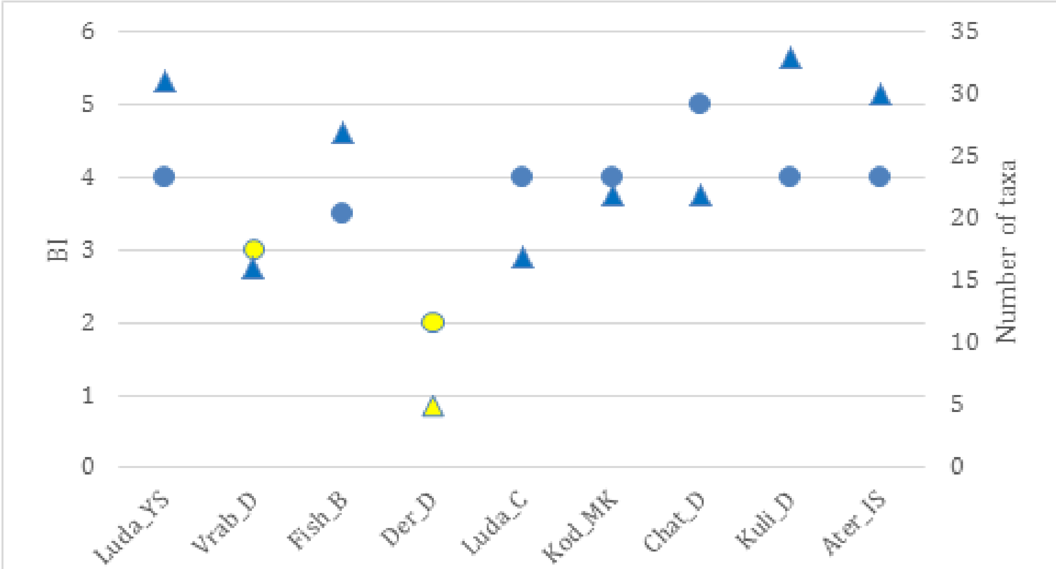

Fig. 8. Ecological status at each of the studied sites. Circles denote values of the Biotic index, triangles - of the Total number of taxa per site, blue - high ecological status and yellow - moderate ecological status (for codes see Table 1).

Another possible explanation could be the local anthropogenic impact these sites are subjected to, as suggested by the recorded high conductivity at the former two sites and their moderate ecological status (East Aegean RBMP, 2016-2021; West Aegean RBMP, 2016-2021). In addition, the site in the Vrabcha River is impacted by the Struma Motorway, the nearby Strumyani Town and a factory for the production of marble, limestone and granite. We recorded 27 taxa at the site in the Fishera River, where various substrata were recorded, thus suggesting a relatively high diversity. However, 14 of the recorded taxa were of the family Chironomidae, unlike the other taxon-rich sites where between one and four taxa of Chironomidae were recorded. A combination among various local impacts at these sites and the availability of suitable microhabitats likely resulted in the lower number of the more sensitive taxa.

Wolfram et al. (2016) identified two subtypes within the R14 national river type: R14a and R14b, as discussed in the Introduction. The presence of families of mayflies, caddisflies and stoneflies along with elmids and simuliids suggest that the undisturbed river sites within the Struma and Arda River Basins (Ludata, Chataldzhevitsa, Kulidzhinska, Aterinska and Kodzha Dere) are fast-flowing and resemble R14b. High similarities among the four rivers from the Arda River Basin were recorded also by Wolfram et al. (2016), where based on macrozoobenthos and other biological quality elements the rivers from the basin of the Arda River were classified as R14b river subtype within the Mediterranean middle-sized R-M2 river type. Moreover, typical of this European river type are Chironomidae, Baetidae, Simuliidae, Heptageniidae, Leuctridae, Elmidae, Ephemerellidae, Hydropsychidae, Gammaridae, Oligochaeta, Hydracarina, Nemouridae, Leptophlebiidae, Limoniidae, Limnephilidae (Feio, 2011). Most of those taxa were recorded also during the current study. On the other hand, Fishera and Dereorman Rivers of the basin of the Tundzha River match the description of R14a (sensu Wolfram et al., 2016).

R-selected chironomids was the only group that was present at all nine sites, owing to their high motility and tolerance to dynamic hydrological conditions (Evtimova

& Donohue, 2016; Langton & Casas, 1999). However, according to Puntí et al. (2009), they are potentially good indicators of ecological status in Mediterranean rivers on the Iberian Peninsula and individual taxa have differing requirements to environmental conditions. For instance, *Micropsectra* gr. are found at river sites with good quality (Marziali et al., 2010), as confirmed also by the records of this genera in the Ludata River at Senokos Village. Thus, it is important to explore the taxonomic structure of family Chironomidae. The other abundant group was Ephemeroptera. Mayflies are sensitive to human disturbances but the common and widespread species of the order are also good colonisers (Brittain, 1982). Overall, insect taxa dominated in terms of diversity and abundance. Their life-cycle strategies ensure thriving in undisturbed intermittent rivers and streams with lower intermittence: change between larval aquatic and aerial adult stages promote survival in relatively natural wet/dry periods, respectively (Datry et al., 2014; Stubbington et al., 2011).

We found a high association between the environmental factors and the benthic invertebrate communities (GL) of Bulgarian intermittent rivers. Our RDA identified key taxa, characteristic of this river type and its two subtypes. The distinctness of communities (at TL) was likely a reflection of the high variability in environmental conditions and local human impacts. Over the last century, among the main disturbances in Medditerranean rivers and streams are alteration of land use, river morphology, water management (Aguiar & Ferreira, 2005; Hooke, 2006). Local impacts could trigger the reduction or even disappearance of sensitive taxa and their replacement by more tolerant ones (e.g. amphipods, isopods, hemipterans and tolerant chironomids). According to our analyses, the taxonomic structure of the studied communities could be explained only partially by the measured environmental parameters. While aquatic invertebrate communities of R14 are type-specific under undisturbed conditions, taxonomic structure is altered when the rivers are affected by various anthropogenic pressures. We could speculate that the taxonomic composition of the studied sites was affected by factors that have not been accounted for in the present study, i.e. the great annual fluctuations in river flow, characteristic for R14, or by loading with nutrients or other pollutants.

Conclusions

Under unaffected conditions of the aquatic environment in the studied intermittent rivers, benthic communities are characterised by high taxonomic richness with a dominant representation of Ephemeroptera, Chironomidae and Trichoptera. The influence of different types of local impacts (agricultural activity, local industry and proximity to a highway) had adverse effects on the integrity of the ecosystem, which impacts the taxonomic richness and ecological status assessed through macrozoobenthos.

Further studies incorporating different seasons, hydrological metrics and organic loading would enable us to better explain the structure of the macrozoobenthos inhabiting intermittent rivers and will bring additional clarity regarding this river type and its representative benthic communities.

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Integrated Assessment of the Status of Fish Fauna Reproduction Areas (Integrated IcRH Index)

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Abstract. Fish fauna reproduction areas (FRA) are natural habitats that ensure sustainable reproduction of fish species populations. In order to preserve them from anthropogenic pressures, it is necessary to monitor the integrity of both biotic and abiotic environmental components. In the monitoring studies carried out in September-November 2020, a pilot approach was applied for integrated assessment of the suitability of streams within the catchments of the Iskar and Vit rivers as FRAs. The studies were performed, adopting the following basic principles for integrated assessment: 1) Applying an ecosystem approach by identifying two main ecosystem components in assessing the status of the FRAs (Biotic Component (1) "Ichthyocenose" (Ic) including 6 criteria for the main biotic metrics; Abiotic Component (2) "Reproduction habitat" (HR) including 5 criteria for the basic hydromorphological and physicochemical quality metrics). 2) Equal weight of the two components in the integrated assessment of the state of the potential FRAs; 3) Application of standardized methodologies and available data in the estimation of the two components and the calculation of the integrated IcRH index. The development of the integrated approach was carried out with data from the river type R 2 Mountain rivers in Ecoregion 12 Pontic province. Its application for assessment to other types of aquatic ecosystems needs to be validated with additional monitoring data. The integrated approach was developed and tested for the river type R 2 Mountain rivers in Ecoregion 12 Pontic province. Its application for other types of surface waters (rivers and lakes) will be further validated with additional data.

Key words: integrated assessment, fish fauna reproduction areas, freshwater protected areas, IcRH Index.

Introduction

FRAs are natural habitats that provide appropriate conditions for the sustainable reproduction of the native fish species. In order to preserve them from anthropogenic pressures, it is necessary to monitor the integrity of both biotic and abiotic environmental components, which provide suitable conditions for reproduction of fish. A detailed literature review on research of the water protected areas (Lowry & Lainsley, 2020) found that the emphasis is on marine protected areas, for which a variety of assessment methods are applied, which is not the case for freshwater protected areas.

A complex of factors, such as: climate change (Orr et al., 2015) human activities

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(Vorosmarty et al., 2010), and non-native species (Rahel & Olden, 2008), can compromise the ability of native fish species to reproduce and / or to realize spawning migrations. In order to implement measures for maintaining, conservation or restoration of aquatic ecosystems, should initially be evaluated their ecological status. The approach for condition assessment of surface water bodies using an index of biological integrity (IBI), based on fish community, was developed (Karr, 1981) and then applied to numerous water bodies, after regional modifications of the multi-metric model. Most of developed indices include a complex of population and coenotic metrics of the fish community, as well as some environmental parameters, but not the conservation status. According to WFD, species should be classified in guilds and therefore "sensitive species" and "intolerant species" are the guilds, which better correspond to the higher conservation status.

Specific conservation zones for fish have been established first in marine habitats. In parallel, certain studies have been carried out to develop tools for freshwater biodiversity conservation (Moilanen et al., 2008), and various methods and strategies have been proposed in this area (Suski & Cooke, 2007).

Other studies are focused on the restoration of given spawning habitats, but based on environmental factors only, such as the spawning substrate (Taylor et al., 2019).

In Bulgaria the legislation concerning the protection of water has determined water protection areas transposing requirements of Annex IV of Water Framework Directive 2000/60/EC (Art. 119a, (1), 1-4 of the Bulgarian Water Law, URL 3) According the Approach for defining / updating water protection areas and their environmental objectives (2016) adopted in the period of 2nd Bulgarian River Basin Management Plans (BG RBMPs 2016 - 2021) the achievement of good quality of surface water bodies identifies with areas designated for the protection of economically significant aquatic species.

Currently, this kind of protected areas are mainly delimited based on presence-absence species data and expert knowledge. Environmental parameters and the overall condition of fish communities are in general overlooked by the existing regulations for the purpose, although the biological condition is largely depending on the quality of the physical habitat features, forming the template within which biological communities develop (Southwood, 1977). This defines habitat assessment as the evaluation of the structure of the surrounding physical habitat that influences the quality of the water resource and the condition of the resident aquatic community (Barbour et al., 1996).

The aim of the present study was to develop a multimetric algorithm for the recognition of critical for the fish fauna reproduction river sections, which integrates assessments of both fish community and habitat parameters, in order to update the boundaries of existing areas and designate new ones for protection of fish species according to the Bulgarian Water Law (1999).

Material and Methods

The application of an integrated approach for assessment of biological communities and physical habitats was performed in 23 river monitoring sites, 12 in the Iskar river basin (Fig. 1) and 11 in the Vit river basin (Fig. 2), identified in 10 FRAs according to the second RBMP and 2 new river sections. Most of the monitoring sites are situated on the territory of BG river type R 2 Mountain rivers in Ecoregion 12 and selected for the implementation of the following tasks:

- To ensure validation of the designated areas for the protection of economically significant aquatic species in the RBMP of DBD (2016 - 2021);

- To perform monitoring and status assessment of the selected river sites with fish populations under protection included in the orders of the Ministry of Agriculture, Food and Forestry according art. 3 & art. 30 of BG Law on Fisheries and Aquaculture in the period 2015 - 2020 (2001);
- To propose additional river sections for the designation of FRAs based on the WFD and NATURA 2000 monitoring data and the performed integrated assessment.

The studies were performed, adopting the following basic principles for integrated assessment:

1) The integrated assessment is based on the definition of an ecosystem (Odum, 1971) and corresponds to the ecosystem approach (Shepherd, 2004) identifying two main components in assessing the status of the FRAs:

- Biotic Component (1) "Ichthyocenosis" (Ic) included 6 criteria for the main biological metrics with an emphasis on fish fauna;
- Abiotic Component (2) "Reproduction habitat" (RH) included 5 criteria for the basic hydromorphological and physicochemical quality metrics.

2) The two components have equal weight in the integrated assessment of the status of the FRAs, assuming that the maximum (reference) number of points for High status =100 points divided equally for the two components (Ic_{max} = RH_{max} = 50 points);

3) Application of standardized methodologies and available public data for the estimation of the two components and the calculation of the integrated IcRH index.

Following these principles, 6 criteria for evaluation of the component Ic (Table 1) and 5 criteria for evaluation of the component RH (Table 2) were defined. The classification scale for assessment of the status of FRA was unified with 5 degrees ecological status scale in Annex V of WFD. The maximum number of points for all criteria is determined when the monitoring data confirms reference conditions (without deviation of the natural conditions) or close to them according to the adopted methodologies. The number of points for each criterion is distributed proportionally between the 5 levels as in normalized rating scales.

The calculation of the Index for assessment of ichthyocenosis reproduction habitats (Index IcRH) is performed using the following mathematical relation:

$$\text{Index IcRH} = \sum Ic\ 1 - 6 + \sum RH\ 1 - 5, \text{ value range: } 6 - 100$$

$$\text{EQR IcRH: } (\sum Ic\ 1 - 6 + \sum RH\ 1 - 5) / 100, \text{ value range: } 0{,}06 – 1{,}00$$

Classification scale for assessment of the status of FRA (Index IcRH) is presented in Table 3.

The ratio of the two components in the Index for assessment of ichthyocenosis reproduction habitats determines the Coefficient of ichthyocenosis & reproduction habitats Integrity–CoIn of the FRA:

$$CoIn = \sum Ic\ 1 - 6 / \sum RH\ 1 – 5$$

The coefficient shows the degree of integrity between the ichthyocenosis and its habitat and its value varies in the following range (Table 4).

Fig. 1. Map of the studied FRAs in the Iskar river basin.

Fig. 2. Map of the studied FRAs in the Vit river basin.

Table 1. Status determination of the component Ichthyocenosis (Ic) - weight 50%, max. Points – 50. Legend: [1] - Classification method TsBRI for assessment of the ecological status of BQE fish fauna (Apostolou et al., 2016). [2] - Refers to identified indicator, sensitive and less tolerant fish species according to the Fish Based Index (Mihov, 2010). [3] - Information system for protected areas from ecological network Natura 2000 (2000). [4] - Adopted methods in Regulation № H-4/14.09.2012 for characterization of the surface waters. *State gazette 22, 5.03.2013, last amend. SG 13, 16.02.2021.* (2012). [5] - Refers to identified indicator, sensitive, less tolerant and introduced fish species according to the Fish Based Index (Mihov, 2010). [6] - BG Executive Agency on Fishery and Aquaculture.

Criteria	Number of points on the adapted 5-point scale (unified with the WFD ecological status scale)				
Criterion Ic 1 WFD ecological status of BQE fish fauna[1]	Bad	Poor	Moderate	Good	High
	1	4	8	12	16
[2]**Criterion Ic 2** Age structure of fish species populations	Absence of ichthyofauna	1 age group / single individuals	2 age groups	3 age groups	> 3 age groups
	1	4	8	12	16
[2]**Criterion Ic 3** Presence of protected fish species (Directive 92/43/EEC, Natura 2000, IUCN, Red Book of Europe and Bulgaria, BG Law for the Biological Diversity)	Absence of protected fish species	1 species - reductant protected according 2 documents	1 species - eudominant or 2 species with less presence protected in 2 documents	2 species in eudominants protected in 2 documents or the 2nd species – in 1 document	2 species or more in eudominants and 1 of them – protected in 3 documents
			If only the genus *Salmo sp.* is presented in the zone ER the assessment is carried out on the basis of Criterion Ic 2		
	1	2	3	5	6
[3]**Criterion Ic 4** Conservation status (CS) and fish species populations density (PD)	Absence of protected fish species / no data available	Unfavorable –bad CS / Very rare-available PD	Unfavorable-inadequate CS /Rare PD	Favorable CS & Common PD	All species in FCS & Common PD
	2	3	4	5	6
[4]**Criterion Ic 5** **WFD BQE ecological status** (BQE PB, MP, Minv, PP - if applicable)	Bad	Poor	Moderate	Good	High
	1	2	3	5	6
[5]**Criterion Ic6** **Restocking** carried out during the last 5	>3 times in the last 5 years	3 times in the last 5 years	2 times in the last 5 years	Ones in the last 5 years	No restocking has been carried out in the last 5 years.

years: official data by the competent authorities[6]	-5	-4	-2	-1	0
Status assessment of the component Ichtyocenosis (Ic) / Total number of points	**Bad (min)**	**Poor (max)**	**Moderate (max)**	**Good (max)**	**High (max)**
Ic 1 (points)	**1**	**4**	**8**	**12**	**16**
Ic 2 (points)	**1**	**4**	**8**	**12**	**16**
Ic 3 (points)	**1**	**2**	**3**	**5**	**6**
Ic 4 (points)	**2**	**3**	**4**	**5**	**6**
Ic 5 (points)	**1**	**2**	**3**	**5**	**6**
Ic 6 (points)	**- 5**	**- 4**	**- 2**	**- 1**	**0**
Ic value (∑Ic 1 - 6)	**1 - 9**	**10 - 20**	**21 - 31**	**32 - 42**	**43 - 50**

Table 2. Status determination of the component Reproduction habitat (RH) – weight 50%, max. Points – 50. Legend: [1] - Standard EN 14614: 2020 Water quality - Guidance standard for assessing the hydromorphological features of rivers (2020). [2] - Standard EN 15843 : 2010 Water Quality – Guidance standard on determining the degree of modification of river hydromorphology (2010). [3] - An approach for assessing the impact of migration barriers, riverbed condition and effectiveness of proposed measures in 2[nd] RBMP of EARBD, Final report on public procurement (Vasilev et al., 2017). [4] - Adopted methods in Regulation № H-4 / 14.09.2012 for characterization of the surface waters. *State gazette 22, 5.03.2013, last amend. SG 13, 16.02.2021.* (2012). [5] - Adopted methodology and standards in Regulation for EQS of priority substances and some other pollutants. (2010). *SG 88, 9.11.2010, last amend. and suppl. SG 97, 11.12.2015.* (2010). [6] - In case of "moderate or worse" status according to criterion RH 4 and / or "bad" according to criterion RH 5, an additional analysis is performed for the sources of pollution on the territory of FRA. If the pollution cause poor or bad status of the biological elements, the number of points for the RH component is reduced: when IC is in poor status - RH is reduced by 1/3 (1/3 ∑ RH) and when IC is in bad status - RH is reduced by 1/2 (1/2 ∑ RH) of the total number of points. In case the total value of RH is a decimal number, it is rounded according to the standard mathematical rule.

Criteria	**Number of points on the adapted 5-point scale (unified with the WFD ecological status scale)**				
Criterion RH 1. Hydromorphological status in the monitoring transect[1,2]	Very heavily modified status	Heavily modified status	Moderate modified status	Good status (slight modification changes)	The status is close to the natural
	1	4	8	11	12
Criterion RH 2. Hydromorphological status of the habitat in the FRA (Barbour et all., 1996a)	Bad	Poor	Moderate	Good	The status is close to the natural
	2	4	8	10	12

Criterion RH 3. Impact from existing migration bariers[3]	Very strong impact	Strong impact	Moderate impact	Weak impact	The status is close to the natural
	2	8	14	18	20
Criterion RH 4. WB ecological status in FRA (physicochemical substances and specific pollutants[4]).	Bad / Moderate & Bad Ic	Poor / Moderate & Poor Ic	Moderate/ Unknown & Moderate Ic	Good	High
	0	0	1	2	3
Criterion RH 5. WB chemical status in FRA (Annex V of WFD and Directive 2013/39/EU)[5]	Bad & Bad Ic	Bad & Poor Ic	Unknown / Bad & Moderate Ic	Good / Good or worse Ic	Good / High Ic
	0	0	1	2	3
[6]Status assessment of the component Reproduction habitat (RH) / Total number of points	**Bad (min)**	**Poor (max)**	**Moderate (max)**	**Good (max)**	**High (max)**
RH 1 (points)	**1**	**4**	**8**	**11**	**12**
RH 2 (points)	**2**	**4**	**8**	**10**	**12**
RH 3 (points)	**2**	**8**	**14**	**18**	**20**
RH 4 (points)	**0**	**0**	**1**	**2**	**3**
RH 5 (points)	**0**	**0**	**1**	**2**	**3**
RH value (∑ RH 1 - 5)	**(3)[5] 5 - 8**	**9 - 16**	**17 - 32**	**33 - 43**	**44 - 50**

Table 3. Classification scale for assessment of the status of FRA (Index IcRH).

Classification scale for assessment of the status of FRA Index IcRH	**Status**	**Bad**	**Poor**	**Moderate**	**Good**	**High**
	Ic	**1 (min)**	**20 (max)**	**31 (max)**	**42 (max)**	**50 (max)**
	RH	**3 (min)**	**16 (max)**	**32 (max)**	**43 (max)**	**50 (max)**
	Index IcRH	**4 - 17**	**18 - 36**	**37 - 63**	**64 - 85**	**86 - 100**
	EQR IcRH	**0.04 - 0.17**	**0.18 - 0.36**	**0.37 - 0.63**	**0.64 - 0.85**	**0.86 - 1.00**

Table 4. Range of the values of the coefficient of degree of integrity between the ichthyocenosis and its habitat.

CoIn	**Degree of integrity**	**Measures**
CoIn ≈ 1	The status of ichthyocenosis in FRA corresponds to the habitats capacity for reproduction. In high, good and moderate status of IcRH and CoIn ≈ 1 the habitat has a good to moderate capacity for determination of FRA. In case of poor and bad status of IcRH, the capacity for reproduction of the fish fauna is low or absent and it is not appropriate to determine FRA.	When the status of IcRH is high and good a surveillance monitoring of the abiotic criteria (RH 1 - 5) is planned. In moderate status, obligatory measures are applied for improvement for the indicated abiotic criteria. The recommended monitoring frequency for assessment of the effect of applied measures is 2 times during the 6 years RBMP period.

CoIn > 1	The status of the ichthyocenosis exceeds the capacity of the habitats for its reproduction, which may due to the formation of local fish fauna communities, isolated from each other and disproportionately distributed in the FRA.	Measures for monitoring and if necessary re-stocking are recommended in case of adverse events and destructuring of fish populations, when they could not recover naturally. If it is economically efficient - removal of migration barriers is appropriate.
CoIn < 1	The status of the ichthyocenosis does not reach the capacity of the habitats for reproduction of the fish fauna due to specific factors: poaching, fish diseases, unidentified pressures etc.	Specific measures depending on the identified reasons / pressures for deterioration of fish fauna status.

Results and Discussion

The summarized results from the conducted study in the FRAs in the Iskar and Vit river basins for the two components Ichthyocenosis (Ic) and Habitat for reproduction (RH), the assessment by the IcRH index, as well as the values of the Coefficient of Integrity (CoIn) are presented in Table 5 and Table 6.

In the calculation of the Component Ichthyocenosis (Ic) a corrective effect of Criterion Ic2 Age structure of fish species populations is observed, which partially reduced the value for Criterion Ic1 Assessment of the ecological status of ichthyofauna (Index TsBRI, Apostolou et al., 2016, URL10). This effect is analyzed and verified in the process of validation of the TsBRI method for the other BG surface water types that have not participated in the EU process of intercalibration. The most significant difference is observed in the assessments of Criterion Ic1 Ecological status and Criterion Ic4 Conservation status: 92% FRA are in good and high ecological status and 100% FRA are in less than good conservation status. The reasons for this essential difference are the significantly larger territorial scope of the conservation status assessment and the timeliness of the data used (due to the limited number of national monitoring points for conservation status assessment, they are often absent in the surveyed FRAs). Therefore, when calculating Component Ic, a possibility was ensured to adjust the assessment of the Criterion Ic4 Conservation status in connection with the actual data from monitoring of fish fauna in the studied FRAs. The Criterion Ic6 Re-stocking according to official data is applied only with the species of river trout (*Salmo trutta fario L.*). During the last 5 years, permanent stocking has been carried out in the catchment of the Beli Vit River and the assessments of the fish fauna show a positive effect on its ecological status.

Table 5. Summarized results from the integrated status assessment[1] of Component Ic in FRAs in the Iskar and Vit river basins. Legend: [1] - Status assessment color (unified to WFD ecological status): High Good Moderate Poor Bad

№	FRA names	Ic 1 Ts BRI	Ic 2 Age str-re	Ic 3 Prot. species	Ic 4 Cons. status	Ic 5 BQE status	Ic 6 Resto king	Ic	RH	IcRH	CoIn
1	BG1FSWIS300R019 GABROVNITSA	16	12	5	4	5	0	42	36	78	1.17
2	BG1FSWIS300R1018 ISKRETSKA	12	8	3	4	3	0	30	41	71	0.84
3	BG1FSWIS300R1017	16	16	6	2	5	0	45	43	88	1.05

	BATULIJSKA										
4	BG1FSWIS700R1107 BISTRITSA VITOSHKA	4	4	2	3	3	0	16	19	35	0.84
5	Iskar River after the inflow of Beli Iskar River into Cherni Iskar River	12	16	6	2	5	-1	40	38	78	1.05
6	Beli Iskar River before the inflow into Cherni Iskar River	16	12	6	3	5	-1	41	38	79	1.08
7	BG1FSWIS900R1103 LEVI ISKAR	16	12	6	3	6	0	43	29	72	1.48
8	BG1FSWIS900R1203 CHERNI ISKAR	16	8	6	3	5	0	38	42	80	0.90
9	BG1FSWVT800L1004 SOPOT DAM (Toplya river)	12	16	6	4	1	0	39	42	81	0.92
10	BG1FSWVT900R1001 BELI VIT	16	16	6	4	5	-5	41	28	69	1.48
11	BG1FSWVT900R1101 RIBARITSA	12	4	5	3	5	-2	29	26	55	1.12
12	BG1FSWVT900R1002 CHERNI VIT	12	8	4	4	5	0	33	29	62	1.14

Table 6. Summarized results from the integrated status assessment[1] of the Component RH in FRAs in the Iskar and Vit river basins. Legend: [1] - Status assessment color (unified to WFD ecological status): High Good Moderate Poor Bad

№	FRA names	RH 1 HM status	RH 2 Habitat status	RH 3 River contin.	RH 4 Ecol. status	RH 5 Chem. status	RH	Ic	IcRH	CoIn
1	BG1FSWIS300R019 GABROVNITSA	11	8	12	2	3	36	42	78	1.17
2	BG1FSWIS300R1018 ISKRETSKA	12	9	16	1	3	41	30	71	0.84
3	BG1FSWIS300R1017 BATULIJSKA	12	7	20	1	3	43	45	88	1.05
4	BG1FSWIS700R1107 BISTRITSA VITOSHKA	4	3	8	1	3	19	16	35	0.84
5	Iskar River after the inflow of Beli Iskar River into Cherni Iskar River	12	9	12	2	3	38	40	78	1.05
6	Beli Iskar River before the inflow into Cherni Iskar River	12	9	12	2	3	38	41	79	1.08

7	BG1FSWIS900R1103 LEVI ISKAR	11	7	6	2	3	29	43	72	1.48
8	BG1FSWIS900R1203 CHERNI ISKAR	12	9	16	2	3	42	38	80	0.90
9	BG1FSWVT800L1004 SOPOT DAM (Toplya river)	11	7	20	1	3	42	39	51	0.92
10	BG1FSWVT900R1001 BELI VIT	11	7	6	1	3	28	41	69	1.48
11	BG1FSWVT900R1101 RIBARITSA	10	6	6	1	3	26	29	55	1.12
12	BG1FSWVT900R1002 CHERNI VIT	12	9	4	1	3	29	33	62	1.14

There is a clear correlation between the values of the components Ichthyocenosis (Ic) and Habitat for reproduction (RH) with Criterion RH 3 Impact from existing migration barriers (to the greatest extent) and Criterion RH 2 "Hydromorphological status of the habitat in the FRA". For river type R 2 "Mountain rivers", the impact on river continuity and runoff as a result of established migration barriers is of major importance for the hydromorphological state of biotopes. The fact that this river type is represented by relatively short sections enhances the limiting effect of this impact. Criterion RH 2 is also secondarily affected by the disturbed river continuity, taking into account negative changes in some of the parameters that form it. CoIn > 1 values have usually been found in FRAs with lower Criterion RH 3 values, indicating that, as a result of physically limited migration, fish fauna form local communities.

Total in 10 of the studied FRAs (83%) the values of the integrated index IcRH determine good or higher status (1 FRA – in High status; 7 designated FRAs and 2 additional river sections – in Good status). The status of 1 FRA is moderate (BG1FSWVT900R1101 RIBARITSA) and it was proposed for inclusion in the boundaries of FRA Beli Vit as its tributary and feeding habitat for the young individuals of *Salmo trutta fario L.* with measures to ensure greater runoff and restore the connectivity of the river. The status of 1 FRA is poor (BG1FSWIS700R1107 BISTRITSA VITOSHKA) and it was proposed for exclusion of the list of designated FRAs. The analysis of the hydromorphological conditions in the last two FRAs in moderate and poor status derives from significant fragmentation, disturbed river continuity, runoff regulation, riverbed correction and other local physical changes leading to compromise of the river sections.

The values of the component ichthyocenosis (Ic) in most cases corresponds to and exceeds the capacity of the habitats for its reproduction (values of RH), as in 42% of the zones this is happening due to the formation of local fish communities, isolated from each other and disproportionately distributed in the FRA.

The achieved results ensured the validation of the designated areas for protection of economically significant aquatic species in the RBMP of DBD (2016 - 2021). Determination of a common FRA including all defined FRAs in the RBMP together with two new river sections have been identified as a measure necessary to protect fish species populations in the upper part of the Iskar river basin. Their hydraulic connectivity, common river type (R 2 Mountain rivers) and the established good integrated status (IcRH) ensure appropriate conditions for successful reproduction of the fish fauna in a large river area for which protection is required. The absence of fish species populations in the designated FRA of the Vitoshka Bistritsa River before the inflow into Iskar River caused by significant hydromorphological pressure from migration barriers is the reason for determination of a poor integrated status and exclusion of this river section as a protected area.

In some tributaries of Beli Vit River a disturbed river connectivity and significantly reduced runoff because of the pressure from water abstraction was established (mouth of the Stara Ribaritsa River) which determine moderate IcRH status. During the study it was found that these river sections are successfully used by young individuals of the species Salmo trutta fario L. as a habitat for feeding and protection, although they develop in small and isolated populations. This fact led to the conclusion that the disturbed river habitats also have to be included in the boundaries of the FRA when they provide appropriate conditions for young individuals of fish populations until they reach breeding age.

For all river sections where the assessment of the RH component found deviations from the good status, measures to improve the river connectivity and the condition of the hydromorphological elements were determined. The integrated assessment has allowed these measures to focus on improving the condition of fish fauna where deviations have been identified or are expected, as well as to make changes to the monitoring carried out to provide reliable data.

The results from the integrated assessment (Index IcRH) were summarized in the information passports, containing specific data for the surveyed FRAs and measures necessary to be taken to protect them.

Conclusion

Based on the definition of the ecosystem (Odum, 1971) as a main functional unit in the ecology, the presented index for integrated assessment of the fish faun reproduction areas (FRA) apply principles of the Ecosystem approach (Shepherd, 2004) introducing a mechanism for monitoring and conservation of the ecosystem structure and functioning. Integrating the assessments of WFD status of biological and abiotic quality elements in aquatic ecosystems (Annex V, WFD) with the assessments of the conservation status of fish fauna (Directive 92/43/EEC, Natura 2000, etc.) the IcHR index uses a wide range of criteria (11) related to 2 main components: (1)Biotic Component „Ichthyocenosis" (Ic) including 6 criteria with emphasis on fish fauna; (2) Abiotic Component "Reproduction habitat" (RH) with 5 criteria, including basic hydromorphological and physicochemical quality metrics. Application of standardized methodologies and available public data for the calculation of the integrated IcRH index and determination of the status of the FRAs ensure representativeness and confidence of the obtained results. The integrated index is developed and applied with data from the river type R 2 Mountain rivers in Ecoregion 12 Pontic province and it needs to be further validated with additional monitoring data for application to other types of aquatic ecosystems.

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Microsatellite Markers Reveal Genetic Diversity among Honey Bee Populations from Some Balkan Peninsula Regions and Distinctive Characteristics of the Local for Bulgaria Apis mellifera rodopica

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Abstract. The genetic polymorphism in honey bee populations from some Balkan peninsula countries was investigated using microsatellite DNA analysis. The local for Bulgaria *A. m. rodopica,* Petrov, 1991 population was compared with *A. m. macedonica* and *A. m. carnica* populations, originating from Greece and Serbia, respectively. In total, 88, 64 and 58 alleles were found for the nine microsatellite loci in the gene pool of the studied Bulgarian, Greek and Serbian populations. Genetic parameters and relationships between the investigated honey bee populations from some Balkan peninsula regions were analyzed. Twenty private alleles were detected for the population of the local Bulgarian *A. m. rodopica*, eight – for the Greek population of *A. m. macedonica* and 11 – for the *A. m. carnica* population from Serbia. Clear diagnostic markers, appropriate for distinguishing the local Bulgarian honey bee were found and described. It was concluded that together with the other molecular, biochemical, morphological and ethological indicators, described earlier, they could be taken into consideration when conduct activities for the conservation of the local Bulgarian honey bee *A. m. rodopica*.

Key words: *A. m. rodopica,* microsatellites, polymorphism, genetic distinction.

Introduction

The conservation of genetic polymorphism and the gene pool of local populations is a priority goal of many biological studies. In recent years, different activities have been focused on the relationship between genetic variability (Meixner et al., 2013; Rahimi et al., 2019) and the vitality of honey bees (Francis et al., 2014) and are related to proving the hypothesis that the health of the bee colonies depends on their genotypes and adaptation to the conditions of the particular environment, which includes not only climate and vegetation, but also prevalent diseases, pesticide pollution and breeding activities (Meixner et al., 2014).

In this aspect, for the period 2008 – 2014, different scientific teams developed and published standardized methods for genetic and breeding analysis (Costa et al., 2012; Meixner et al., 2014), investigated and analyzed comparatively populations with various European origins of *Apis mellifera*, studied their genetic characteristics and selectively significant productive and behavioral traits (Büchler et al., 2014; Hatjina et al., 2014a, b; Uzunov et al., 2014a).

The local Bulgarian honey bee show high queen fertility, high honey productivity in local conditions, good wintering ability, low defensive behavior, good hygienic behavior (Ivanova, 2018). These valuable qualities are confirmed by comparative studies of European populations of *A. mellifera*. The results obtained show that the local Bulgarian honey bee has the highest comparative survival rates (Büchler et al., 2014; Hatjina et al., 2014b; Meixner et al., 2014b), low swarming tendency, high level of gentleness and highest level of hygiene behavior (Büchler et al., 2014; Uzunov et al., 2014a). Previous relative molecular-genetic studies show that the local Bulgarian honey bee belongs to the *A. m. macedonica* subspecies, but can be clearly distinguished from the populations of this subspecies that inhabit the territories of the Republics of Northern Macedonia and Greece (Francis et al., 2014; Uzunov et al., 2014b).

At present, the National program for breeding of *A. m. rodopica* is applied in Bulgaria and a law on beekeeping is in force, which prohibits the import and breeding of honey bees with foreign origin (Petrov & Ganev, 2013). Despite this fact, many beekeepers, for commercial reasons, disrupt the law and carry out uncontrolled imports of honey bee queens with foreign origin (mainly *A. m. carnica*, but also some hybrids), which affects the composition of the gene pool of the local populations. All bases of the National Bee Breeding Association, as well as the established gene bank in Bulgaria, store, protect and distribute the local honey bee *A. m. rodopica*.

Having in mind the valuable characteristics of the local Bulgaria honey bee, a system of genetic markers (enzymatic, DNA microsatellite and mitochondrial) was presented by Ivanova (2018). These markers, in concert with morphometric and ethological indicators, could be used for distinguishing the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991 from the other origins of *A. m. macedonica*, as well as from the populations of other *A. mellifera* subspecies distributed in Europe.

The present study aims to update the genetic marker system for distinguishing the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991 in accordance with its valuable biological and productive characteristics.

Materials and Methods

Worker bees (N=330) from *A. m. rodopica*, *A. m. macedonica* and *A. m. carnica* populations were sampled for this study. The honey bees from the Agricultural University – Plovdiv and the gene bank of National Bee Breeding Association in Sliven belong to the local *A. m. rodopica*, used in Bulgaria as a basis for a National bee breeding program (Petrov & Ganev, 2013). Populations of *A. m. macedonica,* originating from Greece and A. *m. carnica*, originating from Serbia were also included in the study and compared to the local Bulgarian honey bee.

The collected honey bees were frozen in containers at -20°C and then moved into tubes with absolute alcohol until ready for DNA extraction process. DNA extraction, PCR protocol and microsatellite DNA analysis were done as it was described by Nikolova (2011).

Total DNA was isolated from a single worker bee with prior rinsing in insect buffer for one hour followed by mechanical disruption of the tissue, using NEW Omni TH_Q. DNA yields of all extractions were estimated by DNA spectrophotometry according to the manufacturer's instructions.

PCR yields were estimated by comparison of band intensity to a DNA Mass Ladder on a 2% agarose gel stained with ethidium bromide by blinded reviewers who had not participated in DNA extraction.

PCR amplifications were carried out in 10µL of a mixture containing 5-10 ng of DNA template, 400 nM of each primer, 1.2–1.5 mm MgCl2, 1 X QIAGEN Multiplex PCR reaction buffer and 1 X Q-Solution buffer. After denaturating step of 15 min at 95°C, samples were processed through 30 cycles consisting of 30 sec at 94°C, 90 sec at an optimal annealing temperature and 60 sec at 72°C. The last elongation step was lengthened to 30 min at 72°C. Aliquots of fluorescently labeled amplified DNA were mixed with formamide solution and GENESCAN-400(ROX) Size Standard and genotyped on the ABI 3130 Genetic Analyzer using GeneScan™ Analysis Software.

All honeybee samples were analyzed for nine microsatellite loci: Ac011; A024; A043; A088; Ap226; Ap238; Ap243; Ap249 and Ap256.

Population-genetic analyzes have been computed using GenAlEx v.6.42 (Peakall & Smouse, 2006). GenAlEx assignment test was applied to determine the logarithmic probability and the degree of affiliation of a given genotype to a population studied.

Results

Based on the microsatellite DNA analysis performed, allelic diversity in the composition of the gene pool of the studied populations was characterized. The highest number of alleles (23) was diagnosed at the Ap256 locus and the smallest (8) – at the Ap024 locus.

Table 1 presents information concerning the number of alleles per each microsatellite locus in the studied populations of *A. m. rodopica*, *A. m. macedonica* and *A. m. carnica*.

The results demonstrate that the total numbers of alleles for the studied 9 microsatellite loci in the investigated populations varies between 58 (for *A. m. carnica* from Serbia) and 88 (for the local Bulgarian *A. m. rodopica*), which reveals the presence of significantly greater allelic diversity in the gene pool of the Bulgarian honey bee population compared to that of the other investigated populations from Greece and Serbia (Table 1).

Table 2 contains data concerning the identified private alleles in the studied populations.

A total of 39 private alleles have been identified in the comparative analysis of the studied Balkan peninsula populations as follows (Table 2): 20 – for the local Bulgarian bee *A. m. rodopica* (Petrov, 1991), 8 – for *A. m. macedonica* from Greece, and 11 – for *A. m. carnica* from Serbia).

The comparative analyzes indicate that four alleles of the Ac011 locus are diagnostic: 117 – for *A. m. rodopica*; 118 and 112 – for *A. m. macedonica* from Greece; 109 – for *A. m. carnica* from Serbia. Four alleles of the A024 locus are also diagnostic: 102 and 104 – for *A. m. rodopica*; 97 – for *A. m. macedonica* from Greece, 83 – for *A. m. carnica* from Serbia. The A043 locus is represented by a total of three diagnostic alleles: 123 and 126 for *A. m. rodopica*; 129 – for *A. m. carnica* from Serbia. Three alleles of the A088 locus can also be used as diagnostic: 128 – for *A. m. carnica* from Serbia; 130 – for *A. m. macedonica* from Greece; 142 – for *A. m. rodopica*. With respect to the A226 locus, the allele 240 also appears to be diagnostic for *A. m. macedonica* from Greece.

Locus A238 is represented by a total of five diagnostic alleles: 210, 252, and 260, occurring in populations of *A. m. rodopica*; 244 – found in populations of *A. m. macedonica* from Greece and 250 – in *A. m. carnica* population from Serbia. Concerning A243 locus, seven alleles are diagnostic, 6 of which characterize the local Bulgarian honey bee – these are the alleles 200, 210, 237, 240, 253 and 280. The allele 231 is found to be diagnostic for *A. m. carnica* originating from Serbia.

Table 1. Number of alleles per a locus in the studied populations.

Population	Ac 011	A 024	A 043	A 088	Ap 226	Ap 238	Ap 243	Ap 249	Ap 256	Total
A.m. rodopica, Bulgaria	7	7	7	14	9	10	11	10	13	88
A.m. macedonica, Greece	7	6	4	7	9	8	6	7	10	64
A. m. carnica, Serbia	10	5	5	4	5	9	5	3	12	58

Table 2. Private alleles and their frequencies in the populations studied.

Population	Locus	Allele	Allele frequency
A. m. rodopica, Bulgaria	Ac011	117	0.483
	A024	102	0.077
	A024	104	0.077
	A043	123	0.077
	A043	126	0.063
	A088	142	0.063
	Ap238	210	0.063
	Ap238	252	0.129
	Ap238	260	0.092
	Ap243	200	0.056
	Ap243	210	0.056
	Ap243	237	0.068
	Ap243	240	0.156
	Ap243	253	0.202
	Ap243	280	0.058
	Ap249	227	0.061
	Ap249	260	0.109
	Ap249	290	0.065
	Ap249	300	0.060
	Ap256	245	0.060
A. m. macedonica, Greece	Ac011	118	0.500
	Ac011	112	0.500
	A024	97	0.150
	A088	130	0.500
	Ap226	240	0.430
	A238	244	0.250
	Ap256	168	0.150
	Ap256	208	0.132
A. m. carnica, Serbia	Ac011	109	0.125
	A024	83	0.225
	A043	129	0.100
	A088	128	0.455
	Ap238	250	0.250
	Ap243	231	0.300
	Ap249	225	0.370
	Ap256	195	0.079
	Ap256	202	0.312
	Ap256	212	0.063
	Ap256	217	0.125

The A249 locus is represented by five diagnostic alleles: 227, 260, 290 and 300 – for the local Bulgarian honey bee; 225 – for *A. m. carnica* from Serbia. Locus A256 is presented by seven diagnostic alleles. The alleles 168 and 208 are found in populations of *A. m. macedonica* from Greece. Alleles 195, 202, 212 and 217 are diagnostic for *A. m. carnica* from Serbia and 245 – for the local Bulgarian honey bee.

The average calculated value for the gene flow (*Nm*) is 1.723 (varying between 0.402 for Ap243 to 3.282 for A043) and for the fixation index (*Fst*) is 0.152 (varying between 0.071 for A043 to 0.249 for Ap226).

The differentiation between the studied honey bee populations from Bulgaria, Greece and Serbia is presented in Figure 1 by grouping together and in pairs with the local Bulgarian *A. m. rodopica* based on the GenAlEx assignment test.

Discussion

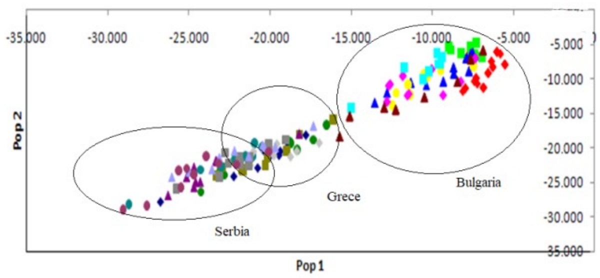
The local Bulgarian honey bee is most adapted to the specific conditions of the country and also characterized by a high level of survival, high fertility and productivity, with a strong hygienic behavior, with a low tendency to swarming and other significant biological features (Büchler et al., 2014; Hatjina et al., 2014a, b; Meixner et al., 2014; Uzunov et al., 2014a). Its gene pool must be preserved through science-based selection and conservation activities in Bulgaria. In this aspect, the microsatellite DNA analysis carried out in this study provides new information on additional diagnostic genetic markers for its discriminating.

A high level of genetic similarity among the studied populations from all over the country based on isoenzyme and microsatellite analyses was found (Georgieva et al., 2016; Ivanova, 2018), which has also demonstrated high consolidation among the selectively controlled and uncontrolled honey bee populations in Bulgaria. This is a serious circumstance giving a reason to assume that a significant part of the bee populations on the Bulgarian territory has the characteristics of the local honey bee *A. m. rodopica*.

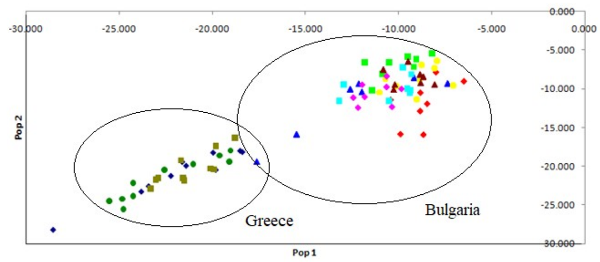
The presence of private alleles in populations is an indicator for their specific population-genetic characteristics or for clear differences between them. The search for diagnostic markers among the more widespread alleles in the population is considered as successful if these alleles are detected in one population while absent in another one, with a frequency above 5%. Based on the results of our study, it becomes clear, that all the 39 identified private alleles in the studied populations from Bulgaria, Greece and Serbia could be used as successful diagnostic markers for discriminating the studied origins as their frequency is above the proposed one (5%) – Table 2.

In this study, the average calculated value for gene flow (*Nm*) is 1.723. *Nm* value varies from 0.402 (Ap243) to 3.282 (A043) for the studied loci, which indicates moderate to high levels of genetic differentiation between the studied populations from Bulgaria, Greece and Serbia. In accordance with the *Nm* data, the results obtained for *Fst* values calculated in pairs indicate also moderate to high levels of genetic differentiation for the studied populations.

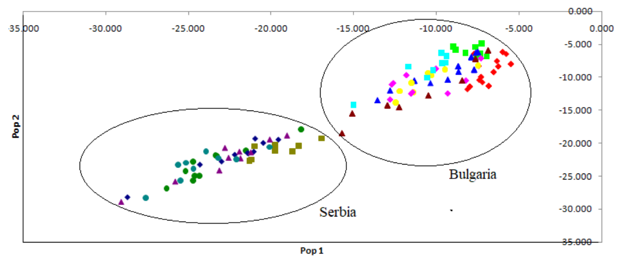
Comparative analyses of the studied honey bee populations from some Balkan peninsula regions show distinct characteristics that could be successfully used for their discrimination. Data from the assignment tests done, demonstrate that the populations of the local Bulgarian honey bee *A. m. rodopica* are grouped separately from *A. m. macedonica* originating from Greece, as well as from *A. m. carnica* originating from Serbia (Figure 1).



1a



1b



1c

Fig. 1. Distribution of individuals in the honey bee populations studied: a) grouping the populations from Bulgaria (local *A. m. rodopica*), Greece (*A. m. macedonica*) and Serbia (*A. m. carnica*) together; b) grouping of the local Bulgarian honey bee *A. m. rodopica* (to the right) and *A. m. macedonica* originating from Greece; c) grouping of the local Bulgarian honey bee *A. m. rodopica* (to the right) and *A. m. carnica* originating from Serbia.

These results could be taken as further proof that the local Bulgarian honey bee could be distinguish by the other European *Apis mellifera* populations, as well as by the Balkan peninsula honey bee populations, based on different genetic approaches, which is in agreement with previously reported data (Ivanova et al., 2012; Francis et al., 2014). Simultaneously with the findings of other authors (Nedić et al., 2014; Uzunov et al., 2014b), this study provides data on the genetic diversity of *Apis melifera* populations in the Balkan Peninsula, as well as possible approaches for their distinction.

Conclusions

Together with the previously described valuable biological and productive characteristics, morphometric, ethological, isoenzymatic and other DNA markers, the characterized here genetic markers update the system of criteria and activities for the protection and conservation of the gene pool of the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991.

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Additions to Moss Flora of Kosovo from "Bjeshkët e Nemuna" Mountains

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Abstract. The moss flora of Kosovo is the least explored in South East Europe, with 346 moss taxa known so far. The aim of the present study is to further increase the knowledge of the moss flora of Bjeshkët e Nemuna Mountains (Prokletije) in Kosovo, which are a hot spot for plant biodiversity in the Balkans. In several field trips conducted in Bjeshkët e Nemuna, about 1140 moss samples were collected and identified. From the current investigation of the moss flora in Bjeshkët e Nemuna, 13 taxa are new records for Kosovo and 41 species are recorded for the first time in Bjeshkët e Nemuna. Therefore, this study brings to 359 the number of moss species for Kosovo and 239 for Bjeshkët e Nemuna. Concluding, much more efforts are needed towards a better knowledge of mosses of Kosovo, and thereafter the protection of potential threatened species.

Key words: Plant biodiversity, moss flora, Bjeshkët e Nemuna, Prokletije.

Introduction

The bryophyte flora of Kosovo is poorly known. The main reason is the lack of local bryologists and low investigation by foreigner bryologists as well. Recently, Pantović & Sabovljević (2017) have summarized the checklist of Kosovo bryophytes based on the review of 13 literature sources – reporting 303 mosses and 28 liverworts. However, these 303 moss taxa are reported based on ca. 700 records, which is a very low number. In addition, new moss records are reported for Kosovo from Germia Regional Park and Sharr Mountain (Krasniqi & Marka, 2018, 2019). Hence, the actual number of moss taxa for Kosovo has reached up to 346 taxa. However, this number is still very low compared with the neighboring Balkan countries from one side (Sabovljević et al., 2011), and especially the presence of two important mountain ranges present in Kosovo – and hot spots for plant biodiversity – namely Sharr Mountains (also known as Šar planina) and Bjeshkët e Nemuna (also known as Prokletije) from the other side. In the check list of Kosovo bryophytes for Bjeshkët e Nemuna are reported 198 moss taxa (Pantović & Sabovljević, 2017). The aim of the present study is to further increase the knowledge of the moss flora of Bjeshkët e Nemuna, in addition to those reported mainly by Martinćić (2006).

Bjeshkët e Nemuna is one of the most

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attractive and beautiful mountain massifs in Europe (Muratagic, 1975). They are a continuation of the Dinaric mountain system and are part of the system of new mountains which are mainly composed of limestone-dolomite rocks. In Kosovo territory it has an area of 63,027.75 ha, which in administrative terms belongs to several municipalities: Istog, Peja, Deçan, Junik and Gjakova (Çadraku et al., 2014). The altitude varies from 500 to 2656 m (Maja Gjeravica) and the average altitude is 1600 m. Bjeshkët e Nemuna include the western and northwestern part of Dukagjini region which is a continuation of the mountains from Liqeni i Shkodrës lake (Albania), Plavë and Guci (Montenegro) to the Ibar tectonic valley. There are found variable forms which are created by fluvioglacial processes, and as a consequence of which is the formation of deep gorges. Erosion is quite pronounced in these mountains, due to the structural composition of the soil and heavy rainfall that makes the rocks washed away (Sherifi et al., 2005). Bjeshkët e Nemuna Mts. are characterized by gorges, valleys, karstic, glacial, fluvioglacial and denudation phenomena (Sherifi et al., 2005). Rugova Gorge and Deçan Gorge by Lumbardhë River represent the most important geomorphological elements (Pasha, 1975).

In Bjeshkët e Nemuna dominates the continental Mediterranean and mountain climate intertwined with micro climates conditioned by the alternation of river valleys (Pllana, 1991). Average monthly temperatures range from -0.33 ° C to 21.37 ° C in Peja Locality and from 0.3 ° C to 20.8 ° C in Istog Locality (Instituti për Hidroekonomi, 1983). In terms of seasons, it turns out that the coldest month is January, while the hottest month is August. The average annual rainfall varies from 650 mm (plain part) to over 1400 mm (mountain part) (Instituti për Hidroekonomi, 1983).

The hydrographic network is quite developed. The main rivers are: Lumbardhë River of Peja, Deçan, Lloqan, and Erenik River. All waters flow east and southeast and are discharged into the Drini i Bardhë River (Çadraku et al., 2014). Underground water sources are present in almost the entire area of the mountains, but have the highest density in carbonate formations. Based on existing data within the boundaries of the Bjeshkët e Nemuna there are about 730 underground water sources (Çadraku et al., 2014).

Material and Methods

A total of 1140 moss samples were collected in different localities in Bjeshkët e Nemuna during several field trips carried out in 2015 and 2019 (Table 1). The identification of the material was done jointly by both authors. The voucher specimens are preserved in the herbarium collection at Faculty of Education, University of Pristina. Nomenclature of the moss taxa follows Hodgetts et al. (2020). The conservation status of moss taxa at European and regional level, as well as their distribution in South East Europe, follows Hodgetts & Lockhart (2020). The floristic element for each taxon is retrieved from Smith (2004).

Table 1. The collecting site details in Bjeshkët e Nemuna.

No.	Locality name	Coordinates and altitude
1	Lagjia e Kaprojve	42°42′52″N, 20°9′3"E, 1103 m
2	Reka e Allagës	42°43′60″N, 20°9′26"E, 1403 m
3	Hajla	42°44′24″N, 20°9′7"E, 1650 m
4	Hajla	42°44′37″N, 20°9′33"E, 1703 m
5	Hajla	42°45′2″N, 20°9′13"E, 2027 m
6	Hajla	42°44′58″N, 20°8′51"E, 2081 m
7	Hajla	42°44′32″N, 20°8′49"E, 1795 m
8	Liqenat	42°40′29″N, 20°5′11"E, 1671 m
9	Liqenat	42°40′6″N, 20°5′24"E, 1865 m
10	Bogë	42°45′34"N, 20°3′25"E, 1543 m
11	Bogë	42°46′3"N, 20°3′32"E, 1619 m
12	Bogë	42°46′25"N, 20°3′34"E, 1659 m
13	Bogë	42°46′44"N, 20°3′36"E, 1767 m
14	Hajla	42°45′25"N, 20°8′22"E, 2362 m
15	Hajla	42°45′34"N, 20°8′3"E, 2350 m
16	Hajla	42°45′35"N, 20°7′47"E, 2249 m
17	Hajla	42°45′40"N, 20°6′55"E, 2240 m
18	Hajla	42°45′33"N, 20°5′43"E, 1809 m
19	Hajla	42°44′58"N, 20°6′38"E, 1615 m

In the Results section is given the list of moss taxa found in Bjeshkët e Nemuna. Taxa marked with one asterisk (*) are new records for Kosovo, whereas those with two asterisks (**) are new records for Bjeshkët e Nemuna. Each moss taxa is followed immediately with the number of locality where it was found and its substrate, floristic element, conservation status and distribution comments in the context of SE European countries. Abbreviations used for conservation status are: CR - critically endangered, EN – endangered, VU – vulnerable, NT – neat threatened and DD – data deficient.

Results and Discussion

Altogether 124 moss taxa are given in the following list. 13 are new records for Kosovo (*) and 41 (**) are new records for Bjeshkët e Nemuna:

Abietinella abietina (Hedw.) Fleisch. – 3, 4, 5, 12: on soil and limestone rocks. This Circumpolar Boreo-arctic Montane species is common and widespread in SE Europe.

Barbula unguiculata Hedw. – 3: on limestone rocks. This Circumpolar Wide-temperate species is common and widespread in SE Europe.

Bartramia ithyphylla Brid. – 4, 5, 14, 15, 19: on basic soil. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

Brachytheciastrum velutinum (Hedw.) Ignatov & Huttunen - 5, 7, 9, 13, 14, 15, 17, 18: on soil, limestone rocks. This Circumpolar Temperate species is very common and widespread in SE Europe.

****Brachythecium albicans* (Hedw.) Schimp. – 13: in grassland. This Circumpolar Boreo-temperate species although is recently reported for Kosovo (Krasniqi & Marka, 2018, 2019) is common and widespread in SE Europe.

****Brachythecium geheebii* Milde. – 9, 13: on acidic rocks. This is an endemic species of Europe and red list species (VU) (Hodgetts & Lockhart, 2020). It is reported for almost all SE European countries and red listed as follows: EN-Bulgaria, CR-Romania and NT-Slovenia. As for Kosovo, it has been recently reported in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

Brachythecium glareosum (Bruch ex Spruce) Schimp. – 4, 6, 7, 11, 12, 13, 14, 15, 17, 18, 19: on soil, on limestone rocks. This Eurasian Boreo-temperate species is common and widespread in SE Europe.

Bryoerythrophyllum recurvirostrum (Hedw.) P.C.Chen – 6, 14, 15: on basic soil. This Circumpolar Boreo-temperate species is common and widespread in SE Europe.

Calliergonella cuspidata (Hedw.) Loeske – 4: on spring. This Circumpolar Temperate species is very common in SE Europe.

**Calliergonella lindbergii* (Mitt.) Hedenas – 12: on wet rocks by stream. This Circumpolar Wide-boreal species is a new record for Kosovo; it is known from many SE European countries but, however, its conservation status is not clear (e.g. DD-Montenegro, Serbia).

**Campyliadelphus chrysophyllus* (Brid.) R. S. Chopra – 14, 15, 16: limestone grassland. This Circumpolar Boreo-temperate species is a new record for Kosovo. However, it is known and common from all SE European countries.

Campylium stellatum (Hedw.) Lange & C.E.O.Jensen – 3, 4, 6, 7, 14, 15, 16: humid soil, wet rocks. This Circumpolar Boreo-temperate species is known from all SE European countries except for Albania.

Campylophyllum halleri (Hedw.) M.Fleisch. – 6, 8: on wet limestone rocks. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe .

Ceratodon purpureus (Hedw.) Brid. – 2, 3, 4, 6, 7, 8, 9 , 11, 12, 14, 17: on acidic soils and rocks. This Circumpolar Wide-boreal species is very common in SE Europe.

Cirriphyllum crassinervium (Taylor) Loeske & M.Fleisch. – 8: on limestone humid rock. This European Temperate species is very common in SE Europe.

Cirriphyllum piliferum (Hedw.) Grout – 5: on wet rocky soil. This Circumpolar Boreo-temperate species is known from all SE European countries except for Albania.

Cratoneuron filicinum (Hedw.) Spruce – 3: on spring. This Circumpolar Wide-temperate species is common in SE Europe.

Ctenidium molluscum (Hedw.) Mitt. – 6 , 7, 8, 9, 14, 15, 18, 19: on limestone rocks. This European Boreo-temperate species is common in SE Europe.

**Cyrtomnium hymenophylloides* (Huebener) T.J.Kop. – 7: on soil. This Circumpolar Boreo-temperate species is a new record for Kosovo. It is recorded in many SE European countries but, however, its conservation status is not clear (EN-Romania, DD-Montenegro, Serbia).

**Dialytrichia mucronata* (Brid.) Broth. – 5: on rock beside stream. This Mediterranean-Atlantic species is a new record for Kosovo. However, its presence in high altitudes is not a normal situation for the species.

**Dicranella varia* (Hedw.) Schimp. – 6, 15: on wet rocks. This Circumpolar Boreo-temperate species is common and widespread in SE Europe.

Dicranum scoparium Hedw. – 3, 4, 5, 7, 8, 9, 14, 15, 16: on silicate rocks. This Circumpolar Wide-boreal species is widespread in SE Europe.

Dicranum tauricum Sapjegin –3, 7, 8, 9, 14, 15, 16: on coniferous and oak bark. This European Temperate species is widespread in SE Europe. It is red listed for Slovenia (EN) and Serbia (VU).

Didymodon fallax (Hedw.) R. H. Zander – 8: on calcareous rock. This Circumpolar Southern-temperate species is common and widespread in SE Europe.

***Didymodon rigidulus* Hedw. – 5, 7, 9, 13, 15: on limestone rocks. This Circumpolar Boreo-temperate species is widespread in SE Europe.

***Didymodon spadiceus* (Mitt.) Limpr. – 4: on spring. This European Temperate species is widespread in SE Europe.

***Didymodon vinealis* (Brid.) R.H.Zander – 7, 14: on limestone rocks. This European Southern-temperate species is widespread in SE Europe.

Distichium capillaceum (Hedw.) Bruch & Schimp. – 6, 7, 8 , 9, 15, 19: on limestone rocks. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

***Distichium inclinatum* (Hedw.) Bruch & Schimp. – 6, 8, 9, 18: on limestone rocks. This Circumpolar Boreo-arctic Montane species is widespread in SE. It is red listed for Bulgaria (VU)

Encalypta affinis R. Hedw. – 3, 17: on rocks. It is known from several SE European countries and red listed for Romania (CR).

Encalypta ciliata Hedw. – 6: on calcarious soil. This Circumpolar Boreal-montane species is known from all SE European countries and red listed for Serbia and Montenegro (VU).

Encalypta rhaptocarpa Schwägr. – 7, 14, 15: on limestone rocks. This Circumpolar Boreo-arctic Montane species is known from many SE European countries.

Encalypta streptocarpa Hedw. – 6, 7, 8, 9, 10, 12, 14, 16: on limestone rock crevices. This Euroasian Boreo-temperate species is the commonest *Encalypta* in SE Europe.

Eurhynchiastrum diversifolium (Schimp.) J.Guerra – 6, 7, 11, 14, 15, 18: on wet rocks, on soil. This Circumpolar Temperate species is known from many SE European countries and red listed for Slovenia (EN).

Eurhynchiastrum pulchellum (Hedw.) Ignatov & Huttunen – 8, 9: on wet rocks. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

Fissidens taxifolius Hedw. – 7, 15, 16, 18: on rocks and soil. This European Southern-temperate species is common and widespread in SE Europe.

Flexitrichum flexicaule (Schwägr.) Ignatov & Fedosov – 6, 7, 8, 9, 10, 13, 15, 17: on limestone rocks and soil. This Circumpolar Wide-temperate species is very common and widespread in SE Europe.

Flexitrichum gracile (Mitt.) Ignatov & Fedosov – 5, 7, 8, 9, 10, 13, 17, 18. This Circumpolar Boreo-temperate species is widespread in SE; however, not as common as *F. flexicaule* and red listed for Romania (CR).

**Grimmia anodon*. Bruch & Schimp. – 7, 16: on limestone rocks. This Circumpolar Wide-temperate species is known from many SE European countries; however, not very common and it is red listed for Romania (VU).

Grimmia hartmanii Schimp. – 1, 2, 3, 4: on acidic rocks. This European Boreal-montane species is widespread in SE Europe and often locally abundant.

***Grimmia muehlenbeckii* Schimp. – 7, 16: on acidic rocks. This species is known from many countries in SE Europe, and it is also red listed for Bulgaria and Romania (VU).

Grimmia ovalis (Hedw.) Lindb. – 1, 3: on acidic rocks. This Circumpolar Boreal-temperate species is widespread in SE Europe.

***Grimmia ramondii* (Lam. & DC.) Margad. – 1, 2, 3, 4, 7, 8, 15, 16: on dry acidic rocks. This European Boreal–montane species is widespread in SE Europe. It is locally abundant in high altitudes. It is red listed for Romania (EN) and Bulgaria (VU).

***Hedwigia ciliata* (Hedw.) P.Beauv. – 2: on acidic rock. This species is widespread in SE Europe.

**Hedwigia emodica* Hampe ex Müll.Hal. – 3: on acidic rock. This Oceanic Temperate species is known from several countries in SE Europe and it is red listed for Slovenia (VU).

Heterocladiella dimorpha (Brid.) Ignatov & Fedosov – 9, 15: on rocks. This European Boreal-montane species is widespread in SE Europe.

***Homalothecium lutescens* (Hedw.) H.Rob. – 6, 7, 15, 16: on limestone rocks and soil. This European Southern-temperate species is very common and widespread in SE Europe.

Homalothecium philippeanum (Spruce) Schimp. – 7: on soil. This species is common and widespread in SE Europe.

Hylocomiadelphus triquetrus (Hedw.) Ochyra & Stebel. – 8, 9: on rocks. This Circumpolar Boreo-temperate species is widespread in SE Europe.

Hylocomium splendens (Hedw.) Schimp. –. 6, 7, 8, 9: on limestone rocks. This Circumpolar Wide-boreal species is widespread in SE Europe.

Hymenoloma crispulum (Hedw.) Ochyra – 7: on acidic rock. This Circumpolar Boreal-Arctic Montane species is widespread in SE Europe.

**Hypnum andoi* A.J.E.Sm. – 1: on acidic rock. This Suboceanic Temperate species is known from many SE European countries, and it is red listed for Serbia (VU), near threatened for Romania and data deficient for Montenegro.

Hypnum cupressiforme Hedw. – 1, 2, 3, 4, 5, 6, 13: on rocks, on fir bark. This Circumpolar Wide-temperate species is common and widespread in SE Europe.

Hypnum cupressiforme var. *lacunosum* Brid. – 3: on wet rock. This Circumpolar Boreo-temperate species is widespread in SE Europe.

***Kiaeria starkei* (F.Weber & D.Mohr) I.Hagen – 7, 16: on silicate rocks. This Circumpolar Arctic-montane species is known from several SE European countries, it is red listed for Slovenia (EN). As for Kosovo, it has been recently reported in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

Lescuraea incurvata (Hedw.) E.Lawton – 7, 8, 9, 10, 11, 13: on rocks. This European Boreal–montane species is common and widespread in SE Europe.

Lescuraea patens Lindb. – 13: on limestone rock. This European Boreal-montane species is widespread in SE Europe but, however, not very common.

Lescuraea plicata (Schleich. ex F.Weber & D.Mohr) Broth. – 6, 7, 8, 9: on rocks. This

European Boreo-arctic Montane species is widespread in SE Europe and red listed for Romania (VU).

***Lewinskya striata* (Hedw.) F.Lara, Garilleti & Goffinet – 13: on beech bark. This European Boreal–temperate species is widespread in SE Europe. As for Kosovo, it has been recently reported in the region of Prishtina and the Sharr Mountain National Park (Krasniqi & Marka, 2018, 2019).

Mnium spinosum (Voit.) Schwagr. – 7, 18, 19: on humid rocks and soil. This Circumpolar Boreal–montane species is widespread in SE Europe.

Mnium stellare Hedw. – 7, 8, 9, 14: on rocks. This Circumpolar Boreo-temperate species is widespread in SE Europe.

Mnium thomsonii Schimp. – 8, 9, 11: on rocks. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

***Orthotrichum anomalum* Hedw.– 9, 11: on limestone rocks. This European Wide-temperate species is common and widespread in SE Europe.

***Oxyrrhynchium speciosum* (Brid.) Warnst. – 13: on beech bark. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe. As for Kosovo, it has been recently reported in the region of Prishtina and the Sharr Mountain National Park (Krasniqi & Marka, 2018, 2019).

Palustriella commutata (Hedw.) Ochyra – 5, 6, 7, 10, 11, 12, 16: on limestone rocks near stream, water flows and creeks. This Circumpolar Boreal-temperate species is widespread in SE Europe.

Palustriella decipiens (De Not.) Ochyra – 4: near springs. This European Boreo-arctic Montane species is widespread in SE Europe.

***Palustriella falcata* (Brid.) Hedenäs – 3, 12: on limestone rocks and near stream. This Circumpolar Boreal-temperate species is widespread in SE Europe.

***Paraleucobryum longifolium* (Hedw.) Loeske – 4, 7, 16: on limestone rocks. This Circumpolar Boreal-montane species is known from many SE European countries and it is red listed for Serbia (VU). As for Kosovo, it has been recently reported in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

Philonotis calcarea (Bruch & Schimp.) Schimp. – 3, 4, 10, 12, 13: near springs and on wet calcarious rocks. This European Boreo-montane species is widespread in SE Europe. It is near threatened in the European red list of bryophytes (Hodgetts & Lockhart, 2020).

Philonotis fontana (Hedw.) Brid. – 7, 15: on wet rocks. This Circumpolar Wide-temperate species is widespread in SE Europe.

Philonotis seriata Mitt. – 4: in acidic spring. This Eurosiberian Boreo-arctic Montane species is widespread in SE Europe and red listed for Slovenia (EN).

Plagiomnium affine (Funck) T.J.Kop. – 7, 15: on soil. This European Temperate species is widespread in SE Europe.

***Plagiomnium ellipticum* (Brid.) T.J.Kop.–7, 14, 16: on shaded and humid soil. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe and red listed for Romania (VU).

Plagiomnium medium (Bruch & Schimp.) T. J. Kop. – 1: on soil in rock crevices. This Circumpolar Boreal-montane species is widespread in SE Europe.

Plagiopus oederianus (Sw.) H.A.Crum & L.E.Anderson – 8: on limestone rock. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

Plagiothecium denticulatum (Hedw.) Schimp. – 7, 16: on acidic shaded rocks. This Circumpolar Boreal-temperate species is widespread in SE Europe.

**Pleuridium acuminatum* Lindb. – 6, 18: on poor soil. This European Temperate species is widespread in SE Europe.

Pleurozium schreberi (Willd. ex Brid.) Mitt. – 7: on wet soil. This Circumpolar Boreal-temperate species is widespread in SE Europe.

Pogonatum urnigerum (Hedw.) P.Beauv. – 6, 7, 11, 12, 13, 15: on acidic rocks, on disturbed soil. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

Pohlia cruda (Hedw.) Lindb. – 6, 7, 16, 18, 19: on rock crevices and on soil. This Circumpolar Boreo-arctic Montane species is common and widespread in SE Europe.

Pohlia elongata (Hedw.) – 9: on rock crevices. This Circumpolar Boreal-montane species is widespread in SE Europe.

Polytrichastrum alpinum (Hedw.) G.L.Sm. – 7, 14: on poor soil. This Circumpolar Boreo- arctic Montane species is widespread in SE Europe.

Polytrichum commune Hedw. – 13: on bogs. This Circumpolar Wide-boreal species is widespread in SE Europe.

Polytrichum formosum Hedw. – 3, 5: on wet soil. This Circumpolar Boreal-temperate species is widespread in SE Europe.

Polytrichum juniperinum Hedw. – 4, 9, 14: on acidic rocks. This Circumpolar Wide-boreal species is widespread in SE Europe.

**Polytrichum strictum* Menzies ex Brid. – 7, 9, 13, 14, 15, 16: in bogs. This Circumpolar Wide-boreal species is known from many SE European countries and it is red listed for Serbia (VU).

Pseudoleskeella catenulata (Brid. ex Schrad.) Kindb. – 7, 8, 9, 10, 14, 15, 16: on limestone rocks. This European Boreal-Montane species is widespread in SE Europe.

Pseudoleskeella nervosa (Brid.) Nyholm. – 7, 10, 12, 13, 14, 15, 16: on beech barks and on rocks. This Circumpolar Boreal-montane species is widespread in SE Europe.

Pterigynandrum filiforme Hedw. – 1, 2, 3, 4, 5, 7, 13, 15: on bark, on limestone rocks. This Circumpolar Boreal-montane species is common and widespread in SE Europe.

***Ptychostomum capillare* (Hedw.) Holyoak & N.Pedersen – 1, 6, 7, 9, 15, 16, 19: on soil and on rocks. This Circumpolar-temperate species is widespread in SE Europe.

***Ptychostomum elegans* (Nees) D.Bell & Holyoak – 9, 10, 11, 12, 13, 14: on limestone rocks. This European Boreal-montane species is widespread in SE Europe, it is also red listed for Bulgaria and Slovenia (VU). It has been recently reported for Kosovo in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

***Ptychostomum moravicum* (Podp.) Ros & Mazimpaka – 7, 9, 13, 14: on tree bark and on limestone rocks. This Circumpolar–temperate species is widespread in SE Europe. As for Kosovo, it has been recently reported in the region of Prishtina (Krasniqi & Marka, 2018) and in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

Ptychostomum pseudotriquetrum (Hedw.) J.R.Spence & H.P.Ramsay ex Holyoak & N.Pedersen – 4, 7: in humid situations near springs. This Circumpolar Wide-boreal species is widespread in SE Europe.

Ptychostomum pseudotriquetrum var. *bimum* (Schreb.) Holyoak & N.Pedersen – 7: in spring. This Circumpolar Wide-boreal species is known from many SE European countries.

***Racomitrium affine* (F.Weber & D.Mohr) Lindb. – 2: on acidic rock. This European Temperate species is known from many SE European countries and it is red listed for Bulgaria (VU). It has been recently reported for Kosovo in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

***Racomitrium canescens* (Hedw.) Brid. – 3, 4, 6, 9, 10, 11, 12, 13 15, 17, 18, 19: on rocks and on soil. This Circumpolar Boreal-arctic Montane species is widespread in SE Europe.

Racomitrium elongatum Ehrh. ex Frisvoll – 7, 16: on silicate rocks. This Suboceanic Boreo-temperate species is known from many SE European countries.

***Racomitrium ericoides* (Brid.) Brid. – 3, 5: on rocks. This Suboceanic Wide-boreal species is known from many SE European countries and it is red listed for Romania (EN).

****Racomitrium macounii* subsp. *alpinum* (E.Lawton) Frisvoll – 2, 7, 16: on silicate rocks. This European Boreal-montane species is rare in SE Europe and it is red listed for Romania (CR) and Bulgaria (VU). It has been recently reported for Kosovo in the Sharr Mountain National Park (Krasniqi & Marka, 2019).

Racomitrium sudeticum (Funck) Bruch & Schimp. – 7, 16: on silicate rocks. This Circumpolar Boreo-arctic Montane species is known from many countries in SE Europe.

Rhytidiadelphus loreus (Hedw.) Warnst. – 7, 15: on root bark. This Suboceanic Boreo-temperate species is known from many countries in SE Europe.

****Rhytidiadelphus squarrosus* (Hedw.) Warnst. – 9, 10, 11, 12: on rocks. This European Boreo-temperate species is widespread in SE Europe.

Rhytidium rugosum (Hedw.) Kindb. – 7, 16, 18: on calcareous rocks. This Circumpolar Boreo-Montane species is widespread in SE Europe.

Roaldia revoluta (Mitt.) P.E.A.S.Câmara & M.Carvalho-Silva – 6, 7, 9, 15, 16, 17: on limestone rocks. This Circumpolar Arctic-montane species is known from several countries in SE Europe and it is red listed for Romania (EN) and Bulgaria (VU).

Sanionia uncinata (Hedw.) Loeske. – 4, 7, 8, 9, 13, 18, 19: on rocks, on soil. This Circumpolar Boreo-arctic Montane species is widespread in SE Europe.

Schistidium apocarpum (Hedw.) Bruch & Schimp. – 12, 13: on limestone rocks. This Eurasian Boreo-temperate species is widespread in SE Europe.

****Schistidium confertum* (Funck) Bruch & Schimp. – 12, 13, 14, 15, 16, 17, 18: on limestone rocks. This Circumpolar Boreo-temperate species is widespread in SE Europe.

**Schistidium helveticum* (Schkuhr) Deguchi – 7, 15: on limestone rocks. This species is known from many SE European countries; however its conservation status is still uncertain for many countries.

****Schistidium papillosum* Culm. – 11: on acidic rock. This Circumpolar Boreo-arctic Montane species is known from many SE European countries, but its status is data deficient for many countries.

**Schistidium rivulare* (Brid.) Podp. – 13: on humid rock. This species is known from many SE European countries and it is red listed for Montenegro (CR) and Slovenia (EN).

Sciuro–hypnum reflexum (Starke) Ignatov & Huttunen – 6, 7, 14, 15: on *Juniperus* bark. This Circumpolar Boreal-montane species is widespread in SE Europe.

**Sphagnum centrale* C.E.O. Jensen – 5: on bog. This species is known from many SE European countries and it is red listed for Montenegro and Serbia (VU) and near threatened for Slovenia.

Stereodon callichrous (Brid.) Lindb. – 13: on limestone rock. This Circumpolar Boreo-arctic Montane species is known from several SE European countries and it is red listed for Bulgaria (VU).

Syntrichia norvegica F.Weber. – 7, 9, 10, 12, 13, 18, 19: on limestone rocks. This Circumpolar Boreo-arctic Montane species is known from most of the SE European countries, it is red listed for Romania (VU) and near threatened for Bulgaria.

Syntrichia ruralis (Hedw.) F.Weber & D.Mohr – 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 18: over rocks and soil. This Circumpolar Wide-temperate species is widespread in SE Europe.

Thuidium assimile (Mitt.) A. Jaeger – 12: on limestone rock. This Circumpolar Temperate species is widespread in SE Europe.

Thuidium delicatulum (Hedw.) Schimp. – 7: on soil. This Circumpolar Temperate species is widespread in SE Europe.

Thuidium tamariscinum (Hedw.) Schimp. – 12: on rock. This European Temperate species is widespread in SE Europe.

Timmia austriaca Hedw. – 6, 7, 8, 9, 17, 18,19: on limestone rocks. This Circumpolar

Boreo-arctic Montane species is widespread in SE Europe.

***Tortella nitida* (Lindb.) Broth. – 9: on limestone rock. This Mediterranean-Atlantic species is known from many SE European countries and it is red listed for Bulgaria (CR) and Romania (EN).

Tortella tortuosa (Hedw.) Limpr. – 4, 5, 8, 9, 10, 11, 12, 13: on limestone rocks. This Circumpolar Boreo-temperate species is common and widespread in SE Europe.

Tortula hoppeana (Schultz) Ochyra – 7, 14: rocks. This species is widespread in SE Europe.

Tortula subulata Hedw. – 3, 7, 9, 13, 16: on soil and rocks.This Eurosiberian Southern- temperate species is widespread in SE Europe.

Weissia controversa Hedw. – 6, 7, 15, 16, 17, 18, 19: on basic rocks and soil. This Circumpolar Wide-temperate species is widespread in SE Europe.

Conclusions

From the current investigation of the moss flora in Bjeshkët e Nemuna, 13 taxa are new records for Kosovo. Some of these (e.g. *Campyliadelphus chrysophyllus*, *Dicranella varia*, *Pleuridium acuminatum* etc.) are common species in the context of the SE European countries, and some are less common or rare and with conservation interest (e.g. *Cyrtomnium hymenophylloides*, *Hedwigia emodica*, *Schistidium rivulare*, *Sphagnum centrale* etc.). In addition, 41 species are recorded for the first time in Bjeshkët e Nemuna. Therefore, this study brings to 359 the number of moss species for Kosovo and 239 for Bjeshkët e Nemuna. In the European level, *Brachythecium geheebii*, an endemic species of Europe, is red listed in the vulnerable threatened category. Concluding, much more efforts are needed towards a better knowledge of mosses of Kosovo, and thereafter the protection of potential threatened species.

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Chronic Exposure to Heavy Metals Induces Nuclear Abnormalities and Micronuclei in Erythrocytes of the Marsh Frog (Pelophylax ridibundus Pallas, 1771)

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Abstract. Amphibians have big potential as bioindicators based on their combined life cycle as aquatic and terrestrial form. They can play the role of prey or predator, making them a key element in toxic substances transfer between aquatic and terrestrial habitats. The nuclear abnormalities (NAs) in amphibians' erythrocytes in recent years have been used as a successful biomarker for anthropogenic pollution. The NAs including micronuclei in erythrocytes of the marsh frog (*P. ridibundus*) have been studied to assess the cytotoxic and genotoxic effect in heavy metal polluted area *in situ*. Here we assess the cyto- and genotoxic potential of the polluted waters (Chaya River) close to the lead-zinc smelter near Plovdiv (Bulgaria) situated in an area that has been contaminated with heavy metals for 60 years. Frogs from Strandzha Natural Park were used as a negative control. Peripheral blood smears have been dyed with acridine orange. NAs of the following types: notched nuclei, nuclear buds and blebbed nuclei have shown the highest frequency. There is no sexual dependence in the formation of different types of NAs. The significant differences ($P \leq 0.0001$) in the mean Total NAs (‰) in erythrocytes of marsh frogs from the polluted area compared to the total NAs from the background region "Strandzha" NP demonstrate the presence of *in vivo* active cytotoxic and genotoxic agents in the impacted area. The obtained results for NAs in erythrocytes of *P. ridibundus* are evidence for successful application of NAs as a biomarker in amphibians for the purpose of biomonitoring.

Key words: genotoxicity, nuclear abnormalities, micronuclei, heavy metals, *Pelophylax ridibundus*.

Introduction

Negative changes in the environment, because of daily anthropogenic activity, are a continuous and irreversible process. Increased anthropogenic pressure leads to changes in the biosphere, which can disrupt the fragile ecological balance, as well as cause several harmful effects on humans and the environment (Ellis, 2015). This requires the application of biological monitoring, which is carried out through a modern integrated approach, research and state assessment as well as forecast of changes in individual organisms, communities, and ecosystems (USEPA, 2000; Şişman et al., 2015).

Heavy metals and their compounds have extremely harmful effects on the biota

(Gall et al., 2015). Therefore, they have been identified as some of the most hazardous toxic substances (The Priority List of Hazardous Substances; ATSDR, 2011). The caused contamination is especially adverse since traces of metals and metalloids are not biodegradable. According to Camizuli et al. (2018) the trace metals from mining sites and metallurgical industry stay bioavailable. They persist in the environment and tend to constant accumulation in plants and animals. Therefore, the local contamination continues to be a serious environmental risk and is subject to many biomonitoring studies.

Stress caused by heavy metals leads to a cascade of biological responses in living organisms, which could be used as a biomarker (Amiard-Triquet et al., 2012). Nuclear abnormalities, including micronuclei, are biomarkers of genotoxicity and chromosomal instability. They have been successfully used as biomarkers in fish, amphibians, and mammals (Fenech et al., 2011; Pollo et al., 2015, 2016; Ivanova et al., 2016). The mechanisms of their occurrence have not been fully studied yet – some authors define them as cytotoxic biomarkers (notched and lobbed nuclei) (Rocha et al., 2011), while others link the appearance of certain NAs not only with the cytotoxic effect of the environment, but also with genotoxic one (nuclear buds) (Bolognesi & Hayashi, 2011). In all cases, their presence is associated with disorders of cell division, apoptosis and genotoxicity or mutagenicity. Therefore, many scientists who have studied the effects of various genotoxicants recommend the use of the NAs method in conjunction with the micronucleus test (Ferraro et al., 2004; Çavaş, 2008; Hoshina et al., 2008). Multiple NAs – including blebbed, notched, lobed nuclei, nuclear buds and binucleated cells – have all been applied as potential biomarkers of genotoxicity (Ayllon & Garcia-Vazquez, 2000; Çavaş & Ergene-Gözükara, 2005; da Silva Souza & Fontanetti, 2006; Muranli & Güner, 2011; Ruiz de Arcaute et al., 2016).

Amphibians are of a great importance for both terrestrial and aquatic communities. They are an extremely important link in food chains and are very sensitive to anthropogenic environmental changes (Alton & Franklin, 2017). In addition, among some aquatic and terrestrial communities, certain amphibian species are the most abundant vertebrates, reaching densities of up to 2,500 for aquatic and 40,000 individuals per hectare for terrestrial communities (Burton & Likens, 1975; Petranka & Murray, 2001). The significant biomass, combined with the typical big appetite of the amphibian larvae (Taylor et al., 1988), their high mass and energy conversion efficiency (Grayson et al., 2005), allows them to play an important role in the transfer of energy and nutrients through food chains (Beard et al., 1998).

Due to their specific characteristics, amphibians can be used as bioindicators to detect toxic waste in water, soil, or bottom sediments (Boone & Bridgs, 2003). Most of their gas exchange takes place through the skin, which leads to an easy absorption of substances that pollute their habitat. In addition, this group of animals cannot take long-distance movements, and this makes them intricately connected to the environment in which they live (Sievers et al., 2018). The sensitivity of anurans as zoomonitors of heavy metals contamination is present in various studies (Leontyeva et al., 1997; Lefcort et al., 1998; Şişman et al., 2015; Zhelev et al., 2015, 2020).

The marsh frog *P. ridibundus* is an unprotected anuran with the widest distribution in Bulgaria. It is a bioindicator of the long-term environmental impact of biological parameters and can be used to assess anthropogenic pollution (Corduk et al., 2018). The aim of the present study is to determine the cyto- and genotoxic effect *in situ* in marsh frog (*Pelophylax ridibundus* Pallas, 1771) from anthropogenically polluted area.

Material and Methods

Study area

The area of study covers two regions listed in the "National Biomonitoring Program of Bulgaria", one as impacted and the other as background (Peev & Gerasimov, 1999). The polluted region includes the area of the lead-zinc smelter (KCM AD) near the city of Plovdiv and the unpolluted – the Strandzha Natural Park (SNP).

The Marsh frogs were captured in a sampling site close to the lead-zinc smelter in the Thracian valley: the Chaya River (synonyms: Chepelare River, Assenitsa River) near the confluence with the Maritza River (42.1561° N, 24.8973° E, 162 m a. s. l.). The place of capture of the studied marsh frogs is near the tailings pond close to the outflow of industrial waters. Industrial pollution with SO_2, NO_2, Pb, Cd, Zn and other toxic substances has been registered. Micro-aggregates of lead, cadmium, and zinc are released into the atmosphere through air emissions (aerosols). They accumulate in soil, spreading over vegetation and aquatic areas. The degree of pollution and the nature of pollutants for the investigated period (2018) are included in the annual reports on KCM and are controlled by the Executive Environment Agency in the Republic of Bulgaria. The areas around the plant are agricultural ecosystems. Studies show that 80% of pollution occurs in the air (pollution torch), and the remaining 20% is due to polluted irrigation water. The physicochemical analysis of the surface waters at the place of sampling is of a highest importance. The data in present study originates from a physicochemical monitoring of the surface water done by the Basin Directorate for Water Management-East Aegean Sea, Region-Plovdiv, Ministry of the Environment and Waters. Table 1 presents information about studied site in Chaya river for a three-year period 2015–2017 (average annual values and lowest and highest measured values) as well as data collected during present study (April 2018). The main pollutants are heavy metals and metalloids (lead, cadmium, zinc, copper and arsenic) as well as nutrients (ammonium nitrogen, nitrate nitrogen, nitrite nitrogen, and total nitrogen).

Table 1. Ecological status of the studied site at the Chaya river for the period 2015–2017, based on the data contained in newsletters of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, Ministry of the Environment and Waters. Physicochemical substances are presented with average annual values and the lowest and highest measured values for each year (for April 2018: recent data at the time of the study). *Legend:* Temperature (Temp), electrical-conductivity (EC), dissolved oxygen (DO), oxygenation (Ox), biological oxygen demand five days (BOD5), chemical oxygen demand (COD), calcium carbonate hardness (CCH), ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), total nitrogen (TN), orthophosphates (PO_4^{3-}), total phosphorus, as P (TP), iron dissolved in water (Fe), lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd), depending on the hardness classes of water, manganese (Mn), nickel (Ni), arsenic (As), mercury (Hg), aluminum (Al), not monitored (NM). Physicochemical data (water): * – The values deviating (< , >) Standards for high water quality according to Ordinance No. H-4 (Ordinance, 2012) on the characterization of surface waters in Bulgaria (State Gazette No.22 of 5.03.2013); ′ – the values above: AAV (Average annual value) and ″ – MPC (maximum permissible concentration) according to Ordinance No. 256 (Ordinance, 2010) for Standards on environmental quality for priority substances and for certain other pollutants (State Gazette No. 88 of 9.11.2010).

Parameters	Standards for high water quality	Polluted site (Chaya River)			
		2015	2016	2017	April 2018
Temp °C	-	13.2 (5.0–22.1)	15.7 (11.0–21.0)	14.5 (6.0–22.0)	10.0

pH units	-	7.5 (7.3–8.1)	7.7 (7.1–8.1)	8.0 (7.9–8.3)	8.03
EC µS/cm	700.0	477.2* (248.0–703.0)	648.7* (253.0–1096.0)	543.0* (250.0–996.0)	390.0*
DO mg/l	9.0–7.0	9.1 (7.2–11.9)	9.3 (6.7–11.3)	9.9 (7.5–12.5)	12.2
BOD_5 mg/l	<2.0	1.47 (0.50–2.0)	1.36 (0.50–3.3)	2.35 (0.9 –4.5)	2.0
COD mg/l	25.0	8.9 6.7–11.1	15.2 6.0–41.0	17.3 6.0–30.0	16.0
Ox %	100–105	88.2 (68.0–92.0)	90.2 (68.0–114.0)	93.5 (76.0–106.0)	114.0
CCH mg $CaCO_3$/l	-	181.7 (105.0–255.0)	218.5 (112.0–357.0)	193.6 (108.0–320.0)	143.0
NH_4^+-N mg/l	<0.10	0.15* (0.12–0.20)	0.21* (0.12–0.31)	0.35* (0.19–0.56)	0.21*
NO_2^--N mg/l	<0.03	0.04* (0.03–0.07)	0.03 (0.02–0.04)	0.05* (0.02–0.07)	0.03
NO_3^--N mg/l	<0.7	1.98* (0.49–4.0)	2.49* (0.47–4.1)	1.42* (0.75–3.2)	0.62
TN mg/l	<0.7	2.78* (1.4–3.9)	2.96* (1.5–4.0)	2.13* (1.3–3.6)	1.3*
PO_4^{3-} mg/l	<0.07	0.05 (0.04–0.06)	0.03 (0.02–0.05)	0.07 (0.02–0.12)	0.03
TP mg/l	<0.15	0.09 (0.06–0.17)	0.07 (0.05–0.12)	0.13 (0.05–0.21)	0.05
Fe µg/l	AAV: 100.0; MPC: not applicable	24.0 (20.0–32.0)	27.0 (20.0–41.0)	34.2 (20.0–69.0)	19.06
Pb µg/l	AAV (1.2); MPC (14.0)	3.24′ (1.12–8.0)	10.17′ (1.12–55.0)	3.87′ (1.55–9.4)	6.4′
Cu µg/l	AAV: 1.0 ($CaCO_3$ 0–50 mg/l); 6.0 ($CaCO_3$ 50–100 mg/l); 10.0 ($CaCO_3$ 100–250 mg/l); 22 ($CaCO_3$ > 250 mg/l); MPC: not applicable	2.31 (1.81–2.6)	2.55 (1.81–3.3)	6.95 (3.8–12.1)	12.2′
Zn µg/l	AAV: 8.0 ($CaCO_3$ 0–50 mg/l); 40.0 ($CaCO_3$ 50–100 mg/l); 75.0 ($CaCO_3$ 100–250 mg/l); 100.0 ($CaCO_3$ > 250 mg/l); MPC: not applicable	143.3′ (54.0–244.0)	651.0′ (186.4–1160.0)	64.0 (52.0–72.0)	57.0
Cd µg/l	AAV: ≤0.08 (class 1); 0.08 (class 2); 0.09 (class 3); 0.15 (class 4); 0.25 (class 5); MPC:≤0.45 (class 1); 0.45 (class 2); 0.6 (class 3); 0.9 (class 4); 1.5 (class 5)	1.92′,″ (0.63–4.2)	8.74′,″ (0.61–49.0)	1.96′,″ (0.18–3.0)	1.11′,″
Ni µg/l	AAV: 4.0; MPC: 34.0	0.61 (0.46–1.41)	0.70 (0.46–2.1)	1.49 (0.47–3.8)	0.67
As µg/l	AAV: 10.0; MPC: 25.0	2.72 (0.47–6.6)	4.89 (0.64–14.0)	5.11 (1.2–13.4)	0.46
Hg µg/l	AAV: not applicable; MPC: 0.07	<0.01	<0.01	<0.01	0.01
Al µg/l	AAV: 15.0; MPC: 10 (pH < 6.5); 25 (pH > 6.5)	5.53 (5.56–5.82)	10.76 (4.5–22.0)	15.6 (5.4–39.0)	1.45

The background region is located in south-eastern Bulgaria and is part of the largest protected area from Strandzha Nature Park. The whole territory of the SNP is included in the network of EU areas of nature protection Natura 2000. There are no important local sources of industrial pollution (Peev & Gerasimov, 1999). No exceedance of the heavy metals soil concentrations has been found in any of the heavy metal monitoring stations in the SNP as well as in the whole surrounding region for the investigated period (Annual report for the activity of RIEW Burgas). The sampling site of the Veleka river was near the confluence with the Black Sea (N42°03′40′′, E27°57′56′′).

Material

Forty animals from KCM area and 39 animals from the SNP, adults (average snout-vent length 83.16 ± 17.91 cm) and sexually mature (Bannikov et al., 1977), were randomly caught. The field research was conducted in the period April - June 2018. Following the Sutherland's (2000) methodology, 1-kilometer-long and 4-meters-wide stretches of shores have been passed. The animals were captured alive at night, blinded by artificial light, and then transported in buckets full of water to the laboratory where the entire laboratory analysis was performed. All individuals (61 males and 18 females) were identified by sex based on secondary sexual characteristics: the presence of "marital corns" on the first finger and resonator bubbles in the corners of the mouth of the males.

The animal handling and laboratory methodology was approved by the Ethics Board for Experimental Animals at the Faculty of Biology at the University of Plovdiv.

Methods

Haematological analyses were done in laboratory conditions, 1 day after the capture (actually less than 24 h after the catch). In order to prepare blood smears, blood was isolated by cardiac ventricular puncture after the anesthesia of frogs. The blood was spread in a thin layer on a glass slide. A minimum of 2 blood smears were prepared from each individual and dried for about 24 hours at a room temperature. The dried slides were fixed for 10 min in absolute methanol (Merk) and stored in a dark and dry place until stained. All prepared blood smears were stained with the fluorescent dye acridine orange (AO) according to Hayashi et al. (1983). AO was prepared first as a 0.1% aqueous solution and then 0.24 mM AO was dissolved in 1/15 M Sörensen phosphate buffer (pH 6.8). Fixed cells were stained with this solution for 3 minutes at room temperature. The slides were rinsed in the buffer three times each 1 to 3 minutes. The samples were studied immediately after AO staining by a Leica DM 1000 fluorescence microscope equipped with a special filter (I3), with a lens 100 × below immersion.

Nuclear abnormalities (NAs), including micronuclei, have been reported according to the criteria of Carrasco et al. (1990), Fenech (2000) and Furnus et al. (2014). Nuclei with a substantial notch into the nucleus were noted as notched nuclei (NotchN); those with larger evaginations (lobe), including those with several lobes, are lobbed (LobeN); the nuclei with relatively small evaginations of the nuclear membrane and contained euchromatin are reported as blebbed (BlebN); those that have relatively small formation connected to the nucleus by a stalk of nucleoplasmic are considered to be nuclei with nuclear buds (NBud); "Eight" shape nuclei (EN) were distinguished according to Furnus et al. (2014) and represented a constriction resembling the shape of the digit eight; kidney shaped nuclei (KN) were reported also; cells in which, in addition to the main nucleus, there is a less isolated nucleus with a round or oval shape, which is not larger than 1/5 of the main nucleus, focuses and fluoresces with the same color as the main nucleus, we consider as erythrocytes with micronucleus (MN). Binucleated cells (BN) were defined as cells with two nuclei of approximately equal sizes. Bridge-like formation (NBr) between two daughter erythrocytes were described according to Anbumani & Mohankumar (2011).

The average frequency of nuclear abnormalities per 2000 scored erythrocytes (polychromatic and normochromatic), expressed in per mille, was calculated for each individual with the following formula:

$$NAs\ Frequency\ ‰ = \frac{Number\ of\ cells\ containing\ NAs}{Total\ number\ of\ cells\ scored} \times 1000$$

Photomicrographs taken with the Leica Application Suite were processed with the ImageJ program (Abràmoff, 2004) with the addition of the "Cell Counter" plugin.

Statistical methods

The data were first tested for both normal distribution (D'Agostino and Pearson omnibus normality test) and homogeneity of variance (Levene, F-test). The MN and NAs data was not normally distributed and therefore the non-parametric Mann-Whitney test was used. For all tests, the level of significance was set at $P \leq 0.05$. All calculations were performed with the software Prism, version 4.02 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Mature erythrocytes of *P. ridibundus* have an oval shape with a centrally located nucleus (Fig. 1A). The nucleus is oval, clearly structured and has a well-defined boundary, which facilitates the identification of fragments in the cytoplasm. Fluorescence microscopy showed different types of nuclear abnormalities – notched nuclei (NotchN), blebbed nuclei (BlebN), nuclear buds (NBud), lobbed nuclei (LobeN), kidney shape nuclei (KN) and eight-shaped nuclei (EN), cells with MN, binuclear cells, mitotic erythrocytes (erythroblasts) (Fig. 1I). The frequency of the first three nuclear abnormalities – NotchN, BlebN and NBud, is many times higher than the other abnormalities. Therefore, all other abnormalities, except micronuclei, were pooled in "Other NAs".

The non-parametric Mann-Whitney test proved the absence of statistically significant differences between sexes in all scored NAs: BlebN (P = 0.3157; U = 1773), NotchN (P= 0.6956; U = 1896), NBud (P = 0.0553; U = 1584); Other NAs (P = 0.7973; U = 1924); MN (P = 0.5573; U = 1909) and Total NAs (P = 0.4574; U = 1824). For the subsequent analyses, we combined males and females per site. The lack of sex dependence in the frequency of NAs in *P. ridibundus* is not surprising, as similar biomonitoring studies also did not report statistical differences between sexes in detection of NAs in erythrocytes of anurans (Pollo et al., 2015; Pollo et al., 2016).

Significant differences in NAs frequency among impact and background sites were found (Fig. 2) with the lowest frequency recorded for SNP.

The highest values of the average frequency of Total NAs at KCM area can be associated with the effect of polymetallic pollution in this area. The obtained average value for Total NAs (49.14 ± 36.59) differs significantly ($P \leq 0.0001$) from the established value in the background region SNP (9.75 ± 13.94) (Fig. 2 F). It is also higher than that recorded by Şişman et al. (2015) in *P. ridibundus* in Turkey. The highest recorded value by the authors for Total NAs (‰) is 11.07 ± 4.06, however only four types of anomalies were reported – LobeN, NotchN, MN and KN. The authors also prove a direct correlation between the frequency of NAs and the concentration of heavy metals in surface waters.

NotchN, BlebN and NBud frequency increased significantly in KCM in respect to SNP ($P \leq 0.0001$). NotchN occurs in the impact regions with the highest frequency, followed by NBud and BlebN. This indicates that a cytotoxic effect is observed in *P. ridibundus* erythrocytes in the impacted region. Although the origin of notched nuclei remains unclear and has not been fully studied, their occurrence in fish and amphibian erythrocytes has been associated with the presence of cyto- and genotoxic agents

(Pollo et al., 2015). Increased frequencies of notched nuclei have been observed in other studies of amphibians inhabiting contaminated areas (Pollo et al., 2015; 2016; Şişman et al. 2015; Raghunath et al., 2017; Corduk et al., 2018). Erythrocytes of individuals of *P. ridibundus* inhabiting a river with pollution of anthropogenic origin (Şişman et al., 2015) have an increased but lower average frequency of NotchN, ‰ (1.78 ± 0.34) compared to our impact region (21.70 ± 15.24). On the other hand, an anomaly of another type (BlebN) prevails with the highest frequency, which shows the potential of different pollutants or their combined impact to predominate the different types of NAs. According to Fenech et al. (2011) NBuds are associated with DNA amplification and repair processes and may be associated with unnecessary chromosomes in aneuploid cells. Shimizu et al. (1998, 2000) used *in vitro* experiments with mammalian cells to show that amplified DNA is selectively localized at specific sites on the periphery of the nucleus and is eliminated by nuclear buds during the S phase of the cell cycle. Amplified DNA can be eliminated from chromosomes by recombination of homologous regions of amplified sequences forming minicircles of centric regions called "double minutes". NBuds are characterized by the same morphology as MN, except that they are connected to the nucleus by a narrower or wider column of nucleoplasmic material, depending on the stage of the process. Bolognesi et al. (2006) reported a strong association between the induction of MN and NBuds in fish erythrocytes. The mean frequency of NBuds (‰) obtained in the present study is the highest in KCM area (Fig.2.B), which may be related to the recorded long-term heavy metal contamination in this region. For example, the observed values (15.32 ± 11.06) are ten times higher than those published by Pollo et al. (2015) for *Rhinella arenarum* inhabiting an artificial water basin in urban conditions (1.14 ± 1.27).

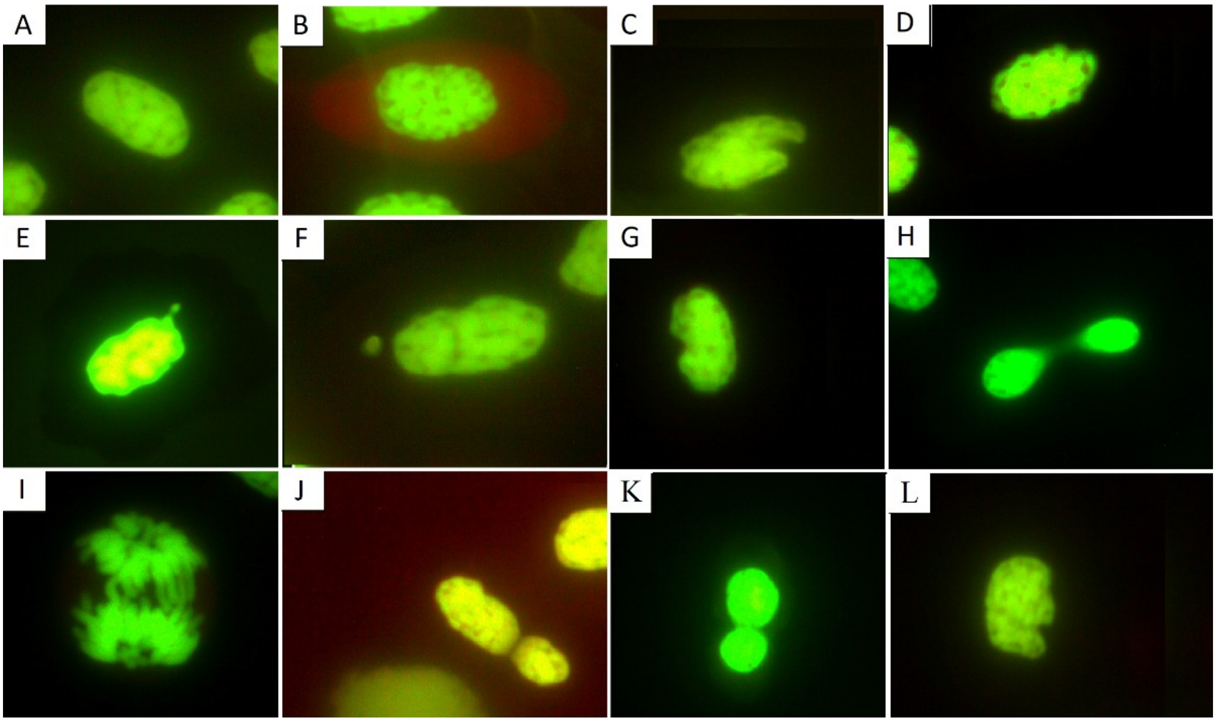

Fig. 1. Different type of NAs in erythrocytes of *P. ridibundus*: A) normal normochromatic erythrocyte without abnormalities; B) normal polychromatic erythrocyte; C) notched nucleus – NotchN; D) blebbed nucleus – BlebN; E) nuclear bud – NBud; F) micronucleus – MN; G) kidney shape nucleus – KN; H) nucleoplasmatic bridge - NBr; I) mitotic erythrocytes; J) binucleated erythrocyte – BN; K) “eight” shape nucleus – EN; L) lobed nucleus – LobeN.

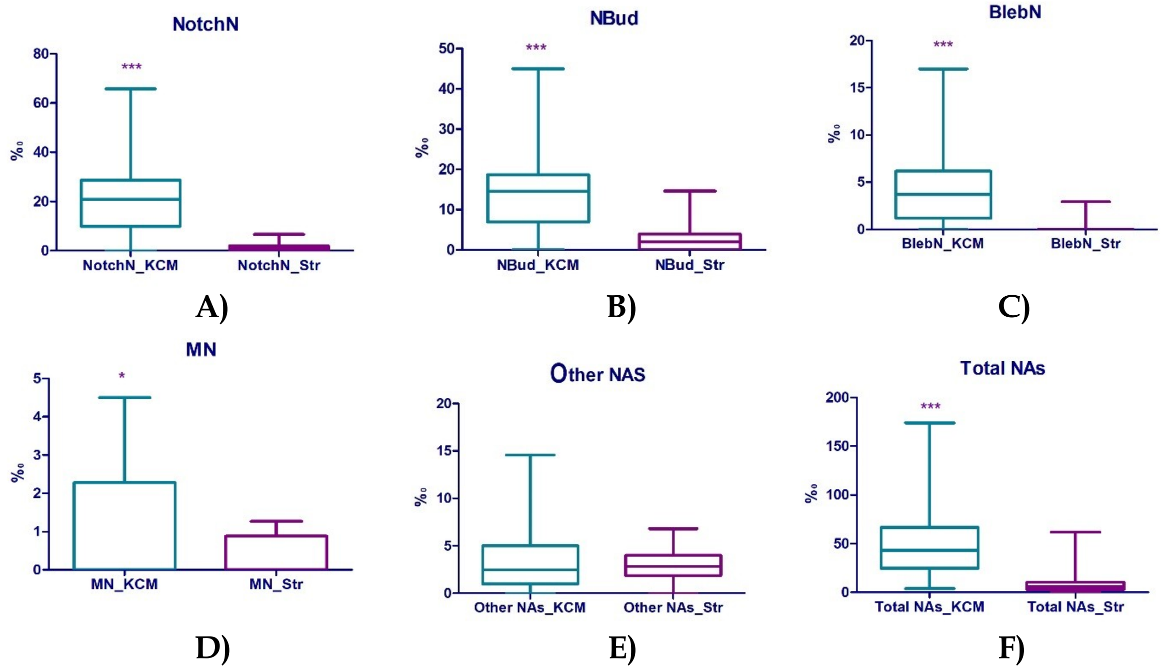

Fig. 2. Comparative analysis of frequencies (‰) of different types of NAs in polluted (KCM, Plovdiv) and background region (Strandzha Nature Park): A) notched nuclei B) nuclear buds; C) blebbed nuclei; D) micronuclei; E) Other NAs - lobbed nuclei, kidney shape nuclei and eight-shaped nuclei, binuclear cells, mitotic erythrocytes (erythroblasts); F) Total NAs. Bottom and top of the box represent 25 and 75% percentile values, respectively, with median values within the box. Error bars indicate minimum and maximum values (*** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$, compared to negative control).

Rare abnormalities reported in single cells of individuals were pooled in Other NAs. No statistically significant differences were found concerning Other NAs between the investigated sites ($P = 0.83$, $U = 367$) (Fig.2 E). The low values of the mean frequencies of Other NAs in the impact and background regions indicate that their values are probably close to the normal values for the species. In the impact region there is greater individual variability than in the background region, which proves the susceptibility of *P. ridibundus* as a zoomonitor species. This variability is probably due to the presence of genotoxic agents in the affected areas, which induce the appearance of rarer abnormalities, such as nucleoplasmic bridges and mitotic erythrocytes, which we included in the general sample.

The induction of NAs and micronuclei in our study is not surprising concerning the heavy metal pollution in the investigated area. Other authors (Zhelev et al., 2020) report also that *P. ridibundus* individuals inhabiting the same site in Chaya River have severely deteriorated general health status, suppressed hemopoiesis and a weakened immunity due to the high levels of toxicants of anthropogenic origin (human industrial activity) combined with nitrate fractions and heavy metals. The levels of cadmium and lead in the liver and muscles of frogs were significantly higher than those in frogs from reference site. In addition, our study proves that chronic exposure to heavy metals in polluted area causes clear cyto- and genotoxic effects on amphibians red blood cells *in situ*.

BlebN as well as LobeN are considered precursors of MN (Shimizu et al., 1998; Anbumani & Mohankumar, 2012) associated with a mutagenic effect. Their increased frequency depends on the dose of exposure and can be explained by the cellular mechanism for dealing with excess chromatin, in which the secreted genetic material is incorporated into micronuclei and can be expelled from the cell as a "double minute" (Shimizu et al., 1998). It is hypothesized that accurate initiation of the Breakage-Fusion-Bridge cycle to separate entangled and attached chromosomes is associated with gene amplification and may lead to the formation of LobeN or BlebN, NBuds, nucleoplasmic bridges, and MN during isolation of amplificated nuclear DNA (Shimizu et al., 1998, 2000; Fenech et al., 2011). In this sense, the nuclear abnormalities registered in the present study in the impact region demonstrate genetic instability in the studied individuals, caused by the presence of cyto- and genotoxic agents in the environment.

Conclusions

The obtained results reveal the greater induction of NAs in erythrocytes of march frog, *P. ridibundus* inhabiting polluted waters (Chaya river) close to the lead-zinc smelter near Plovdiv (Bulgaria). NAs of the following types: notched nuclei, nuclear buds and blebbed nuclei have shown highest frequency. The NAs frequency and micronucleus frequency are significantly higher in the impact area than those from the control region (Veleka river, Strandzha Nature Park). Our findings demonstrate a clear cyto- and genotoxic effect, which shows that the anurans in the polluted region are vulnerable to polymetallic contamination. Our study also confirmed that the assessment of NAs in addition to the MN test determinese the potential cyto- and genotoxic effect of heavy metals on amphibians red blood cells *in situ*.

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Occurrence of Haemoparasites of the Genus Hepatozoon (Adeleorina: Hepatozoidae) in the Marsh Frog (Pelophylax ridibundus Pallas, 1771) in Bulgaria

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Abstract. The distribution of species of *Hepatozoon* from anurans, specifically frogs, and their host-parasite relationships are of great interest. Due to the specifics of the ontogeny of frogs, they form a link between aquatic and terrestrial ecosystems. These amphibians are important in food chains, ensuring the normal functioning of biocenosis. In this study, we present data on the presence of *Hepatozoon* sp. in the marsh frog, *Pelophylax ridibundus* (Pallas, 1771) for the first time in Bulgaria. The blood smears of 137 individuals were investigated by fluorescence microscopy after staining with acridine orange. In three of five studied localities, the presence of apicomplexan haemoparasite from the genus *Hepatozoon* was revealed. Prevalence and parasitaemia values were different in frogs populations inhabiting the Chaya River (27.5% and 11.0%, respectively), Tsalapitsa Rice Fields (6.4% and 9.9%, respectively) and nature wetland Zlato pole (10.0% and 8.1%). It was found that the morphology and morphometric parameters of the parasite gamonts are closest to *Hepatozoon magna*, but molecular tools are required to confirm the genus and species determination. Our findings revealed that acridine orange is appropriate dye for detecting haemoparasites of the genus *Hepatozoon*.

Key words: amphibia, blood parasite, *Hepatozoon, Pelophylax ridibundus.*

Introduction

Apicomplexan haemoparasites of genus *Hepatozoon* Miller 1908, of the family *Hepatozoidae* (Wenyon, 1926) can infect a wide range of vertebrate animals that act as intermediate hosts (Smith, 1996). The gamont stages parasitize red blood cells in reptiles, amphibians, as well as leukocytes in birds and mammals. They are transmitted by a variety of arthropods, especially mosquitoes and ticks, which they use as definitive hosts (Smith, 1996). Their life cycle comprises roughly four stages: merogony and gamogony in the vertebrate host, and fertilization and sporogony in the arthropod host. Gamonts are usually intraerythrocytic and are variable in shape; some with broad shape, others with narrow and elongate shape (Rajabi et al., 2017).

More than 45 species of *Hepatozoon* have been described from amphibians around the world (Smith, 1996). Despite the wide geographical distribution of *Hepatozoon* species the data about this blood parasite found in frogs from certain geographical areas such as Europe and the Balkans are few compared to reports from Africa, North America, and Asia (Smith, 1996; Netherlands et al., 2014, 2018). Currently, despite the biological significance of the genus *Hepatozoon* the ecological and biological characteristics of the species are not completely understood in many areas due to the lack of information (Korzh & Zadorozhnyaya, 2013).

The distribution of blood parasites from anurans, specifically frogs, and their host-parasite relationship are especially important. The frogs form a link between aquatic and terrestrial ecosystems due to their specific ontogeny. These amphibians are important in food chains, preying on a variety of invertebrates, serving as prey for different animal groups, and ensuring the normal functioning of biocenosis (Toledo et al., 2007). At the same time, amphibians are among the most threatened vertebrate groups in the world due to the climate change, habitat destruction, and emerging diseases (Ferreira et al., 2020).

The marsh frog *P. ridibundus* is the anuran with the widest distribution in Bulgaria but the presence of parasites in its blood still remains unstudied. In the last decade, infection with *Hepatozoon magna* in *P. ridibundus* were reported from Russia (Peskova et al., 2018), Ukraine (Korzh & Zadorozhnyaya, 2013), and Iran (Rajabi et al., 2017). Previously, *Hepatozoon ridibundae* was detected in marsh frogs inhabiting Saudi Arabia (Shazly, 2003).

In Bulgaria, there are only three reports on representatives of species of *Hepatozoon*, one in passerine birds (Shurulinkov, 2003) and two in dogs (Ivanov & Kanakov, 2003; Tsachev et al., 2008). The current study is the first record of *Hepatozoon* sp. in amphibians from Bulgaria and the purpose of this paper is to present data about infection prevalence in established *Hepatozoon* reservoirs and morphological characteristics of the observed parasite in the blood of *P. ridibundus*.

Materials and Methods

Totally 137 sexually mature individuals of marsh frogs (*P. ridibundus*) were collected in five sites (Table 1) of Southern Bulgaria: 1) the Chaya River (synonyms: Chepelare River, Assenitsa River) near the confluence with the Maritza River (N42°15'61′′, E24°89′73′′); 2) the Vacha River (N42°03'43′′, E24°47'04′′), south of the town of Krichim, inside "Izgoraloto Gyune" Reserve; 3) the Tsalapitsa Rice Fields (N42°23'30′′, E24°58'42′′); 4) the natural wetland Zlato Pole (N42°02'12", E25°42'55") located several kilometers away from the town of Dimitrovgrad, south of the Zlato Pole village; and 5) the Veleka River near the confluence with the Black Sea (N42°03′40′′, E27°57′56′′). The last three localities are a part of the protected by Natura 2000 wetlands in Southern Bulgaria: protected area “Orizishta Tsalapitsa” (BG0002086), protected area "Martvitsata Zlato Pole" (BG0002103) and “Strandzha” Nature Park (BG0001007).

Table 1. Distribution, sampling period and number of *P. ridibundus* examined.

Site		***P. ridibundus***	**Sampling time**
1	Chaya River	40	April 2018
2	Vacha River	20	April 2018
3	Tsalapitsa Rice Fields	47	June 2017, May 2018
4	Wetland Zlato Pole	10	September 2018
5	Veleka River	20	June 2018

The field studies were conducted during 2018, except in the Tsalapitsa Rice Fields, where the marsh frogs were collected in 2017-2018 (Table 1). The frogs were captured nocturnally in the water using active collecting and a flashlight. Analyses were done in laboratory conditions in line with the ethical standards for research work with live animals according to Beaupre et al. (2004). Animals were transported from the site of capture to the laboratory in buckets full of water. Blood was taken through a cardiac ventricular puncture using small, heparinized needles after frogs were anaesthetized with ether according to Stetter (2001). Thin blood smears were prepared, air dried and fixed in absolute methanol. The presence of *Hepatozoon* was investigated on a Leica DM 1000 LED fluorescence microscope equipped with a special I3 filter (Leica Microsystems, Germany) suitable for AO, with a lens 40 × or 100 × below immersion. The samples were observed immediately after AO staining according to Hayashi et al. (1983). AO prepared first as a 0.1% aqueous solution, which may be available for several weeks, stored at 4 °C. 0.24 mM AO was dissolved in 1/15 M Sörensen phosphate buffer (pH 6.8) and used as working solution. Fixed blood smears were stained with this solution for 3 minutes at room temperature and then the slides were rinsed in the buffer. AO binds with nucleic acids and fluoresces. The fluoresces can easily be observed in contrast to the dark background. DNA bound with the AO fluoresces a bright yellow or apple green while RNA fluoresces red.

To assess the infection, we used prevalence, which characterizes the number of infected individuals as a percentage of the total number of examined frogs, and parasitaemia. Parasitaemia, a measure of infection intensity, of intracellular *Hepatozoon* sp. stages, and in this case was determined by counting the number of parasites per 2000 erythrocytes in randomly selected fields of view of the microscope. ImageJ software was used for microphotographs analysis and measurements. *Hepatozoon* species were identified based on comparative analysis of morphometric parameters of developmental stages (Smith, 1996). All measurements (i.e., mean length and width of the body and nucleus of parasite for established reservoirs) were given in µm. Additionally, the shape index as length/width ratio were calculated for gamonts and their nuclei.

The data were processed statistically using Graph Pad Prism 5 (GraphPad Software, Inc). One-way ANOVA was performed in order to assess significant differences among studied reservoirs.

Results

The presence of haemoparasites of genus *Hepatozoon* were established in the blood of *P. ridibundus* in three of five studied locations: Chaya River, Tsalapitsa Rice Fields and wetland Zlato Pole. The values of prevalence and parasitaemia of these reservoirs were present in Table 2, except for parasitaemia of Zlato Pole, where only one individual with *Hepatozoon* sp. infection was registered (parasitaemia=8.1%).

Table 2. Indices of infection with *Hepatozoon* in erythrocytes of *P. ridibundus*.

Reservoir	Prevalence (%)	Parasitaemia (%)			
		Mean	Min	Max	SD
Chaya River	27.5 (11/40)	11.0	0.5	39.0	7.9
Tsalapitsa Rice Fields	6.4 (3/47)	9.9	0.7	27.5	7.0
Wetland Zlato pole	10.0 (1/10)	-	-	-	-

Table 3. Morphometric characteristics of gamonts of *Hepatozoon* in erythrocytes of *P. ridibundus*. *Legend:* n - number of infected frogs.

	Parameters (µm)	Chaya River (n=11)				Tsalapitsa Rice Fields (n=3)				Wetland Zlato Pole (n=1)			
		Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Gamont	Length	24.08	36.85	30.45	2.90	23.17	36.92	31.38	2.68	25.07	32.71	30.07	1.98
	Width	3.88	6.43	5.25	0.55	3.82	5.68	4.70	0.40	3.56	5.09	5.09	0.47
	Shape Index	4.15	8.13	5.85	0.74	4.80	9.09	6.72	0.79	4.92	8.52	7.11	7.11
Nucleus	Length	3.99	5.88	4.86	0.51	3.39	5.94	4.44	0.65	3.44	6.06	4.49	0.64
	Width	3.47	5.03	4.36	0.38	3.09	4.23	3.76	0.33	2.46	4.37	3.42	0.36
	Shape Index	0.80	1.35	1.12	0.14	0.87	1.58	1.19	0.17	1.00	2.21	1.33	0.28

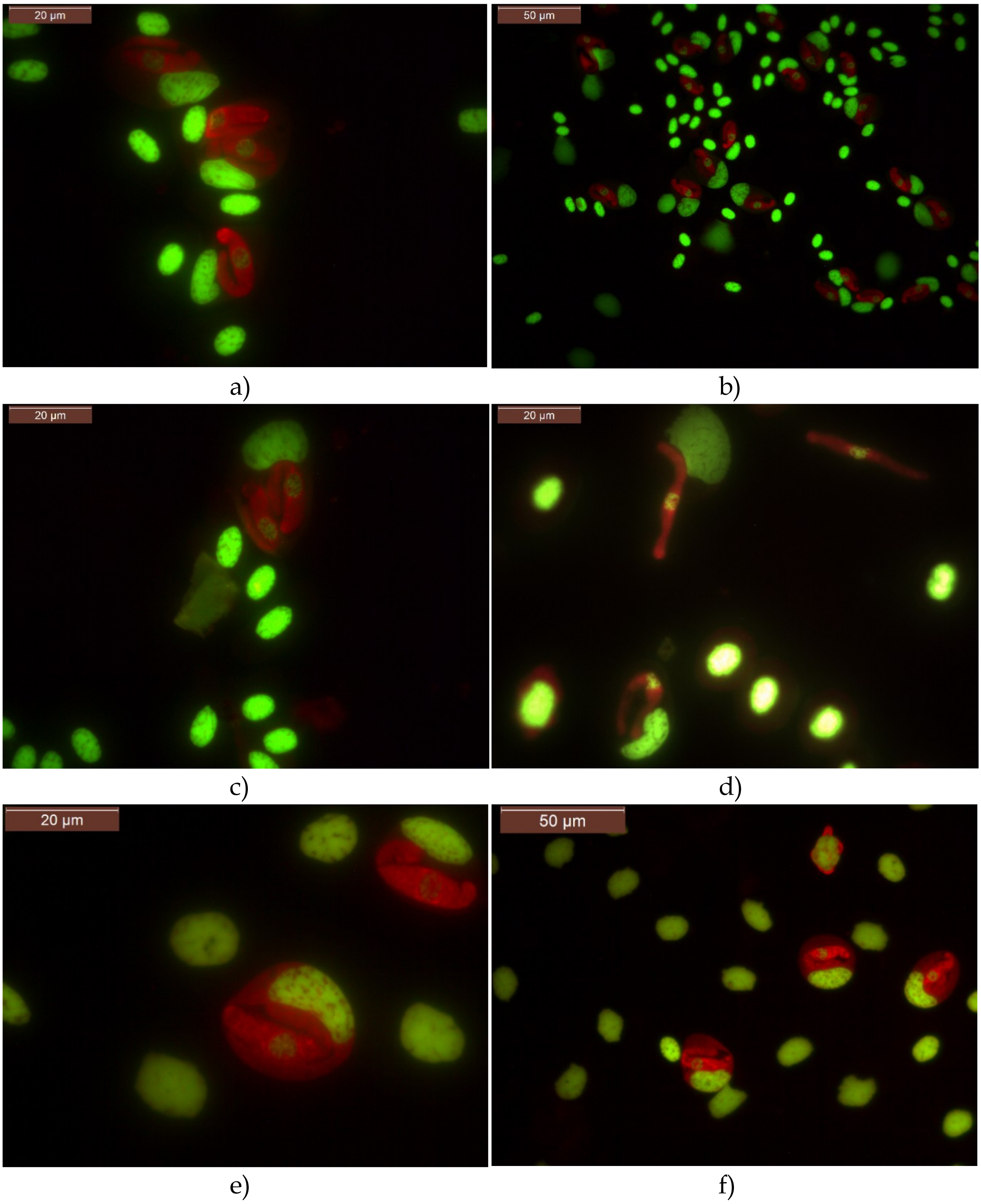

Fig. 1. Photomicrographs of *Hepatozoon* sp. observed from *P. ridibundus*: a) gamonts from Chaya River; b) individual with high value of parasitaemia; c) double infection in one erythrocyte; d) extracellular forms; e - f) red cytoplasm of host cells and increased size of nuclei of infected erythrocytes (a,c,d,e - magnification 1000 ×, in immersion; b, f - magnification 400 ×).

Extracellular forms, as well as intracellular forms (i.e., mature and young gamonts) were found. Most of observed gamonts were found within erythrocytes. Table 3 provides information on the length and width of gamonts, the length and width of their nucleus, and the shape index. The intracellular gamonts have cells bent in the middle so that the "tail" is pressed against the main body exceeding half of the body length (Fig. 1). The body shape was elongated and well differentiated in the anterior with broader rounded end and narrower posteriorly. The parasites occupy a maximum area in the host erythrocytes and were placed either with the concave border of the host cell nucleus, and or sometimes at one pole of the host cell.

Double infection (Fig. 1c) and extracellular forms have been observed only in infected erythrocytes of animals with high parasitaemia value. The extracellular forms of *Hepatozoon* (trophozoites) have an elongated worm-like body; one end of the body is narrower, the other is wider and rounded (Fig. 1d). The mean length (L) of the trophozoites is similar to gamonts, but the width (W) is less (Chaya River: L=30.6 µm, W=3.37 µm, L*nucleus*=4.652 µm, W*nucleus*=3.147 µm; Tsalapitsa rice fields: L=32.99 µm, W=3.654 µm, L*nucleus*=5.415 µm, W*nucleus*=3.436 µm; Wetland Zlato Pole: L=30.62 µm, W=3.37 µm, L*nucleus*=4.65 µm, W*nucleus*=3.147 µm).

The nuclei of parasites and host cells are well differentiated due to the staining with acridine orange. The parasite nucleus occupies almost the entire width of the cell body and has a granular structure. The host cell nucleus was displaced laterally or to one pole and appeared enlarged and frequently lobed (Fig. 1e). Erythrocytes infected with *Hepatozoon* sp. stages were increased in size, and their cytoplasm changed its color to red after staining with acridine orange (Fig. 1e, 1f).

We did not find any significant differences among the three reservoirs for the length of gamonts. However, the mean values of all other parameters (i.e., gamont width, nucleus length, nucleus width, and shape index for gamonts and nuclei) differed statistically between the three reservoirs ($P< 0.0001$).

Discussion

Amphibians are among the most threatened vertebrate groups in the world and anurans have the highest rate of population decline due to human activities, including climate change, habitat destruction, pollution, and emerging diseases (Wake & Vredenburg, 2008). Frogs are known to host a wide variety of haemoparasites, including intracellular apicomplexans from the genus *Hepatozoon* (Smith et al., 1996; Netherlands et al., 2014, 2018; Ferreira et al., 2020).

The prevalence and levels of parasitaemia of *Hepatozoon* sp. infection among marsh frogs, *P. ridibundus,* in Bulgaria was studied for the first time. Our results revealed that prevalence of *Hepatozoon* sp. in the blood of marsh frogs was higher in Chaya River (27.5%) compared to Zlato Pole and Tsalapitsa Rice Fields (10.0% and 6.4% respectively), but lower than values reported from Korzh & Zadorozhnyaya (2013) from two different biotops in Ukraine (44.4% and 66.6%). The individual frog with the highest parasitaemia, at 39.0%, was recorded in Chaya River (Table 2). The mean parasitaemia (%) for this site was 11.0±7.9, which represents a wide variability of this term. A comparison with similar studies showed various levels of parasitaemia (%) of *Hepatozoon* species in the blood of *P. ridibundus*: 10.8±1.2 and 17.3±1.8 from two different populations in Russia (Peskova et al., 2018); 2.8 in Iran (Rajabi et al., 2017); 5.8±1.8 and 10.1%±3.6 from two different populations in Ukraine (Korzh & Zadorozhnyaya 2013).

Based on the morphometrics of the gamont stages, initially we supposed the presence of *Hepatozoon magna* in blood smears of *P. ridibundus.* However, the length of gamonts were much greater in our study (i.e., 30.45±2.90, 31.38±2.68, and 30.07±1.98 µm for the three reservoirs) compared with data from other authors. Rajabi et al. (2017) reported *Hepatozoon magna* from *P. ridibundus* in Iran with gamonts that were remarkably similar in morphology as compared with those observed in our study, but with lower body length of 18.03±1.59 µm. For *H. magna* from *P. ridibundus* in Russia, Peskova et al. (2018) reported also lesser length of gamonts (16.3±0.28 and 17.2±0.58 for two studied sites), but greater width (7.1±0.16 and 7.6±0.12) compared to our results (5.25±0.55, 4.70±0.40 and 5.09±0.47 respectively). The results of Korzh & Zadorozhnyaya (2013) from two localities in Ukraine for length (35.31± 0.48 and 32.18± 0.82) and width (4.16± 0.07 and 3.68± 0.12) of gamonts observed in marsh frog are closest to the morphometrics of the gamonts from our study. Based on morphometric characteristics, the authors considered the larger parasite as *Hepatozoon magna*, and the smaller as *Hepatozoon* sp. In our study, we did not find any significant differences between the three established reservoirs only for the length of gamonts, but the mentioned authors described great variability of this parameter. *Hepatozoon* spp. are more challenging to differentiate than any of the other adeleorinid genera due to low host specificity for either or both their invertebrate and vertebrate hosts (Smith, 1996). Subtle differences in the morphology of species also makes them difficult to identify and differentiate from blood stages alone (Ferreira et al., 2020). Thus, despite the great morphological similarities with *H. magna*, the *Hepatozoon* detected in the blood of *P. ridibundus* in our study should be considered as *Hepatozoon* sp. for the present time, and the genus and species should be determined in a further analysis using molecular tools.

The fluorescent observations showed that infected erythrocytes and their nuclei are enlarged, and often lobed, compared to uninfected erythrocytes, which has also been observed in similar studies of *Hepatozoon* infection in marsh frogs (Korzh & Zadorozhnyaya, 2013; Rajabi et al., 2017; Peskova et al., 2018).

The presence of *Hepatozoon* gamonts in our research was discovered in blood smears stained with the fluorescent dye acridine orange (AO). The AO dye was found to have a high diagnostic capacity to detect different parasites like protozoans and rickettsiales in blood smears because of its higher speed of reading and sensitivity when compared with common bright field microscopy using Giemsa staining, as well as providing the opportunity to detect DNA/RNA through staining in different colors. In addition, for the microscopic observation of intraerythrocytic parasites of Giemsa-stained slides an oil emulsion objective (100 ×) is required, but the dry high lens (40 × or 50 ×) was sufficient for the AO method (Kong & Chung, 1995). The AO technique is recommended for a fast diagnosis, especially in countries with endemic areas of malaria, sleeping sickness, Lyme disease, babesiosis, and spirochetemia, and it is appropriate for detecting even cases of low-level parasitaemia (Ravindran et al., 2007; Kimura et al., 2018). Despite that the parasites reported in this study are quite large and would be easily detectible with more traditional bright-field staining techniques, the use of the AO fluorescent staining allows easy detection of a wide range of parasites in the same blood sample, including other adeleorinid blood parasites that present as much smaller intraerythrocytic forms (e.g. *Babesiosoma stableri*).

Conclusion

Our study revealed the presence of apicomplexan haemoparasite from the genus *Hepatozoon* in erythrocytes of marsh frog, *P. ridibundus*, for the first time in Bulgaria. Three of five studied populations from South Bulgaria were reservoirs of this infection. Prevalence and intensity of *Hepatozoon* was higher in Chaya River (27.5% and 11.0% respectively), followed by wetland Zlato Pole (10.0% and 8.1%) and Tsalapitsa Rice Fields (6.4% and 10.0%). Based on gamont morphology and morphometric characteristics, we suggest that the observed species is the closest to the *Hepatozoon magna* but should be considered as *Hepatozoon* sp. until confirmation in further studies, including molecular detection of the genus and species. Differences in the erythrocyte morphology of the marsh frog, when infected, were identified. Our findings revealed that acridine orange is appropriate dye for detecting haemoparasites of the genus *Hepatozoon*.

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Herbicide Effect of Greek Oregano Essential Oil on Metabolite Profiles of Target Plants

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Abstract. *Origanum vulgare* ssp. *hirtum* (Link) Ietsw. essential oil has been found to possess a wide range of biological activity among which the biocidal properties are particularly valuable in view of the need to use natural products in organic farming. In the present study, attention is focused on the metabolic variations of weeds after treatment with Greek (white) oregano essential oil as a bioherbicide. *Dasypyrum villosum* (L.) Borbás, *Matricaria chamomilla* L., *Sinapis arvensis* L., *Lolium perenne* L., *Trifolium repens* L. and *Trifolium pratense* L. were used as target weeds. The essential oil was applied on weed seedling in the form of an aqueous solution at 5 and 10 μg/mL concentrations by spraying. The effect was reported on the seventh day after treatment and expressed as lethality percentage. The studied Poaceae species were found to be the most resistant, retaining almost 100% of their viability at the both tested concentrations, while the other species at the higher concentration were completely destroyed or significantly damaged. The aerial parts of the surviving individuals of each species were collected and examined by GC/MS for the content of main metabolites. Organic, phenolic, fatty and amino acids, sterols, polyols as well as mono- and disaccharides were identified. Variations in the content of metabolites after treatment with essential oil were observed. The response of treated plants appears to be specific to each species. The results obtained provide data on the use of Greek oregano essential oil as an herbicide in post-emergence stage and complement knowledge of metabolic response of plant to stress factors.

Key words: *Origanum vulgare ssp. hirtum,* phytotoxicity, eco-metabolomics, abiotic stress.

Introduction

Eco-Metabolomics (or "Ecometabolomics") is a new field of study of metabolic response and accumulation to environmental changes or allelopathic interactions (Sardans et al., 2011; Peters et al., 2018; Sardans et al., 2020). Accepting the broader meaning of this concept, metabolic changes arising under the influence of pesticides can be included also. Although Lydon & Duke (1989) summarized the data concerning to effects of pesticides on secondary metabolites of higher plants this type of researchers is limited. In the last decades essential oils are examined as a promising alternative to synthetic herbicides (Campiglia et al., 2007; Cai & Gu, 2016; Nikolova & Berkov, 2018; Synowiec et al., 2019; Frabboni et al., 2019; Verdeguer et al., 2020). The most of the research have been directed to the establishment of inhibitory activity of essential oils against seed germination in *in vitro* assays (Ibáñez & Blázquez, 2017; Hazrati et al., 2018; Grulová

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et al., 2020). Much less studies have been conducted *in vivo* in greenhouse or in the field conditions, as well as those related to the application of essential oils at post-emergence stage (Frabboni et al., 2019; Verdeguer et al., 2020). Despite the demonstrated toxicity of essential oils against weeds, Jouini et al. (2020) have established their safety for soil organisms which confirms their potential such as environmentally friendly herbicide. Essential oils of *Origanum vulgare* and other Lamiaceae species have been proved to possess significantly phytotoxic potential (de Almeida et al., 2010; Atakm et al., 2016; Ibáñez & Blázquez 2017). In a our previous screening study of plant extracts and fractions as inhibitors of seed germination, the essential oil of *Origanum vulgare* ssp. *hirtum* was identified as the most active (Yankova-Tsvetkova et al., 2020). In the present study herbicidal potential of Greek oregano essential oil was assessed by *in vivo* experiment treating seedlings of six plant species with an aqueous solution of the essential oil. In addition, aerial parts of surviving individuals were analyzed for content of main metabolites by GC/MS. Data on metabolic changes complement the assessment of the phytotoxic potential of the essential oil.

Material and methods

Plant materials. Aerial parts of donor plant (*Origanum vulgare* ssp. *hirtum*) were collected from the *ex situ* collection of Institute of Biodiversity and Ecosystem Research (IBER), http://www.iber.bas.bg/sites/default/files /projects/plantscollection/index.html.
Seeds of target species were collected from natural population (*Dasypyrum villosum*) and *ex situ* collection of IBER (*Matricaria chamomilla, Sinapis arvensis*) as well as were purchased from Florian Company - https://brcci.eu/en/florian-ood (*Lolium perenne, Trifolium repens* and *Trifolium pretense*).

Isolation and identification of the essential oil composition. Essential oil of *O. vulgare* ssp. *hirtum* was extracted on Clevenger apparatus by water distillation. The conditions of isolation and identification of the essential oil composition was described by Traykova et al., (2019).

In vivo toxicity test. Number of fifteen seeds per plant species were planted in plastic pot (8 cm diameter) filled with substrate. Pots were placed in a growth chamber with average temperature 23ºC and 30% humidity. The studied plants at second true leaf stage were sprayed with an aqueous solution of essential oils at concentration 5 and 10 µl/mL by hand sprayer. The spraying rate was 50 mL/m^2. Seven days after spraying, the treated plants were checked for visible injury and the vital individuals were counted. The results are reported as the lethality percentage (LP) using the following formula:

$$LP=[N-n/N] \times 100,$$

where: N is number of healthy individuals before treatment; n is number of alive individuals after treatment.

The experiment was repeated three times for each plant species. Aerial parts of the surviving individuals were collected for subsequent analysis by GC/MS.

GC/MS analysis. Air dried, ground plant material of target plants (50 mg) was placed in Eppendorf and was extracted with 1mL methanol for 24 hours at room temperature with added internal standard 3,4 dichloro-4-hydroxybenzoic acid (50 µg/mL) at the beginning of the extraction. The amounts of metabolites were estimated against this standard. For GC/MS analysis 300 µL of each extract was transferred to a vial and evaporated to dryness, then silylated with 50 µL of N, O-Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C. The spectra were recorded on a Thermo Scientific Focus GC combined with a Termo Scientific DSQ mass detector as described previously (Nikolova et al., 2019).

Statistical analysis. Statistical analyzes were performed using Microsoft Excel software. The results are presented as mean with standard deviation (SD).

Results and Discussion

Essential oil composition. Chemical composition of *Origanum vulgare* ssp. *hirtum* essential oils was analyzed by GC/MS. The main components were identified as carvacrol (74,34%), *p*-cymene (9,46%), *γ*-terpinene (4,24%) and *β*-pinene (1,73%). The other components are presented in quantities of less than 1%.

The established composition of the essential oil is in accordance with the previously reported profiles of samples collected from Bulgaria and Hungary (Veres et al., 2003; Konakchiev et al., 2004).

In vivo toxicity test. Seeds of five target species were grown in plastic plots to the second true leaf stage. At this stage seedling were treated with essential oil and after 7 days the results were assessed by the number of surviving individuals and their morphological status. It was established that the treatments had the phytotoxic effect on the target plants. The plant response to essential oil treatment was found to be dose-dependent and specific to each species. Poaceae species – *D. villosum* and *L. perenne* had kept their vitality at 100% at the both tested concentrations, while the other species – *S. arvensis*, *M. chamomilla*, *T. repens T. pratense* at the higher tested concentration were completely destroyed or significantly reduced (Table 1). The treatment of weeds with 5 μg/mL essential oil solution had caused 64% mortality of *T. repens*, 41% of *T. pretense,* 33% of *S. arvensis* and 15 % of *M. chamomilla*. No lethal effect was found for Poaceae species. Application of a solution with concentration at 10 μg/mL resulted to lethality rates 100% of *T. repens,* 94% of *M. chamomilla,* 89% of *T. pretense,* 42% of *S. arvensis* and 33% of *L. perrene.* No lethal effect was found on *D. villosum.* The state of untreated (controls) and the treated *T. pratense* plants with the both concentrations of essential oil, seven days after treatment, are presented at Fig.1

Morphological changes on the treated plants were examined also. On Poaceae species only a slight burn at the top of single leaves was found while in more sensitive species spots of different size and color on the leaves were observed.

Table 1. Lethality of target plant species after treatment with essential oil solution.

Treated weeds	Applied concentration of essential oil [μl/mL]	Lethality of individuals [%] mean±SD
Dasypyrum villosum	5	0±0
	10	0±0
Lolium perenne	5	0±0
	10	34±10
Sinapis arvensis	5	33±6
	10	42±11
Matricaria chamomilla	5	15±3
	10	94±9
Trifollium repens	5	64±11
	10	100±0
Trifollium pratense	5	41±15
	10	89±12

Metabolite analysis of treated plants. Methanolic extracts of aerial parts of surviving individuals were analyzed for metabolite profiles by GC/MS. Organic, phenolic, fatty and amino acids, sterols, polyols (sugar alcohols) as well as mono- and disacharides were identified. Variations in the accumulation of studied metabolites after treatment with essential oil in comparison with the control were observed. A reduction in the amount of phenolic acids was found in *L. perrene* individuals (chlorogenic, quinic and protocatechuic acids) *S. arvensis* (ferulic and 3,4-dimethoxycinnamic acid), *D. villosum* (4(p)-hydroxybenzoic acid). Only in *M. chamomilla* increased quinic acid content was found after treatment. With regard to the content of organic acids, that of malic and succinic acid was found to decreased in the more resistant species (*D. villosum*, *L. perrene*, *S. arvensis*) while in the more sensitive species (*M. chamomilla*, *T. repens*, *T. pratense*) their amount was increased. A similar trend was observed with the accumulation of quinic acid. In the case of fatty acids it was found that the content was increased in *L. perenne*, *T. repens* and decreased in *T. pratense* and *D. villosum*. At both species - *M. chamomilla* and *S. arvensis* the content of octanoic acid decreased and that of hexadecanoic acid increased in treated individuals. The polyol - myoinositol was found to be increased in *D. villosum*, *L. perrene* and *M. chamomilla* but in other studied species its content decreased. The amount of disaccharide - sucrose decreased in all studied species with exception of *M. chamomilla*. Amino acid - prolin increased in *L. perene*, *M. chamomilla* and *T. pratense*. The content of amino acids - serine and threonine increased in *D. villosum*, *L. perrene*, *M. chamomilla*, *T. repens.* The sterol content of the studied species was not affected by treatment with exception of *S. arvensis* and *T. repens*.

Fig. 1. Herbicide effect of *O. vulgare* ssp *hirtum* on *T. pratense* seedling.
A - plants treated with 10 µg/mL essential oil solution;
B - plants treated with 5 µg/mL; C - controls, untreated individuals.

Table 2. Identified metabolites in methanolic extracts of treated weeds with essential oil of *Origanum vulgare* ssp *hirtum. Legend:* c – control; Metabolites quantification was based on internal standard, added in the beginning of the extraction, using the calculated areas for the both components.

Compounds	*Dasypyrum villosum*			*Lolium perrene*			*Sinapis arvense*			*Matricaria chamomilla*		*Trifolium repens*		*Trifolim pratense*	
	Control and applied concentrations of essential oil **[µg/mL]**														
	c	**5**	**10**	**c**	**5**	**10**	**c**	**5**	**10**	**c**	**5**	**c**	**5**	**c**	**5**
Succinic acid				82.1	23.2	11.4	56.8	19.4	5.8	17.8	74.5	9.3	10.3	8.9	10.3
Glyceric acid	13.0	33.5	28.7	4.6	15.4	11.4				72.7	7.3			7.4	5.1
Malic acid	21.5	10.5	9.7	46.5	44.6	39.4	278.6	118	58.7	68.2	133.7	20.6	47.4	10.9	27.1
Pyroglutamic acid	34.8	13.5	12.2	18.0	44.8	51.9	137.2	95.6	33.9			5.0	1		
4(p)-Hydroxy-benzoic acid	1.7	0.3	0.5	0.1	0.6	0.5		0.1	0.7			0.1			
Protocate-chuic acid	0.2			9.8	1.4		0.1		0.1					0.9	0.6
Quinic acid				48.3	33.1	28.6				1.3	6.7	0.1	0.2		
Ferulic acid							1.1	0.2	0.1						
3,4-Dimethoxy-cinnamic acid							13.1	4.3	4.4						
Chlorgenic acid				7.5	5.2	4.8									
Octanoic acid	64.6	35.2	32	34.8	91.2	111.8	137	83.9	44.9	313	228	10.6	17	47.3	17.4
Hexadecanoic acid	131	45	13.3	28.5	101.4	146.9	69.1	128.1	166.7	4.9	12.1	2.9	10.8	192.5	30.8
Octadecanoic acid										1.5	1.1	0.9	2. 1	42.4	30.9
Glycerol	70.5	41.6	38.2	210	120	144	149.3	141	141.2	203.2	227.9	21.8	30.6	225.8	148
Myo-Inositol	1.9	6.9	3.9	63.1	73.4	84.7	173.8	114	35.4	31.8	75.5	20.5	6.5	102.8	65.1
Fructose 1	52.2	37.8	39.5	71.2	38.4	44.6	158.9	138.5	153.5	0.5	0.6	16.1	13.1	20.8	22.4
Fructose 2	107.7	116.1	86.9	101.5	50.1	24.7	43.3	53.7	85.1	0.4	0.7	10.9	0.9	5.7	7.4
Glycose	101.9	122	99.2	42.6	45.1	50.8	6.6	6.1	12.5	7.7	4.2	41.3	50.9	17.6	21.9
Sucrose	321.8	380	255.9	445.3	193	176	564.2	251.9	177.8	43.5	121.3	34.8	11.9	430.3	82.5
Sterol	20.8	10.7	8.9												
Campesterol	0.4	0.2	0.3	0.9	0.5	0.8	16.8	5.7	1.7	6.2	3.5				
Stigmasterol										0.4	0.1				
ß-Sitosterol	6.9	6.5	5.8	10.9	9.2	9.8	83.7	24.2	7.4	1.3	1.2	10.5	2.2		
Proline	383.9	140	104	3.5	55.5	40.4	7.1	0.2		24.1	80.7	12.2	10.6	4.5	10.6
Glycine	7.0	5.5	7.8			1.3	38.7	5.4	8.1						
Serine	122.7	226.8	246.4	3.2	30.7	27.1	122.6	32.2	0.2	0.3	0.7	6.3	6.7	2.4	1.2
Threonine	48.7	55.1	142	7.4	13.1	52	214.1	74.6	12.7	12.1	23.5	2.6	3.4		1.1
Aspartic acid	9.0	9.8		2.9	6.2	17.7	12.6			2.3	2.7		3	0.5	0.1
Glutamic acid	10.0	6.6	6.8	0.1	5.8	1					1.8	0.8	1.4		
Phenylalanine	81.2	81.3	89.5		3.7	11.5	439.9	95.2	8.1	4.1	5.5	0.2	0.7		

The received results from the metabolic analysis showed that treatment with essential oil causes variations in the content of the several metabolites. The response appears to be dose-dependent and species-specific. The most common metabolic response of treated species is a decrease in sucrose levels which can be considered as an indicator of oxidative stress. Reduced sugar levels in treated plants are a sign also of impaired photosynthesis. Increased level of free amino acids often occurring after abiotic stress and probably this is the result of protein degradation. Decreased levels of malic acid are associated with increased activity of enzymes (NADP-depend) which is also established as a plant response to various abiotic stressors (D'Abrosca et al., 2013). The accumulation of proline that is found in *L. perrene*, *M. chamomilla* and *T. pratense* is a non-specific reaction of plants to different types of abiotic stress (Singh et al., 2017). Araniti et al., (2018) shows in experiments with *Arabidopsis thaliana* seedlings that oregano essential oil disrupts the absorption of inorganic nitrogen in amino acids, which destroy the metabolism of glutamine and leads to an excess of ammonia in the leaves, that causes destructive chain processes (oxidative stress, disorders of photosynthesis and etc.). The metabolite variations observed in the present study showed indications for presence of oxidative stress, disorders of photosynthesis and etc. These are processes that occur under the influence of various abiotic stress factors.

Conclusion

The present study provides the data about herbicidal potential of *Origanum vulgare* ssp. *hirtum* essential oil applied on target plants at post-emergence state. Phytotoxic effect of the essential oil on all studied plants was established. Strong impact was found at the highest applied concentration (10 µg/mL) furthermore complete destruction was observed for *Matricaria chamomilla*, *Sinapis arvensis*, *Trifolim repens* and *Trifolium pratense*. The noted metabolic changes in the treated plants with oregano essential oil show that it has a phototoxic activity comparable to the effects of abiotic stress factors. The results obtained clearly characterize Greek oregano essential oil as a potent bio-herbicide for organic farming.

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Polycyclic Aromatic Hydrocarbons in Traditionally Used Medicinal Plants from Varna Region, Bulgaria

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Abstract. Extracts of medicinal plants are often used for preparation of food supplements, pharmaceutical products or for direct preparation of teas, but they may contain toxic substances, such as polycyclic aromatic hydrocarbons (PAHs). The content of PAHs was determined in *Matricaria chamomilla* L., *Thymus serpyllum* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Achillea millefolium* L., collected from urban and rural region near Varna, Bulgaria. The aim of this study was to compare the levels of PAHs in traditionally used medicinal plants from different regions to assess environmental pollution. Benzo[a]anthracene, chrysene, benzo[b]fluoranthene and benzo[a]pyrene are among the 16 priority pollutants pointed out by The United States Environmental Protection Agency. The PAH levels were determined in extracts of medicinal plants by GC-MS after purification. Chrysene was registered as most abundant compound in all the plant species investigated. Benzo[a]pyrene, identified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans, was not detected in the samples analyzed. The levels of investigated PAHs were significantly higher in samples from areas with intensive traffic compared to samples from suburban areas. The sum of the four PAHs in all plant species were found in the range of 0.68 (*Sambucus nigra* L.) to 6.82 μg/kg dw (*Tilia tomentosa* Moench) and was below the permissible limit of the European commission.

Key words: Polycyclic aromatic hydrocarbons, medicinal plants, GC-MS, Bulgaria.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds that consist of two, three or more condensed aromatic rings. Some of them are classified as carcinogens to humans by the International Agency for Research on Cancer (IARC, 2010). The United States Environmental Protection Agency (US-EPA) determined 16 PAHs as priority pollutants based on their incidence and carcinogenicity (EPA, 1984). Vehicle traffic (Napier *et al.*, 2008), coal combustion (Yunker *et al.*, 2002) and other pyrogenic processes (Li *et al.*, 2006) were pointed out as significant sources of PAHs in the atmosphere.

People have benefited from the biologically active components of the herbs for many years. Dried herbs are often used for direct preparation of teas and tinctures

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for oral administration or external treatment of skin disorders. Extracts of medicinal plants are included in food supplements, pharmaceutical products and cosmetics. Some of the herbs are used as spices in food processing (FSAI, 2001; WHO, 2018). Thus, the advantages of herbs are indisputable, but recent research showed that they may contain toxic substances, such as PAHs (Guillen *et al.,* 1994; EC, 2002; Šimko *et al.,* 2005; Jira *et al.,* 2008; Perelló *et al.,* 2008; Lawal *et al.,* 2017; Ilinkin *et al.,* 2020). The PAHs content in unprocessed plants depends mainly on the level of environmental pollution (Ciemniak *et al.,* 2019; EFSA, 2008). Therefore, in the EU, permissible limits for PAHs content in dry herbs were set, e.g. the levels of benzo[a]pyrene should not exceed 10 µg/kg, and the sum of the four PAHs: benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene should not exceed 50 µg/kg (EC, 2015). An introduction of a new regulatory limit for sum of the four PAHs was necessary due to high levels of PAHs found in herbs and spices (EFSA, 2008). Recent studies reported high levels of PAHs in tea leaves (Ciemniak *et al.,* 2019) which were exceeding the regulatory limit (Zelinkova & Wenzl, 2015a; Londoño *et al.,* 2015).

In Bulgaria, there is a long-lasting tradition of harvesting, drying and use of herbs for medicinal purposes and as beverages.

Local studies showed that the most often used medicinal plant species in the area of the North Black Sea coast, Bulgaria are from the Lamiaceae, Asteraceae, Apiaceae and Rosaceae families (Cherneva *et al.,* 2017; Kozuharova *et al.,* 2013; Stoyanov *et al.,* 2018) and the most commonly used plant species were *Valeriana officinalis* L., *Matricaria chamomilla* L., *Tilia tomentosa* Moench, *Ginkgo biloba* L., *Sambucus nigra* L., *Mentha piperita* L. and *Thymus serpyllum* L. (Bachev & Yaneva, 2018).

The aim of this study was to determine and compare the levels of PAHs in traditionally used medicinal plants - *Matricaria chamomilla* L. (chamomile), *Achillea millefolium* L. (white yarrow), *Tilia tomentosa* Moench (linden), *Sambucus nigra* L. (elder) and *Thymus serpyllum* L. (thyme) collected from a rural region and a suburban area (town of Varna).

Materials and Methods

Collection of the plant material

Two regions were selected to assess the impact of intensive traffic on PAH levels in medicinal plants. The urban area was defined in a main city road in Varna, Bulgaria. It is characterized by a heavy traffic – 2124 vehicles/h (MC - Annex A, 2017). The rural region - Fichoza is located about 15 km southeast of Varna. It is characterized by very light traffic – the closest vehicle counting point has registered 120 vehicles/h (MC - Annex B, 2017). The second station was selected in order to compare the levels of PAHs in plant species from both regions.

Plant species *Matricaria chamomilla* L., *Achillea millefolium* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Thymus serpyllum* L. were hand-picked from their natural habitats in the months of May - July 2018 following the guidelines of the Bulgarian Ministry of Environment and Water (MOEW, 2004). The average height of harvested plants was about 25-40 cm (including leaves, stems and flowers). The plant species were determined by "Field guide to the vascular plants in Bulgaria " (Kozhuharov, 1992).

From every plant species was prepared an average sample from at least 5–7 randomly selected individual plants. Collected herbs were dried in a ventilated and dry place at room temperature. The samples were prepared by grinding the leaves, stems and flowers of the dried plants in a mortar. A representative portion of the ground sample (1.5 g) was transferred in a glass centrifuge tube (50 mL).

Extraction, cleanup and chemical analysis

Samples were prepared according to a previously described procedure (Kowalski *et* *al.*, 2015) with some modifications. The dried samples (1.5 g) of each plant species was homogenized with 15 mL distilled water. Next, a mixture of hexane: acetone (8:2, v/v) (10 mL) was added. Samples were then vortexed for 30 minutes. Thereafter 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were slowly added. The resulting mixture was shaken again and centrifuged for 20 minutes at 3000 rpm. The supernatant was subjected to a cleanup by column chromatography.

A chromatographic glass column (10x250 mm) was loaded in the following sequence - 1 g of Na_2SO_4, a pre-prepared mixture of 5 g of silica gel (G60) and 0.4 g of primary-secondary amine (PSA) sorbent (silica gel with polymer-bonded ethylenediamine-N-propyl phase) and 1 g of anhydrous Na_2SO_4. The loaded column was conditioned with hexane. The supernatant was transferred into the chromatographic column and eluted with 20 mL hexane/dichloromethane (7: 3, v/v).

The eluate was concentrated at 40 °C on a rotary evaporator (Hei-Vap Precision Heidolph, Heidolph Instruments GmbH & CO. KG, Germany) and was transferred to a gas chromatography vial. The eluate was evaporated under nitrogen to near dryness and reconstituted in 0.5 mL hexane.

The analytical determination of PAHs was performed by gas chromatograph (GC FOCUS, Thermo Electron Corporation, USA), with a POLARIS Q Ion Trap mass spectrometer and AI 3000 autosampler. Experimental mass spectrometry parameters: transfer line and ion source temperatures were 250 °C and 220 °C, respectively. The injector was in splitless mode, with a temperature of 250 °C. The temperature program of the oven was as follows: 40 °C (1 min), 40 °C/min to 130 °C (3 min), 12 °C/min to 180 °C, 7 °C/min to 280 °C, 10 °C/min to 310 °C with a final hold for 5.0 min. The volume of sample injected was 1 µL. A TG-5ms capillary column with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 µm was used. Helium was used as carrier gas at a flow rate of 1 mL/min.

All measurements were performed in triplicate in order to ensure accuracy of the analytical procedures. The measured compounds were: acenaphthylene (ACL), anthracene (AN), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[k]fluoranthene (BkFA), benzo[ghi]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), dibenzo[a,h]anthracene (DbahA), fluorene (FL), indeno[1,2,3-cd]pyrene (IP), phenanthrene (PHE) and pyrene (PY).

Quality control

Pure reference standard solution (EPA 525 PAH Mix B, 500 µg/mL of each component in acetone, Supelco) was used for instrument calibration, quantification of compounds and recovery determination. Procedural blanks were analyzed between each 5 samples to monitor possible laboratory contamination. The limit of detection (LOD) of the method was calculated from 0.15 to 0.36 µg/kg and limit of quantification (LOQ) from 0.46 to 1.09 µg/kg. Based on the low concentrations of the analytes in plant species the method limits of detection (LOD) were estimated as 3 times the standard deviation and LOQ is the analyte concentration corresponding to ten times standard deviation. LOD for individual PAHs: 0.23 (ACL), 0.16 (FL), 0.36 (PHE), 0.34 (AN), 0.27 (PY), 0.17 (BaA), 0.20 (CHR), 0.31 (BbFA), 0.31 (BkFA), 0.15 (BaP), 0.27 (IP), 0.30 (DbahA) and 0.19 (BghiP) µg/kg.

Data analysis

Concentrations of the 13 investigated PAHs were calculated on a dry weight basis (µg/kg dw). Statistical significant differences in the mean levels of detected PAHs in samples from the two regions were tested at $\alpha = 0.05$ with Student's t-Test (Excel for Microsoft Office Professional Plus 2013).

Results and Discussion

In the present work, the levels of 13 PAHs were determined in five wild herbs: *Matricaria chamomilla* L., *Achillea millefolium* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Thymus serpyllum* L. collected from urban (Varna) and rural (Fichoza) regions in Bulgaria. The concentrations (µg/kg dw) of 13 PAHs in medicinal plants in two different locations are presented in Table 1.

Table 1. PAH levels (µg/kg dw) in the studied medicinal plants. *Legend:* LOD - Limit of detection, PAHs: polycyclic aromatic carbons; ACL: acenaphthylene, AN: anthracene, BaA: benz[a]anthracene, BbFA: benzo[b]fluoranthene, BkFA: benzo[k]fluoranthene, BghiP: benzo[ghi]perylene, BaP: benzo[a]pyrene, CHR: chrysene, DbahA: dibenzo[a,h]anthracene, FL: fluorene, IP: indeno [1,2,3-cd]pyrene, PHE: phenanthrene and PY: pyrene.

	ACL	FL	PHE	AN	PY	BaA	CHR	BbFA	BkFA	BaP	IP	DBahA	BghiP	Sum of 13 PAHs
Tilia tomentosa Moench (n=6)														
rural region	< LOD	88.26 ± 0.14	< LOD	< LOD	< LOD	< LOD	1.43 ± 0.07	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	89.69
urban area	< LOD	379.42 ± 0.72	1239.61 ± 1.00	64.76 ± 0.20	13.95 ± 0.20	0.52 ± 0.10	6.30 ± 0.30	< LOD	< LOD	< LOD	< LOD	< LOD	1.29 ± 0.11	1705.9
Achillea millefolium **L.** (n=5)														
rural region	< LOD	30.94 ± 0.31	< LOD	< LOD	< LOD	< LOD	2.37 ± 0.12	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	33.30
urban area	< LOD	251.48 ± 0.52	945.10 ± 1.01	84.29 ± 0.21	37.66 ± 0.17	< LOD	5.43 ± 0.10	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1324.0
Sambucus nigra **L.** (n=6)														
rural region	< LOD	97.53 ± 0.21	180.43 ± 0.22	< LOD	< LOD	< LOD	0.68 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	278.64
urban area	< LOD	239.50 ± 0.71	779.89 ± 0.24	85.99 ± 0.22	19.81 ± 0.25	< LOD	5.04 ± 0.09	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1130.2
Thymus serpyllum **L.** (n=4)														
rural region	< LOD	105.53 ± 0.10	225.69 ± 0.24	10.66 ± 0.06	< LOD	< LOD	0.82 ± 0.08	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	342.70
urban area	< LOD	292.45 ± 0.23	977.84 ± 0.51	63.37 ± 0.20	20.59 ± 0.17	< LOD	3.96 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1358.2
Matricaria chamomilla **L.** (n=5)														
rural region	< LOD	3.23 ± 0.10	< LOD	< LOD	< LOD	< LOD	0.86 ± 0.08	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	4.10
urban area	< LOD	163.12 ± 0.34	419.33 ± 0.29	4.20 ± 0.09	7.20 ± 0.10	< LOD	1.80 ± 0.07	< LOD	< LOD	< LOD	< LOD	< LOD	0.60 ± 0.08	596.25

Regarding the regulated four PAHs (benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene), chrysene was determined as most abundant compound in all the plant species investigated. The measured concentrations of chrysene varied from 0.68 µg/kg (*Sambucus nigra* L.) to 6.30 µg/kg (*Tilia tomentosa* Moench) and were found lower compared to other studies. Drabova (2012) reported the highest level of chrysene in tea samples - 41.9 µg/kg (black tea) and 21.2 µg/kg (green tea) (Drabova *et al.*, 2012). Zelinkova & Wenzl (2015) determined chrysene in medicinal plants and food supplements in the range <0.25 to 280.1 µg/kg (mean 13.6 µg/kg) (Zelinkova & Wenzl, 2015b). Among the regulated four PAHs chrysene was determined as prevalent compound in a study in Croatia. The mean value of chrysene in dry herbs analyzed was 10.15 µg/kg (Bogdanovic *et al.*, 2019).

Benzo[a]pyrene (BaP) was classified by the International Agency for Research on Cancer as a human carcinogen (IARC, 2010). Therefore, the EU legislation set a maximum limit for BaP of 10 µg/kg in dry herbs. In our research BaP and BbFA were found below limit of detection (<LOD) for both herbs sampled from urban and rural region. Benzo[a]anthracene was found only in *Tilia tomentosa* Moench from urban area.

Among the unregulated PAHs, phenanthrene (PHE) was found as predominant compound in all samples. The maximum concentration of PHE was determined in *Tilia tomentosa* Moench (1239.6 µg/kg in urban area) and the lowest in *Matricaria chamomilla* L. (419.33 µg/kg in urban area) (Table 1). These findings were in accordance with research by Yu et al. (2012) who reported phenanthrene as prevalent contaminant of 16 PAHs investigated in nine Chinese medicinal herbs.

Phenanthrene, fluorene, anthracene and pyrene were added to the list of priority PAHs, due to their high frequency of occurrence and potential for human exposure (US EPA, 1984; Zelinkova & Wenzl, 2015b). The presented PAHs profile with dominant less-toxic PAHs (PHE, FL, AN and PY) was in accordance to results from other studies (Ciemniak *et al.,* 2019; Bogdanovic *et al.,* 2019)

The sum of 4 PAHs in all plant species from rural region was in the range of 0.68 (*Sambucus nigra* L.) to 2.37 µg/kg dw (*Tilia tomentosa* Moench) and were below the permissible limit - 50 µg/kg of the European commission (EC, 2015). The results for herbs from the urban area showed maximum level in *Tilia tomentosa* Moench – 6.82 µg/kg dw and minimal values in *Chamomilla* L. (1.8 µg/kg) – Fig. 1. In recent study by Ciemniak (2019) was shown the highest values of the sum 4 PAHs in linden – mean 213.5 µg/kg, compared to other herbs investigated (Ciemniak *et al.,* 2019).

The sum of the 4 PAHs in medicinal plants was found to be significantly higher in samples from areas with intensive traffic (urban area) compared to samples from rural region (Fig. 1). Potential sources of toxic chemicals and contaminations in herbs and herbal products can be environmental growing conditions, method of drying (FAO, 2009), storage conditions and manufacturing processes (Chan *et al.,* 2003; Kosalec *et al.,* 2009). The higher content of pollutants in herbs from urban areas (Fig. 1) is probably due to heavy car traffic as the main source of PAHs.

The total levels of PAHs (as sum of 13 PAHs) in medicinal plant species from rural areas varied from 4.10 µg/kg (*Matricaria chamomilla* L.) to 342.70 µg/kg (*Tilia tomentosa* Moench). Our results were lower compared to data from recent studies (Ishizaki *et al.,* 2011; Yu *et al.,* 2012). Ishizaki (2011) reported the total content of 15 PAHs (in five teas and 29 medicinal herbs) in the range from 6.5 to 1112.1 µg/kg, and Yu (2012) have investigated 16 PAHs in nine Chinese medicinal herbs and the sum of 16 PAHs varied from 98.2 µg/kg (cassia seed) to 2245 µg/kg (eucommia bark) (Yu *et al.*, 2012).

The distribution pattern of PAHs with 3-6 rings is shown in Figure 2. PAHs with 3 rings (PHE, FL, AN) were predominant in all plant species and account for 96.8-98.7% of the total PAHs content, while PAHs with 4 rings (BaA, CHR, PY) and 6 rings (BghiP) were found below 2% (Fig. 2). Yu (2012), Ciemniak (2019) and Lin (2005) reported similar mass distribution. In all of these studies, low molecular weight PAHs (PHE, FL, AN and PY) were found as the dominant pollutants (Yu *et al.*, 2012, Ciemniak *et al.*, 2019, Lin *et al.,* 2005).

In samples from rural region were found only PAHs with 3 rings (PHE, FL, AN) in levels lower than concentrations in plants species from urban area (Table 1).

The molecular ratio of specific hydrocarbons is often used to investigate the anthropogenic sources of PAHs (Lin *et al.*, 2005). Main emissions of PAHs in atmosphere originated primarily from combustion processes and release of petroleum products (Kucuksezgin *et al.*, 2020).

The molecular distribution of PAHs and specific ratios in environmental and food samples are used for determination of possible sources and processes that generate these pollutants (Table 2) (Lin et al., 2005; Kucuksezgin et al., 2020). The ratios commonly used in the literature are FLUR/PY, IcdP/BPe, PHE/AN, FLU/FLU+PY (Baumard *et al.,* 1998; Yunker *et al.,* 1996; Benlahcen *et al.,* 1997; Kucuksezgin *et al.,* 2020).

The present study evaluated the PHE/AN ratio in medicinal plants from urban areas whereas the results from rural region for AN were found below LOD and no PHE/AN ratio might be calculated. The PHE/AN values of urban samples were higher than 10 (ranged from 9.07 to 19.14) showing prevalence of petrogenic source.

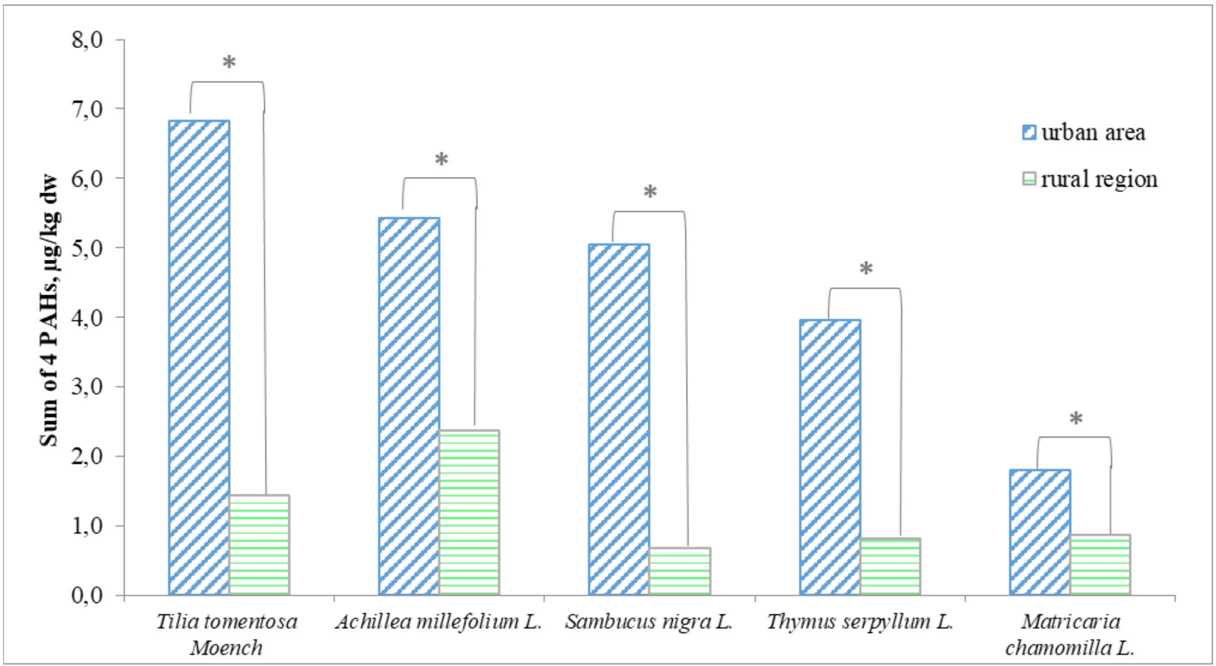

Fig. 1 The sum of 4 PAHs in samples from areas with intensive traffic and from suburban areas (* indicates statistical significant differences of $p < 0.05$).

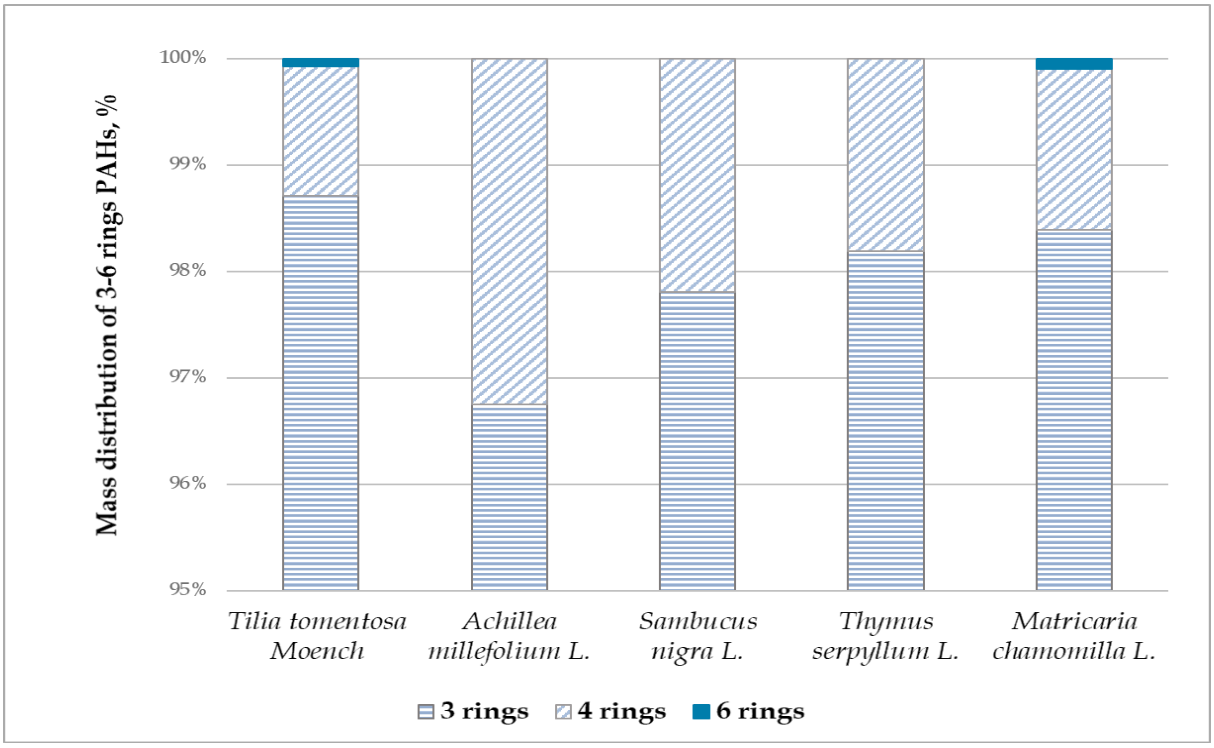

Fig. 2 Distribution pattern of PAHs with 3-6 rings in plant species from urban area.

Table 2 Potential source of pollution depending on values of molecular ratio of specific PAHs.

Ratio	Value	Potential source	Reference
FLUR/PY	> 1.0	pyrolytic origin	Baumard *et al.*, 1998
FLUR/PY	< 1.0	petroleum hydrocarbons	Baumard *et al.*, 1998
IcdP/BPe	> 1.0	combustion sources	Yunker *et al.*, 1996
IcdP/BPe	< 1.0	petrogenic sources	Yunker *et al.*, 1996
PHE/AN	< 10.0	combustion sources	Benlahcen *et al.*, 1997
PHE/AN	> 10.0	petrogenic sources	Benlahcen *et al.*, 1997
FLU/FLU+PY	< 0.4	petrogenic origin	Kucuksezgin *et al.*, 2020
FLU/FLU+PY	> 0.5	wood and coal combustion	Kucuksezgin *et al.*, 2020

Conclusion

Thirteen PAHs were determined in five medicinal plants collected from different areas near Varna. The maximum overall PAH's contamination was found for *Tilia tomentosa* Moench, while *Matricaria chamomilla* L. proved to have the minimum contamination. The levels of PAHs were found to be significantly higher in samples from urban area compared to samples from rural region. The sum of four PAHs in all traditionally used medicinal plant did not exceed the permissible limit of the European commission. The results of the present study showed that the cultivation and collection of medicinal plants from areas close to the roads with heavy traffic leads to a possible risk of accumulation of high concentrations of PAHs. The data from present research can be useful in further studies for assessing the human exposure to PAHs.

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State of the Marine Environment along the Bulgarian Black Sea Coast as Indicated by Acetylcholinesterase Activity of Wedge Clam (Donax trunculus Linnaeus, 1758)

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Abstract. The aim of the study was to assess changes in the acetylcholinesterase (AChE) activity in the wedge clam (*Donax trunculus* L.), gathered in spring (March), summer (June/July) and early autumn (September) from shallow sublittoral sandy habitats at different localities along the Bulgarian Black Sea coast. It is well established that inhibition of AChE in marine organisms is mainly caused by the neurotoxic effects of organophosphate and carbamate pesticides, however recently it was found that a number of other chemical substances have similar effects including heavy metals, polycyclic aromatic hydrocarbons, detergents etc. The activity of AChE in the soft body of the clams in our study showed considerable variation among the studied localities. The most significant AChE inhibition was present in clams from localities close to coastal areas with intensive tourism, such as the big resorts Slanchev Bryag, Duni, Arkutino and Primorsko. Significant seasonal differences in AChE activity were also established. Higher activity of AChE was present in the wedge clams gathered in autumn than in the other seasons. Overall, our results demonstrated for the first time the presence of significant ecotoxicological effects of anthropogenic impact on the shallow sublittoral sandy habitats along the Bulgarian Black Sea coast where *D. trunculus* is a dominant. Hence, this clam species appears to be suitable bioindicator of marine environmental quality.

Key words: Acetyl choline esterase activity; Bulgarian Black Sea; *Donax trunculus* L

Introduction

Anthropogenic pressures are increasingly affecting marine ecosystems, causing significant changes in their state and functionality. The increase of urbanization and intensification of industry, agriculture and transport are leading to increased pollution of the marine environment (Gherras Touahri et al., 2016). The Black Sea is a unique semi-exclosed basin, which is accepted to be one of the highly polluted seas (Oguz & Velikova, 2010; Makedonski et al., 2017). Until recently, the main approach to marine environmental assessment has been the measurements of certain pollutants, both in the environment and in organisms. However, this approach does not provide sufficient information on the reaction of living organisms and their degree of adaptation to environmental changes and deterioration. Moreover, the number of possible pollutants is huge and they can have not only cumulative, but also synergistic effects (Canesi et al., 2014; Vernon et al., 2020), as the reported synergistic effects of global climate change and marine pollution (Cabral et al., 2019). Thus, assessment of the biological responses of susceptible organisms, along with the qualitative and quantitative analysis of chemical contaminants, is becoming a key tool in monitoring programs (Allan et al., 2006; Depledge, 2009). Such integrative approach has been also required to achieve Good Environmental Status in European marine waters as required by the Marine Strategy Framework Directive (MSFD, 2008/56/EC) (Lyons et al., 2010).

Currently, a number of biomarkers, as well their combinations, are used to assess the impacts of pollution, environmental risk and marine environmental management (Galloway et al., 2004; Viarengo et al., 2007). In modern ecotoxicology, two types of biomarkers are conventionally distinguished - those that reflect the exposure of organisms to environmental stressors (Exposure Biomarkers) and those that show the adverse effect of stressors on the state of organisms (Effect Biomarkers) (Hook et al., 2014). Inhibition of acetylcholine esterase (AChE) is used as a marker for both, exposure and effect, mainly of organophosphorus and carbamate pesticides in aquatic organisms (Bocquené et al., 1990; Magni et al., 2006; Blaise et al., 2017). AChE plays an important role in the functioning of the neuromuscular system by determining the nerve impulse transmission. When AChE is inhibited, it may cause continuous muscle contractions with depletion of energy resources, leading to serious dysfunction in organisms, e.g., paralysis and death (Fulton & Key, 2001). However, it has been established that despite pesticides, AChE may be inhibited also by a number of other substances present in the marine environment, i.e. heavy metals (Frasco et al., 2005; Sabullah et al., 2014), PAHs (Fu et al., 2018), PCBs, DDT, detergents (Lionetto et al., 2011; Tsangaris et al., 2011), algal toxins (Lehtonen et al., 2003), and recently microplastics (Tlili et al., 2020). AChE activity has been identified and biochemically characterized in many aquatic invertebrate species (Fulton & Key, 2001). In particular, high levels of AChE activity in the gills, digestive gland and haemolymph have been reported in different bivalves (Srivatsan, 1999; Escartín & Porte, 1997; Galloway et al., 2002), among which mussels from the order *Mytilida*.

The aim of the present study was to obtain a preliminary assessment of the ecological and ecotoxicological risks associated with the contamination of the sublittoral sandy habitats using the wedge clam, *Donax trunculus* L. as a bio-indicator. The spatial and temporal variations of AChE activity were measured in wedge clams collected from different localities along the Bulgarian Black Sea coast.

Materials and Methods

Materials

The reagents acetylthiocholine iodide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and Folin-Ciocalteu reagent were purchased from Sigma Aldrich, owned by Merck KGaA (St. Louis, USA). All other chemicals were of the highest commercially available purity.

Sampling

Adult clams (23-35 mm) were collected from their natural shallow sublittoral sandy habitats (depth 0.5 – 2.5 m) along the Bulgarian Black Sea coast (Fig. 1) in different seasons: spring (March), summer (June-July) and autumn (September) of 2020. A pooled sample of wedge clams from each locality was gathered by hand dredge and was transported to the laboratory in containers with sea water. In the laboratory, the clams from each location were put in dry ice for further transportation and thereafter were stored at –80°C. For the biochemical analyses subsamples of different number of clams (n=6-12) were randomly picked up from the corresponding pooled sample.

The biochemical analyses of the total clam soft tissues were carried out in the Laboratory of Free Radical Processes of the Institute of Neurobiology-Bulgarian Academy of Sciences.

Locality	N	E
Sv. Konstantin & Elena	43.2294	28.0146
Rodni Balkani	43.2026	27.9243
Pasha Dere	43.1212	27.9284
Shkorpilovtsi	42.9603	27.8970
Irakli	42.7498	27.8901
Sveti Vlas	42.7091	27.7595
Slanchev Bryag	42.6906	27.7137
Chernomorets	42.4468	27.6422
Dyuni	42.3662	27.6927
Arkutino	42.3334	27.7312
Primorsko	42.2738	27.7565
Tsarevo	42.1744	27.8504

Fig. 1. Sampling localities along the Bulgarian Black Sea coast with geographical co-ordinates of the sampling sites.

Tissue preparation

On the day of measurements, the soft tissue of each individual wedge clam to be studied was carefully extracted and homogenized, using a Potter Elvehjem homogenizer fitted with a Teflon pestle (Thomas Scientific, USA) in chilled potassium phosphate buffer (100 mM, pH 7.5). Homogenates were centrifuged at 10 000 rpm for 20 min at 4°C using a K-24 centrifuge (Janetsky, Germany). The obtained supernatants were used for the measurement of AChE activity.

Determination of acetylcholinesterase activity

The AChE activity was assayed by the method of Ellman (Ellman et al., 1961), based on the production rate of thiocholine. The reaction mixture contained 0.1M K-PO_4 buffer pH 8.0, 0.045 M acetylthiocholine iodide, 0.008 M 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and the appropriate amount of clams' tissue homogenate. The thiocholine, resulted from acetylthiocholine hydrolysation by AChE, reacted with DTNB and yield a yellow colored product, 5-thio-2-nitro-benzoic acid. The rate of change of absorbance at 412 nm was recorded and calculated as U (μM ACh/min)/mg protein. Protein content was measured by the method of Lowry (Lowry et al., 1951) and was calculated from a standard curve, prepared with bovine serum albumin. The assays were performed using Spectrophotometer AE-450N (ERMA Inc., Japan).

Statistical analyses

All measurements are presented as mean ± standard error (SEM). Significance of differences between groups was estimated using t-statistic and ANOVA, at $p < 0.05$ significance level. The analyses were carried out using the STATISTICA 10 software package (StatSoft Inc., Tulsa, USA).

Results

There was significant variation in the measured AChE activity both among seasons and among localities. The activities of AChE in the soft tissue of *D. trunculus* gathered in March and in June were significantly ($p<0.05$) lower than the activities in the samples from July and September (Fig. 2).

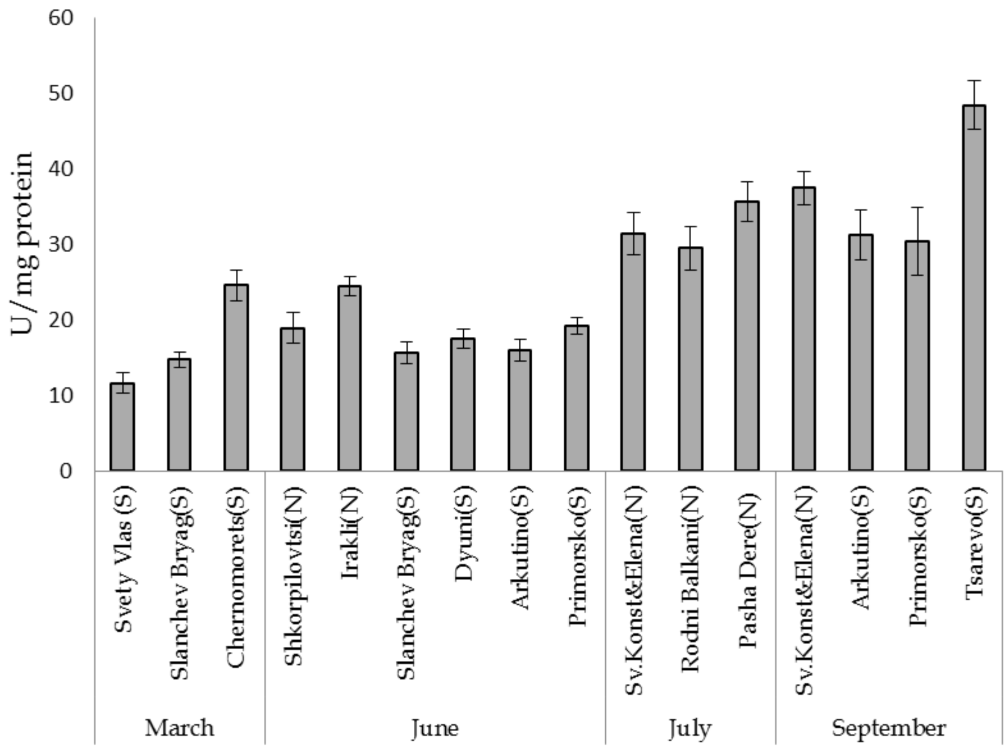

Fig. 2. Acetylcholine esterase activity in soft tissue of *D. trunculus* from different localities and seasons (S – Southern localities; N – Northern localities) in Bulgaria.

In spring the AChE activities, measured in the samples from Sveti Vlas and Slanchev Bryag were significantly lower than that from Chermomotrets. In summer, the lowest AChE activities were observed in wedge clams sampled from Slanchev Bryag and Arkutino (15.71±1.41 and 16.01±1.38 U/mg protein, respectively). It is noteworthy that the activity of the enzyme in the samples from July was almost twice as high as those from June. The AChE activities in the clams from the northern localities Sv. Konstantin & Elena, Rodni Balkani and Pasha Dere, were 31.48±2.84, 29.56±2.90 and 35.74±2.60 U/mg protein, respectively (Fig. 2). The AChE activity in the samples, collected during the autumn varied in the range from about 30 to 48 U/mg protein. The lowest average values were measured in the samples from Arkutino and Primorsko (31.30±3.27 and 30.48±4.54 U/mg protein, respectively) (Fig. 2). The highest activity of AChE was detected in the September samples from Tsarevo (48.49±3.19 U/mg protein).

It is well established that AChE activity in aquatic animals depends on the temperature of the environment. The enzyme activity in the wedge clams from

Arkutino and Primorsko was significantly higher at higher temperature of the marine water - the average water temperature in the localities for the time of sampling in June was +20.8°C, and in September +27.4°C (https://www.stringmeteo.com/synop/sea _water. php? year = 2020 # 03).

In order to confirm the observed overall patterns in AChE activity in the wedge clams from the studied localities in different seasons we applied factorial ANOVA analysis and the results are presented in Table 1. The main effect of "locality" alone was highly significant and confirmed the significance of the variations observed in the AChE activity in the wedge clams inhabiting the different localities studied (Table 1). In addition, the interdependent effect of "locality" and "season" proved to be also highly significant (Table 1). This suggested that the effects of the state of the marine environment in a given locality on the AChE in the wedge clams living there can be different depending on the season.

Table 1. Results of ANOVA analysis of the effects of locality and season on the AChE in *D. Trunculus.*

Effects	df Effect	MS Effect	F	p
Locality	7	572.82	13.43	0.000000
Season	2	4028.19	94.47	0.000000
Locality* Season	8	564.59	13.24	0.000000

Concerning the localities, it is noteworthy that lower AChE activity was present in wedge clams from sites located near to sources of higher coastal anthropogenic impact, i.e. larger resorts. For instantce the lowest values of the enzyme were present in the samples from Sveti Vlas and Slanchev Bryag in March (Fig. 2). Similarly, lower AChE activities were measured in the tested individuals from Arkutino, Slanchev Bryag, Duni and Primorsko sampled in June, while in the clams from Irakli, an area falling within the protected sites "Emine" (for protection of wild birds) and "Emine-Irakli" (for conservation of natural habitats), significantly higher AChE activities were measured. A similar pattern was observed in the September samples, where the clams from Arkutino, Primorsko and Sv. Konstantin & Elena had significantly lower AChE activities than those from Tsarevo which, in turn, were over 30% higher than those from Arkutino and Primorsko, and over 20% higher than those from Sv. Konstantin & Elena. Although on average in the wedge clams gathered from the northern regions higher activities of AChE were found compared to those from the southern localities, the differences were rather individual and seemed to be due to the state of the marine environment at the concrete locality.

Discussion

Inhibition of AChE is considered as a specific indicator reflecting the pollution with organophosphorous and carbamate compounds either in aquatic or terrestrial environments. Recent evidence that the AChE activity depends not only on neurotoxins, but on a number of other factors (Fu et al., 2018), incl. oxidative stress (Lionetto et al., 2011), suggests that it may be more widely used as an indicator for assessing ecosystem health. In addition, it has been shown that apart from the catalytic function in neurotransmission and muscular contraction, different forms of AChE are involved in cell proliferation, differentiation, and responses to various stresses (Lionetto et al., 2011), which can affect the general condition of individuals.

Our data, although preliminary, clearly showed that AChE activity significantly varied among seasons. The enzyme activity in the clams sampled from Arkutino and Primorsko in September was significantly higher compared to the June samples. Furthermore, the activities of AChE in general were lower in the samples of March and June compared to those of July and September. These results strongly suggested that seasonal temperature differences could have played a significant role. There are published data, which show that the water temperature could affect the AChE activity (Bocquene & Galgani, 2004; Pfeifer et al., 2005). In *Mytilus* sp. from the south-western Baltic Sea seasonal differences in the AChE activity were observed with a maximum during the summer and minimum in the winter period, and these changes had a positive correlation with water temperature (Pfeifer et al., 2005). Presumably in wedge clams, as cold-blooded animals, lower ambient temperatures are associated with reduced vital functions and lower AChE activity.

Activity of AChE is proved to be sensitive to various types of pollutants in the natural environment, mainly organophosphorous and carbamate chemicals. Our preliminary results indicated that the enzyme activity was lowest in wedge clams sampled from localities adjacent to areas known as sources of relatively high coastal anthropogenic load such as the large resorts Slanchev Bryag, Sveti Vlas, Arkutino, Duni. Given that in the immediate vicinity of these complexes there is no intensive agriculture and industry, it is most likely that the wastewater influx in the sea from these resorts could produce the main effects on AChE activity in wedge clams. Similarly, it has been shown that in *Ruditapes decussatus*, exposed to treated municipal effluents (TME), the AChE activity has been reduced in both gills and digestive gland (Kamel et al., 2012). Exposure to TME was also found to affect the pro/antioxidant balance in clams' cells and oxidative stress indicators were shown to correlate with AChE activity (Kavitha & Rao, 2008; Lionetto et al., 2011). It should be noted that the tourist flow to the Black Sea resorts in Bulgaria in 2020 was extremely limited due to the Covid-19 pandemic, which reduced tourist numbers and consumption. Consequently, the inflow of wastewaters was reduced, so the differences between the AChE activities we established in this preliminary study may be not typical and very expressive. Another factor that can affect the results obtained is the constant high water input into the northern part of the Black Sea by the big rivers and the pollutants they carry (Dineva, 2011; Dar & Bhat, 2020).

In recent years, the problem of marine pollution with plastic waste, incl. microplastics (MPs) becomes obvious (Hidalgo-Ruz et al., 2012; do Sul & Costa, 2014). The small size (<5 mm in diameter) of the MPs makes them bioavailable to marine organisms and transferable through the food web. MPs have been found in the Black Sea and their concentration in the Bulgarian part is similar to that found in other parts of the Black Sea, as well as in the Baltic Sea, and the Mediterranean Sea (Berov & Klayn, 2020). MPs have been shown to affect AChE activity and inhibition of the enzyme was observed by exposure to them alone or in combination with organic/metallic pollutants (Oliveira et al., 2013; Ribeiro et al., 2017; Tlili et al., 2020). Preliminary studies we have carried out showed the presence of microplastics in *D. trunculus* from areas with intensive tourism – Slanchev Bryag and Gradina (unpublished data). It is highly possible that the presence of MPs could have also affected the AChE activity in the studied wedge clams.

Conclusions

The preliminary results, obtained from the present study, demonstrated that AChE activity is sensitive to environmental pressure by various types of pollutants and variations in the conditions of the wedge clam habitats. The reaction of AChE activity in *D. trunculus* towards the changes in the marine environmental quality suggested that this clam species, representative for the shallow sublittoral sandy habitats of the Black Sea, is a sensitive and suitable bioindicator for the state of the marine ecosystems.

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Population Status and Natural Localities of Rhodiola rosea in Rila Mts., Bulgaria

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Abstract. *Rhodiola rosea* L., a member of Crassulaceae family, is an alpine plant that grows in specific and sensitive high-mountain habitat and is, therefore, a very suitable for studying the effect of climate change. Although the natural area of *R. rosea* is wide and includes most of the boreal and temperate parts of the Northern hemisphere, urgent measures for conservation of natural resources of the species are necessary. In Bulgaria, the species occurs on stony and rocky places, often on screes, in habitats near late-melting snowdrifts, which provides sufficient soil moisture (2280 - 2600 m above sea level) in Pirin, Rila and Stara planina Mts. It is protected by the Biodiversity Act of Bulgaria and is listed in the Red List of Plants in Bulgaria. The species is included in the Red Data Book of the Republic of Bulgaria (Peev, ed., 2015). According to the IUCN criteria, its threat status is Critically Endangered. This study aimed to evaluate the population size and the conservation status of six natural localities of *R. rosea* in Rila Mts: 1) Seven Rila Lakes; 2) Skakavitsa's waterfall; 3) Kalin Dam; 4) Rusaliite; 5) Belmeken; 6) Musala hut. The best characteristics of both population size and conservation status were recorded in the localities situated in remote and hardly accessible areas with limited or no tourists' access. Based on thorough evaluations of trade levels and trends compared to population sizes, *R. rosea* must be considered to face serious threats from overexploitation and growing international trade due to its increasing use in herbal medicine.

Key words: Golden root, conservation, natural localities.

Introduction

R. rosea (synonyms: *Sedum rosea* (L.) Scop., *Rhodiola roanensis* Britton, *Rhodiola arctica* Boriss., *Rhodiola iremelica* Boriss. *Sedum rosea* var. *roanense* (Britton) A. Berger, *Sedum roseum* (L.) Scop., *Sedum roseum* var. *roanense* (Britton) A. Berger, *Sedum rosea* var. *roanensis* (Britton) A. Berger) is a member of Umbilicinae subtribe, Sedeae tribe, Sedoideae subfamily, Crassulaceae family (Engler, 1964). The plant's name can be traced back to the ancient Greek physician Dioscorides, who first recorded medicinal applications of *rodia riza* in 77 C.E. in De Materia Medica (Mell, 1938). Linnaeus renamed it *Rhodiola rosea*, referring to the fresh-cut rootstock's rose-like fragrance (Linnaei, 1749). Based on the reproductive systems' specifics, Linnaeus first differentiated the two close genera – *Rhodiola* and *Sedum.* Most of the species of the genus *Rhodiola* are dioecious, *R. rosea* including. Its

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flowers are unisexual, rarely hermaphrodite, yellow to orange, with reddish nuance (Hegi, 1963, Lindman, 1964, Sandberg & Göthberg, 1998) and pleasant scent (Mossberg & Stenberg, 2003). Male flowers are more extensive and prominent during the flowering period (Dragland, 2005), lasting from June to August (Hegi, 1963; Mossberg & Stenberg, 2003), and male plants tend to be larger and heavier than female ones (Galambosi et al., 2006). *R. rosea* is an insect pollinating species. Therefore, it depends on many environmental factors that could affect its pollinators. Due to the severe climate condition in the alpine belt, the pollinator species occupy specific microhabitats. *R. rosea* is an alpine plant that grows in specific and sensitive high-mountain habitat and is, therefore, a very suitable for studying the effect of climate change. It is one of the priority species for conservation in many European countries: Finland, Lithuania, Sweden, Norway, Iceland (Cuerrier et al., 2015). Today it is not red-listed in the Scandinavian countries, except in the Swedish counties Västra Götaland (Kylin, 2010, Alnarp, Sweden – pers. comm.), Göteborg and Bohuslän (Mossberg & Rydberg, 1995). *R. rosea* is one of the protected species in the Czech Republic, Bosnia and Herzegovina (Lange 1998). In these countries it is endangered while in Slovakia it is considered vulnerable (Galambosi et al., 2006). It occurs in most European countries with wider distribution in the northern latitudes, while in the southern part of Europe its distribution is restricted mostly to high-mountain habitats. Genetic surveys revealed differentiation among the geographically distinct populations (György et al., 2016).

It is a subject of protection in many former Soviet Union Republics: Borodin, 1978; Malyshev & Sobolevskaya, 1980; Harkevich & Kachura, 1981; Takhtajan, 1988; The Red Book of the Murmansk region (Konstantinova et al., 2003), as well as the Komi Republic (Taskaev, 1999), the Central Urals, Arkhangelsk, Nenetz and Khanty Mansiysk Autonomous Area of the Russian Federation, including the Republic of Karelia (Kotiranta et al., 1998). *R. rosea* is one of the 202 rare species listed by the Red Data Book of Ukraine (Didukh, 2009). Ziman and Derbak (2013) studied the species' population status in the high mountain flora of the Ukrainian Carpathians and Balkan. As a result of long-standing monitoring, they determined *Rhodiola* populations' participation as a part of "hot spots" characterized by including 5 to more rare species. Authors recommended for conservations and protection of these hot spots. The largest population of the species in the world is found in Altai, south Siberia (Galambosi, 2006). The conservation status of the species in North America differs between the states and regions, but it is under protection in most of them (Booker et al., 2015). The same author stated that species status is better in Canada due to the more suitable habitats related to the northern climate.

Although the natural area of *R. rosea* is wide and includes most of the boreal and temperate parts of the Northern hemisphere, urgent measures for conservation of natural resources of the species are necessary. *R. rosea* is protected by the Biodiversity Act of Bulgaria (2002) and is listed in the Red List of Plants in Bulgaria. The species is included in the Red Data Book of the Republic of Bulgaria (Meshinev, 2015). According to the IUCN criteria, its threat status is Critically Endangered [CR A4d; B2ab(iv)]. In Bulgaria, the species occurs on stony and rocky places, often on screes, in habitats near late-melting snowdrifts, which provides sufficient soil moisture (2280 - 2600 m above sea level) in Pirin, Rila and Stara planina Mts. It is one of the characteristic plants of the priority habitats of Directive 92/43/EEC. The species' occurrence in Stara planina is very scarce, and the size of populations in Pirin is considered small (Meshinev, 2015). Therefore, its populations in Rila Mts are considered more important and deserving more attention.

The aim of this study was to evaluate the population size and the conservation status of six natural localities of *R. rosea* in Rila Mts: 1) Seven Rila Lakes; 2) Skakavitsa's waterfall; 3) Kalin Dam; 4) Rusaliite; 5) Belmeken; 6) Musala hut. The results obtained are part of long-term monitoring on the population status of the species in Bulgaria.

Materials and Methods

The objects of the study included six localities of the species in Rila Mts. They were selected based on preliminary information about the species' distribution and the attempt was to cover different parts of the mountain.

The climate conditions in the region of study are typical for the high mountain regions in Bulgaria. Because climate data are available from only one climate station (Musala), they could be considered, with some approximation, as valid for the whole region of study. The mean annual temperature is -3 ºC, with the coldest month being January (-10.9 ºC) and the warmest one – August (5.3 ºC). The average monthly temperatures are positive only in June, July, August and September. Total annual precipitation is 1176 mm, predominantly snow, with maximum value 130 cm in March.

The field observations took place in the period June-September, 2016 – 2020. Transects were used for preliminary assessment of the regions of study and for determining the exact locations of the scoring plots. The plot size was 100 m^2, which was convenient enough considering the stony habitats with lower plant cover. Depending on the size of the locality, 2 to 3 scoring plots were established. The target characteristics of *Rhodiola rosea* localities were: population size, average density, and the status of the populations. The area covered by populations was determined by the positions of the peripheral plants of each locality. During the field observations GPS coordinates and altitude were scored, and some other peculiarities of the locality have been described. The data from the control populations were included in particular "Terrain form" accepted by "National System for biomonitoring" (Gussev & Bancheva, 2016).

Results and Discussion

Generally, the populations of *R. rosea* in the studied region are represented by a small number of individuals. The most numerous is the population Belmeken with about 130 individuals growing on an area of approximately 1800 m^2. The poorest was the locality near Kalin dam, consisting of only 4 individuals, and Rusaliite (12 individuals). However, the places where the species occurs are sometimes difficult to reach, and one could skip few individuals, especially if *Pinus mugo* thickets grow on the screes. The average number of individuals per 100 m2 varied between 4 and 7, which means that the population density is not strongly related to population size. There is insufficient information concerning the population size of the *Rhodiola rosea* populations in Bulgaria. Most studies focused on the chemical composition, medicinal properties and cultivation (Marchev et al., 2016), while few studies aimed at studying the population status and conservation (Meshinev, 2015).

The localities near the tourist roads are Seven Rila Lakes, Skakavitsa's waterfall, and Musala hut. They are characterized by a relatively large population area, but the number of individuals is small, and the distance between them is long. This makes pollination difficult, and there were no seedlings. Besides, we have registered violations related to the sale of the golden root in the locality of Panichishte, which is the start point of many tourist roads leading to Seven Rila Lakes and Skakavitsa's waterfall.

The best characteristics of both population size and conservation status were recorded in the localities situated in remote and hardly accessible areas with limited or no tourists' access. In relatively good condition is the population in the region of Belmeken (Table 1). The locality can be accessed only by walking 8 km on rough terrain.

The other two localities (Kalin Dam and Rusaliite) have a small population area. The reason for the populations' poor state should be attributed to climate change and loss of habitats. Also, these two localities can be accessed by off-road vehicles and are thus more exposed to anthropogenic pressure.

The main reason for the small population size can be traced in its ecological requirements to the specific type of habitats. *R. rosea* occurs on stony and rocky, wet places on screes and along the mountain streams, above the treeline. The species could be found on areas close to the late melting snowdrifts, which provides sufficient soil moisture. Although *R. rosea* has succulent morphology (Fig. 1), it is highly dependent on relatively stable water supplies.

Fig. 1. *R. rosea* in its natural locality Belmeken (photo: Ina Aneva).

Therefore, collection and trade of this species from natural localities is a violation of the legislation and is a subject to punishment. However, we have observed in some popular tourist destinations (near the regions of Seven Rila Lakes and Skakavitsa's waterfall) that roots of *R. rosea* have been offered by private retailers, albeit in small quantities (up to 100-200 g in a package). Concerning trade abroad, to other countries, we do not have information. Officially it is not possible because of the protection status of the species. However, in some cases, collections from the wild could be declared as originating from cultivation and offered to the international market. It is possible, but we do not have information whether it happens frequently or not. Based on thorough evaluations of trade levels and trends compared to population sizes, *R. rosea* must be considered to face serious threats from overexploitation and growing international trade due to its increasing use in herbal medicine.

The localities of *R. rosea* in Bulgaria are part of three priority habitats: 8110 – Siliceous screes of the montane to snow levels, 8220 - Siliceous rocky slopes with chasmophytic vegetation, and 4070 - Bushes with *Pinus mugo* of Directive 92/43/EEC (Table 1). Also, the locality Skakavitsa waterfall borders another priority habitat – 95A0 Forests of Macedonian pine (*Pinus peuce* Griseb.) and Bosnian pine (*Pinus heldreichii* Christ.). They harbour many rare and endemic species, like *Primula deorum* Velen., *P. minima* L., *Geum bulgaricum* Pančić, *Leontodon rilaensis* Hayek, *Pinguicula balcanica* Casper, *Soldanella pusilla* Baumg. Thus, the conservation measures taken to protect *R. rosea* natural habitats have multiple effects, by conserving the other rare and endangered plants in these habitats.

The conservation of the studied localities is insured by the fact that all they are part of protected territories: Rila National Park and Rila Monastery Nature Park. Also, part of the territory falls into the nature reserves "Ibar" and „Skakavitsa", which have more strict protection status, including prohibition of collecting of any plant species on the territory.

Table 1. Characteristics of the studied localities. *Legend:* 1) According to Directive 92/43 EEC.

Locality	**Habitat type[1)]**	**GPS coordinates**	**Altitude (m a.s.l)**	**Area of the locality (m^2)**	**Number of *R. rosea* plants**	**Mean number of individuals per 100 m^2**
Seven Rila Lakes	8110	42°11'47.62"N 23°19'37.25"E	2400	1200	63	5
Skakavitsa's waterfall	8220	42°13'16.37"N 23°18'21.10"E	2050	600	48	6
Kalin Dam	8220	42°10'28.18"N 23°14'48.77"E	2400	100	4	4
Rusaliite	8110	41°58'46.33"N 23°23'56.20"E	2020	200	12	6
Belmeken	8110 and 4070	42°11'27.72"N 23°44'56.41"E	2200	1800	130	7
Musala hut	8110	42°11'30.74"N 23°35'22.20"E	2400	600	28	4

Conclusion

R. rosea is a species with high conservation value worldwide. Further active protection measures aimed at achieving long-term conservation of the populations of *R. rosea* and the unique nature as a whole must be taken. Regular monitoring needs to be carried out. The including of the species in CITES Appendix II should be done.

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Status of Conserved Local Plant Biodiversity in Bulgaria – New Challenges and Research Priorities

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Abstract. Bulgaria is one of the countries with the greatest plant biodiversity in Europe, as well as with a significant number of unique ecosystems. The study aims to present the documented local plant genetic resources, conserved in the Bulgarian seed genebank, and to address the new challenges, created by climate change. The research priorities of the national program on plant genetic resources in Bulgaria are focused on expanding the role of biodiversity in agricultural food systems to increase the sustainability of agriculture and livelihood of farmers and to offer consumers the traditional taste of agricultural produce. The survey is based on the national register of plant genetic resources and the international databases such as FAO WIEWS, GENESYS, EURISCO and AEGIS. The results show that the national collection compares 65,015 accessions with passport descriptors. Among those, 16,010 accessions are characterized with Bulgarian origin. Through expeditions 10,687 seed samples of local varieties have been collected within the country. The overview of plant genetic resources originating from Bulgaria is an indication of the wealth of crop plant species and their wild relatives, and the national register may assist in identifying gaps and needs for further collecting. It may also be a good starting point for compiling checklists of cultivated plant species and help define future research activities. The results of this study will integrate description of local origin of landraces, taking into account consumers' perceptions and farmer's needs for creation of rural thematic networks aimed to promote the durable use of local plant genetic resources. The research was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Healthy Foods for a Strong Bio-Economy and Quality of Life", approved by DCM № 577/17.08.2018.

Key words: landraces, *ex situ* collections, databases, climate change, sustainability.

Introduction

Plant genetic resources for food and agriculture are vital to food security, nutrition and crop productivity, resilience and adaptability of production systems in the crop sectors (Pilling et al., 2020).

Plant genetic diversity disappears in parallel with the entry of intensive agriculture and the introduction of high-yielding cultivars into the farm. As a result of the modern plant breeding, the genetic base of new crop varieties and hybrids is significantly narrowed. This fact turns genebanks preserving *ex situ* plant genetic diversity into a major factor limiting the genetic erosion of agrobiodiversity. Therefore, it is extremely important to fill gaps and continuously enrich the genetic diversity of the preserved collections by collecting additional local plant genetic resources from the wild and cultivated land, eventually leading to their sustainable use. The foci of the collecting missions should be on local traditional varieties and crop wild relatives, having valuable genes for the breeding process, organic production and sustainable agriculture (Ulian et al., 2020).

Climate change, as well as the restructuring of the breeding criteria towards high biological quality of crop production, directs the researchers to the inexhaustible source of useful genes – the local gene pool (Borrell et al., 2020; Maggiore et al., 2020). Originating from a long-term selection process by farmers based on valuable traits and qualities in wild and domesticated crop populations, and adapted to the specific conditions of the area in which they are grown, the landraces are a unique source material for breeding to restore the traditional taste of crop varieties (Knüpffer, 2002; Kehlenbeck et al., 2007).

The availability of comprehensive information on the composition of conserved germplasm is an important prerequisite to further develop genebank collections. Easy access to plant genetic resources and to traditional knowledge about their cultivation and application contributes to enhancing their sustainable use and increases the capacity of breeders to respond to climate change. Conservation of genetic resources of crop genepools, including landraces, crop wild relatives, breeding and research materials are the pillars of modern plant breeding, as well as of localized breeding efforts by farmers and farming communities (Ebert & Engels, 2020).

Bulgaria is one of the countries with the greatest plant biodiversity in Europe, as well as with a significant number of unique ecosystems (Knüpffer, 2016). The preservation of the plant biodiversity from the wild and cultivated flora is the main priority in the research work of the Institute of Plant Genetic Resources in Sadovo serving as a national coordinator of plant genetic resources and as a member of the European Programme for Plant Genetic Resources (ECPGR). The main mission of the national seed genebank is the conservation of plant genetic resources, and ensuring their availability and distribution to local and foreign users in relation with the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA, 2009) and Nagoya Protocol (CBD, 2011).

This study aims to present the documented local plant genetic resources, conserved in the Bulgarian seed genebank, and to address the new challenges, created by climate change.

Material and Methods

The Computer center, established in 1982 at the Institute of Plant Genetic Resources "K. Malkov " – Sadovo, maintains a specialized database Phyto'2000 in Microsoft Access format. It contains information about all seed accessions, preserved in the national genebank.

The documentation system optimizes the management of plant genetic resources in relation to their targeted storage, study, reproduction, free exchange and use. All accessions are described by 33 descriptors: catalogue number, taxonomic description, biological status of the genotype, donor and geographical origin, type of conservation – long term, medium term or working collection, availability for exchange, safety duplication in another genebank, etc. according to the Muli-crop passport descriptors of FAO/Bioversity (2017) and the international genebank standards (FAO, 2014).

The taxonomic description of the crops is under the nomenclature of the USDA Genetic Resources Information Network (GRIN, 2015).

The national genebank in Sadovo has been nominated by ECPGR as a focal point for Bulgaria in the European Searching Catalogue on Plant Genetic Resources – EURISCO (http://eurisco.ecpgr.org), providing information about *ex situ* collections, maintained in Europe (Weise et al., 2017).

Through EURISCO the information about the Bulgarian National Inventory is accessible in other international databases, such as A European Genebank Integrated System - AEGIS of ECPGR (Engels & Maggioni, 2013), World Information and Early Warning System – WIEWS (FAO, 2020) and online platform about Plant Genetic Resources for Food and Agriculture, conserved in genebanks worldwide - GENESYS (2015).

Results and Discussion

During the period 1982-2020 the germplasm stock of the national seed genebank in Sadovo was enriched with 53,338 additional accessions, comprising 122 botanical families, 3,583 taxonomical descriptions, including 36,675 accessions, received through international free exchange, 10,687 accessions - collected during expedition missions and 5,976 entries of – breeding materials with Bulgarian origin.

The *ex situ* collections of the national genebank comprise cereals (62 %), grain legumes (16 %), oil and industrial crops (8 %) forages (3 %), vegetables (10 %), medical and aromatic plants (1 %).

The foreign genotypes in the collection are being introduced via professional contacts with about 195 genebanks, plant genetic resources centers and botanical gardens worldwide. The main partners of the institute in the free germplasm exchange are research centers such as USDA (USA), ICARDA (Syria), VIR (Russia), NordGen (Sweden), IPK (Germany), INRA (France), John Innes Centre (UK). The requested foreign germplasm is investigated under the country's environmental conditions and used as a donor of valuable traits in breeding programs.

In the database, 5,976 breeding materials are registered, composed of – lines and improved new varieties from the institutes of the Agricultural Academy, Bulgarian Academy of Science, Agricultural University, and others. The access to the accessions of those institutes is regulated in accordance with the principles for the protection of breeders' intellectual property rights.

Currently, accessions with local Bulgarian origin comprise 30% of the genebank holdings, their conservation and sustainable preservation is a top priority in the activities of enrichment of the collections, in accordance with the new national-level aims. The accessions from collecting missions are amounting to 10,687, composed of – local varieties and populations from home gardens and crop wild relatives obtained in their natural habitats. The organized data sets show significant enrichment of the *ex situ* collections with local plant genetic resources in recent years (Fig. 1).

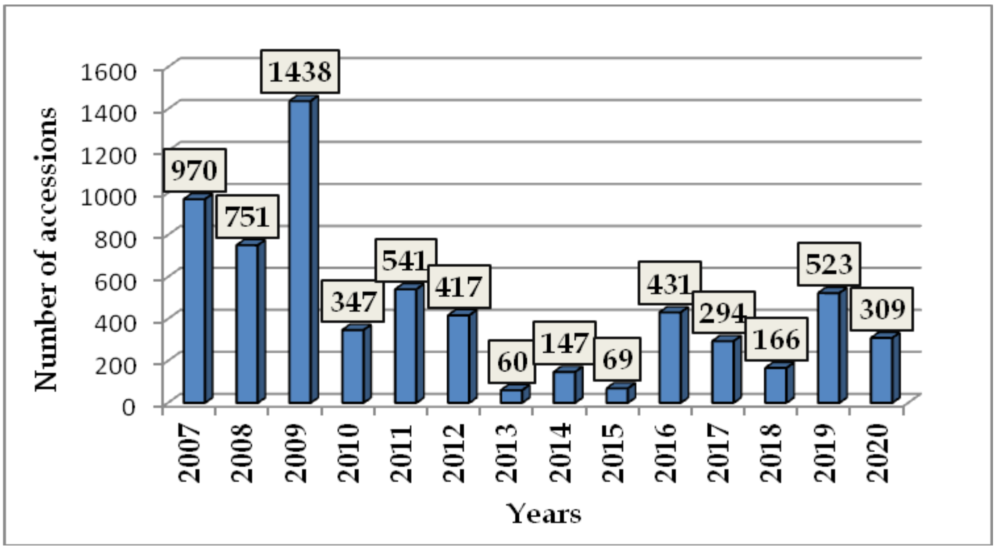

Fig. 1. Enrichment of the Bulgarian *ex situ* collection with local plant genetic resources.

The germplasm samples - species and varietal diversity of landraces from small farms and crop wild relatives obtained in their natural habitats, have been collected through expeditions, funded by projects. The routes of the collecting missions are determined on the basis of prior awareness of the specifics of the respective production areas or local habitats.

The first direction of any local expedition activity is the collection of cultural forms from field and vegetable crops. In small farms and home gardens traditional samples of tomatoes, peppers, cucumbers, pumpkins, melons, watermelons, onions, salads, potatoes were found, perfectly adapted to specific agro-environmental conditions, with valuable qualities and properties such as early maturity, resistance to biotic and abiotic stress, high content of biologically active substances, etc. (Krasteva et al., 2013). Regarding the cereals and legumes, the attention is focused on collecting samples of ancient and primitive forms of wheat, of old and local populations of corn, beans, cowbeans, lentils, and others. Of particular interest is the species diversity of some forgotten spices and medicinal plants, presently rediscovered for the purposes of dietary and healthy nutrition, used in therapies for alternative treatment of several diseases.

Another approach of collection missions is the protection of wild, semi-domesticated diversity and crop wild relatives of cultivated plants. The high urbanization, developed transport infrastructure and environmental threats put a large number of wild species from different botanical families at great risk. On the one hand, their conservation is important for biodiversity, and on the other - they have valuable qualities for breeding, such as being sources of high protein, starch, resistance to fungal, bacterial and viral diseases, high adaptability in connection with global warming and climate changes (Dempewolfetal., 2014).

As a result of the enrichment of germplasm collections and the passport description of the accessions made accessible in a well-organized database, suitable conditions have been created for mapping and zoning of the local gene pool. These are necessary prerequisites for expanding the expeditionary activity in specific regions of the country, which contain valuable plant resources as a result of primitive breeding, aimed mainly in selection by taste, fruit size, and disease resistance.

Another direction of the expedition activity is searching for varieties and species that are suitable for cultivation in semi-mountainous and mountainous areas, on poor productive lands, for areas with regular droughts, as well as for organic farming.

In recent years, the local accessions became associated with more complete passport information, because of the use of a GPS system during expeditions. For older germplasm samples the lack of eco-geographical characteristics could be partially restored through the modern satellite systems.

As a result of enrichment of *ex situ* collections, a rich plant diversity of field, vegetable, medicinal and aromatic species has been collected in the genebank. Core collections within evaluation and characterization data in accordance with the perspective of the source material for its inclusion in breeding programs, as well as a selection of samples for improving the species and varietal structure in Bulgarian agriculture have been created.

The wheat breeding started in 1902 as a part of the first programme of the Agricultural experimental station in Sadovo. During its long history 46 new common wheat varieties have been created and successfully introduced in agriculture. The main priority of the breeding is creation of high-yielding and characterized with good quality varieties of common wheat and triticale with wide ecological plasticity and resistance to biotic and abiotic factor. The focus of the resent years is the new varieties to be acceptable for low inputs cultivation in relation to sustainable and conservation agriculture. The new wheat varieties are suitable for South and North Bulgaria climate conditions, as well as for Turkey and North Greece.

During the period 1982-2020 in the Institute of Plant Genetic Resources through the multi-year study and selection in populations of local plant genetic resources as an initial material in plant breeding 27 new varieties from durum wheat, barley, rye, oat, pea, chickpea, cowpea, bitter vetch, garden bean, tomato, pepper, eggplant, kohlrabi, and lettuce were created. All these varieties possess the traditional taste of landraces and they are very well adapted to the specific conditions in the country. Seeds from these new varieties are distributed annually to farmers, farmer communities, NGOs, etc.

The peanut and sesame breeding is aimed at creating lines and varieties with a complex of valuable economic qualities such as high productivity, early maturity, complex resistance to fungal diseases, balanced chemical composition, good taste, opportunities for mechanized harvesting, etc. Both crops are thermophilic, and the sesame falls into the category of plant species that are successfully grown in hot and dry climate.

Organic farming is a system of farming that promotes environmental, social and economical sustainability of food production. As the awareness about the harmful effects of chemicals on health, soil, environment etc., is increasing inorganic farming is slowly shifting towards organic farming. Bulgaria with diverse agro climatic conditions and plenty of diverse local varieties has great potential for organic farming. Organic agriculture contributes to the social well-being by reducing the losses of soil, water contamination, biodiversity erosion, food loss, and pesticide poisoning. By using local plant genetic resources and associated, local knowledge, and by connecting farmers, consumers and their markets, the economic conditions and the well-being of rural people can be improved. Organic agriculture stresses diversification and adaptive management to increase farm productivity, decrease vulnerability to weather vagaries, and consequently improves food security, either with the food the farmers produce or the income from the products they sell. The increasing demand for organic products creates new research and economic opportunities.

The national genebank of Bulgaria implemented free germplasm exchange by providing samples to national and foreign users. The exchange collection contents 2,989 accessions of 42 genera and 89 plant species. According to the annual reports in the period 2017-2020, 258 seed samples from cereals, oil crops, legumes and vegetables were provided to researchers in Bulgaria for research and breeding activities. By seed requests totally 709 seed samples from 27 plant species were sent to scientific organizations as genebanks, research institutes, etc. within EU countries (549 samples), Japan (61 samples), China (50 samples), India (20 samples), Israel (11 samples), Mexico (11 samples), and New Zealand (7 samples). The scientific interest of the partners abroad is focused mainly on the local germplasm from genus *Triticum*, *Hordeum*, *Avena*, *Zea*, *Pisum*, diverse forage grasses, vegetables, as well as the new varieties of *Arachis hypogaea* and *Sesamum indicum*, related to the climate change. The germplasm is distributed internationally complying with the quarantine requirements of the recipient country.

The free access to the conserved germplasm accessions by international networks has direct impact on sustainable and efficient utilization of plant biodiversity and genetic resources related to the new challenges, created by the climate change.

The Bulgarian National Inventory comprises the richest *ex situ* collection in Southeast Europe. According to the EURISCO (data check February 2021) it comprises 69,435 accessions from three Bulgarian institutes with diverse geographical origin (Table 1).

The Bulgarian National Inventory is the 7th biggest in Europe and has a share of 3,4 %, after the National Inventories of the Great Britain, Russia, Germany, Ukraine, Poland and Spain. It consists of genotypes of various geographical origins, with 26% of the samples (17,843 accessions) being of Bulgarian origin. The highest group of accessions is from the genera *Triticum*, *Hordeum*, *Zea*, *Phaseolus*, *Avena*, *Vicia*, *Capsicum*, *Pisum*, *Linum*, *Arachis* (Fig. 2). The species with more than 1,000 accessions are shown in table 2.

The status of the Bulgarian National Inventory in the AEGIS database (data check February 2021) includes information about 341 local accessions with Bulgarian origin of six plant species (Table 3).

Table 1. Status of the Bulgarian National Inventory in EURISCO (February 2021).

FAO INSTCODE	Institutes	Number of accessions	With BGR origin
BGR001	IPGR Sadovo	65 015	16 010
BGR005	IREMC Kazanlak	563	4
BGR029	DAI General Toshevo	3 857	1 829
	Number of accessions	69 435	17 843

Table 2. Species with the highest number of accessions in the Bulgarian National Inventory.

Taxonomy	Number of accessions	With BGR origin
Triticum aestivum	12,959	2,821
Hordeum vulgare	6, 365	303
Zea mays	4,827	1,939
Phaseolus vulgaris	3,488	1,698
Avena sativa	2,476	149
Triticum durum	2,370	1,193
Capsicum annuum	1,885	1,408
Pisum sativum	1,744	241
Triticosecale	1,461	532
Linum usitatissimum	1,461	77
Arachis hypogaea	1,373	444
Lycopersicon esculentum	1,371	534
Secale cereale	1,300	827
Cucumis sativus	1,031	95

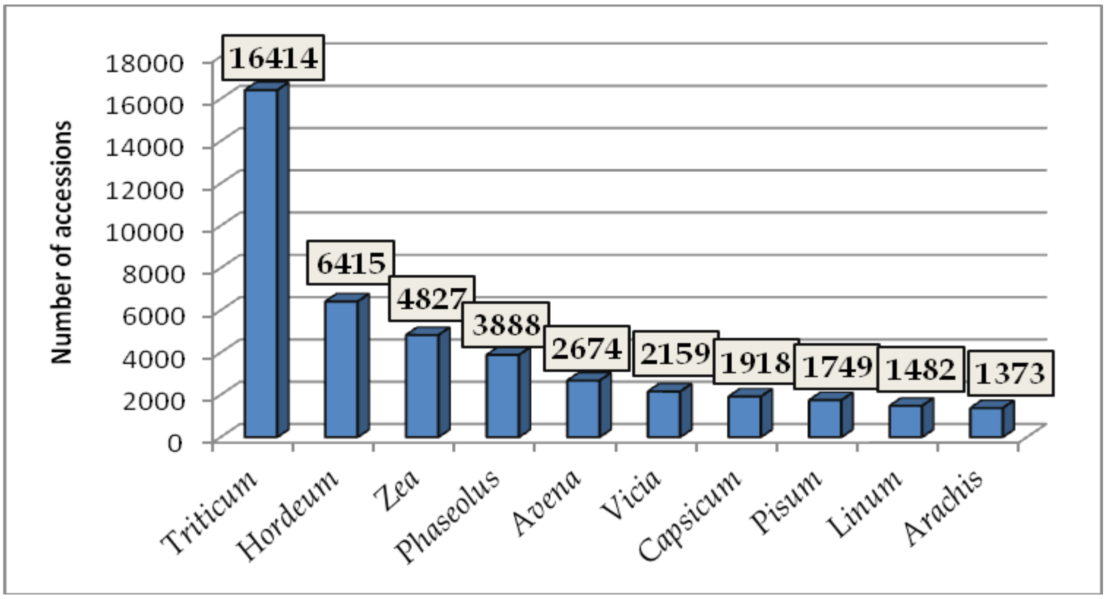

Fig. 2. Genera with the highest number of accessions in the Bulgarian National Inventory.

Table 3. Bulgarian accessions in AEGIS database (data check February 2021).

Taxonomy	Country of origin	Sample status	Number of accessions
Triticum aestivum	BGR	local	135
Triticum dicoccon	BGR	local	26
Triticum durum	BGR	local	126
Triticum monococcum	BGR	local	32
Triticum spelta	BGR	local	7
Secale cereale	BGR	local	15
		Number of accessions	**341**

The beneficiaries of the international networks of plant genetic resources are all stakeholders at regional, national and international level – scientists, breeders, farmers (including bio-producers), NGOs, environmental organizations, students in the field of agricultural sciences, etc.

Ex situ collections can be considered as a backup copy of the plant diversity in nature and thus their preservation is guaranteed. According to FAO WIEWS (http://www.fao.org/wiews/data/) and GENESYS (https://www.genesys-pgr.org/) 1,750 genebanks and more than 2,500 botanical gardens are currently actively working in the world, and they are responsible for conservation of more than 7,4 million plant germplasm accessions. Through EURISCO, the information about the Bulgarian National Inventory is presented in the world databases FAO WIEWS and GENESYS. It comprises one of the richest *ex situ* collections in Europe and the richest conserved plant diversity in Southeast Europe. Conservation and targeted use of plant genetic resources is a national responsibility and priority, the successful implementation of which is directly dependent on the good coordination among all partners and stakeholders: storage and documentation specialists, curators of collections, breeders, scientists, farmers, NGOs, politicians etc.

By signing of the International Treaty for Plant Genetic Resources (ITPGRFA, 2009) and the Nagoya Protocol (CBD, 2011) free access to the conserved germplasm for scientific, public, ecological and other organizations has been regulated for fair and equitable distribution of the benefits arising from their use.

At international level, the EU plays an active role, contributing to ensure the achievement of the global appointments for biodiversity and conservation. In relation to access to the plant diversity, transferring data about Bulgarian *ex situ* collections to international networks requires highly specialized information that is made available to all users of plant diversity to ensure guaranteed and equitable access. One of the priorities of ECPGR is creation of equal mechanisms in databases construction in connection with the inclusion of the national collections.

Conclusions

The Bulgarian national genebank maintains one of the largest *ex situ* collections in Europe and the richest conserved plant diversity in Southeast Europe.

Its documentation system, according to the international standards, optimizes the management of plant genetic resources in relation to their sustainable conservation and target use.

The free electronic access to the information about the Bulgarian *ex situ* collection is achieved through participation in the international databases, such as GENESYS, EURISCO, AEGIS, and FAO WIEWS.

International networks provide free access to potential users regarding to conserved genotypes according to the principles of the International Treaty on Plant Genetic Resources for Food and Agriculture and the implementation of the Nagoya Protocol on equitable distribution of their benefits.

The overview of plant genetic resources originating from Bulgaria is an indication of the wealth of local crop plant species and their wild relatives, and this may assist in identifying gaps and needs for further collecting. It may also be a good starting point for compiling checklists of cultivated plant species and to define future research activities.

The research priorities of the national program on plant genetic resources in Bulgaria are focused on expanding the role of biodiversity in agricultural food systems to increase the sustainability of agriculture and the livelihood of farmers and to offer consumers the traditional taste of agricultural products.

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Is the Marine Environment of the Black Sea Stressful for Organisms: A Pilot Assessment of Oxidative Stress in Bulgarian Coastal Fish Species

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Abstract. The present study is the first assessment of oxidative stress (OS) in fish species inhabiting the Bulgarian Black Sea coastal zone. The fish were caught during trawl selectivity experiments from different localities of the northern and southern coastal regions. The pro/antioxidant status of fish individuals was assessed by measuring standard OS biomarkers (lipid peroxidation, glutathione concentration, activities of superoxide dismutase and catalase) in gills and liver. Differences in the concentration and activity of OS biomarkers in the studied organs were clearly demonstrated. It was found that the level of OS in the studied fish species differed depending both on the species and on the coastal region they inhabit. Our results demonstrated for the first time the presence of OS in fish inhabiting coastal ecosystems of the Bulgarian Black sea sector with different quality of the marine environment. Obviously, further studies are needed for the assessment of multiple stressor effects on the ecology of Bulgarian Black Sea fish populations.

Key words: Black Sea, Bulgaria, coastal fish, oxidative stress.

Introduction

Marine ecosystems are under the increasing influence of numerous stressors, including climatic, oceanographic, environmental and anthropogenic, causing significant changes in their functioning and the services they provide. The Black Sea is a unique semi-exclosed basin accepting a high river inflow from the rivers Danube, Dnieper, Dniester, and Southern Bug (Zaitsev & Mamaev, 1997). Because of the significant ecological deterioration, the Black Sea has been declared as a highly polluted sea (Oguz & Velikova, 2010; Makedonski et al., 2017). However, intensive pollution does not affect the entire Black Sea but, rather, its northwestern part and also the marginal habitats where marine, terrestrial, and freshwater organisms interact (Zaitsev, 2008).

Fish inhabit a broad range of ecosystems where they are subjected to many different aquatic contaminants. Fish responses to stress can be polymorphic depending on the species, age, diet and on the stressors severity (Pimentel et al., 2015;

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Hamilton et al., 2017; Zielinski & Pörtner, 2000). Different biomarkers are recently becoming an integral part of health assessments and management of marine ecosystems, in addition to the more routine water chemical analyses (Valvanidis et al., 2006; Hook et al., 2014). Fish are traditionally used as bioindicators and play an important role in marine monitoring programs. In many cases, the detrimental effects of water contaminants have been connected to induction of oxidative stress in fish (Lushchak, 2016). The adaptive response of marine fish to environmental changes can be expressed at the cellular level through changes in their pro/antioxidant status. Oxidative stress (OS), as a disturbance of the oxidation/reduction balance in the cell, is characteristic of all aerobic organisms where reactive oxygen species (ROS) are generated together with the antioxidant processes in which they are neutralized (Birnie-Gauvin et al., 2017). The Black Sea ecosystems are especially vulnerable to pressures by various anthropogenic activities and fish, as a key component of these ecosystems, are exposed to multiple stressors of the changing marine environmental conditions. Application of biomarkers for assessing the biological impact of pollutants and xenobiotics, and the relationship between antioxidant responses and susceptibility to oxidative stress in different species of Black Sea fish were studied by a number of authors (Rudneva et al., 2010; Kovyrshina & Rudneva, 2016; Skuratovskaya et al., 2017; Chesnokova et al., 2020; Sigacheva et al., 2020).

At present no research has been carried out on the OS in marine fish in Bulgaria. The aim of this preliminary study was to make an initial assessment of the activities of a battery of OS biomarkers in several common fish species from the Bulgarian Black Sea coastal zone.

Material and Methods

Sampling

The fish selected for the study are common demersal species, including three benthic forms - *Platichthys flesus* (Linnaeus, 1758), *Neogobius melanostomus* (Pallas, 1814), *Trachinus draco* (Linnaeus, 1758), and two bentho-pelagic forms - *Mullus barbatus* (Linnaeus, 1758) and *Merlangius merlangus* (Linnaeus, 1758) (BSFishList, 2020; FishBase ver. 2021). Fish were randomly sampled from trawl catches using pelagic Midwater otter trawl (7x7 mm mesh size of the codend) from 4 localities of the northern and 3 localities of the southern Bulgarian Black Sea coast (Table 1).

Table 1. Trawling localities along the Bulgarian Black Sea coast with geographical co-ordinates and sampled fish species. *Legend:* *N – northern locality; S – southern locality.

Code	Trawling locality	Trawling start point	Trawling end point	Fish species
*N1	Tyulenovo	43.521269 28.728255	43.481384 28.717535	*Platichthys flesus, Merlangius merlangus*
N2	Kaliakra cape	43.365251 28.428852	43.371263 28.405309	*Platichthys flesus, Neogobius melanostomus*
N3	Batova bay	43.378802 28.157542	43.343525 28.142787	*Platichthys flesus, Neogobius melanostomus, Mullus barbatus*
N4	Shkorpilovtsi	42.976214 27.966946	42.952405 27.940966	*Platichthys flesus, Merlangius merlangus*
S1	Nessebar bay	42.599613 27.791345	42.625721 27.832556	*Neogobius melanostomus, Trachinus draco, Mullus barbatus*
S2	Pomorie bay	42.569317 27.791159	42.609556 27.796726	*Trachinus draco, Mullus barbatus*
S3	Sozopol bay	42.439217 27.914862	42.433054 27.887407	*Platichthys flesus, Merlangius merlangus, Neogobius melanostomus, Trachinus draco*

Tissue preparation

The fish samples were shock frozen on board for best preservation (Secci & Parisi, 2016) and transported to the laboratory. The fish were dissected and their liver and gills were extracted following available protocols (Stoyanova et al., 2020 a,b). The organs were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and thereafter centrifuged at 3000 g for 10 min. The post-nuclear fraction was used for determination of lipid peroxidation (LPO) and glutathione (GSH) levels. For obtaining a post mitochondrial supernatant used for measurement of the antioxidant enzymes activities, a portion of the post-nuclear fraction was re-centrifuged at 12 000 g for 20 min at 4°C.

Measurement of oxidative stress biomarkers

Lipid peroxidation was determined using MDA assay kit (Catalog No: MAK085), purchased from Sigma-Aldrich Co. LLC (USA). The assay is based on the reaction of thiobarbituric acid (TBA) with end-products of the LPO. The absorption of the formed malone dialdehyde (MDA) was read at 532 nm and was calculated as nmoles/mg protein using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

Glutathione concentration was measured according to Rahman et al. (2006). The reduced glutathione (GSH) reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) giving a color compound with absorption peak at 412 nm. The GSH amount was calculated using a reference standard and expressed as ng/mg protein.

Superoxide dismutase activity was measured according to Peskin & Winterbourn (2017). The inhibition of water-soluble tetrazolium (WST-1) reduction by superoxide radicals is a measure for enzyme activity. The values were expressed in U/mg protein as one unit is defined as the amount of enzyme needed to inhibit the WAT reduction by 50%.

Catalase Activity was assayed by the method of Aebi et al. (1984), based on the decrease of absorption at 240 nm that corresponds to the enzymatic decomposition of H_2O_2. Enzyme activity was expressed as U/mg protein.

Protein concentration was measured according to Lowry et al. (1951) and calculated from a standard curve, obtained using bovine serum albumin as a standard.

Statistical analyses

The software package used was STATISTICA (Data analysis software system), StatSoft Inc. (2010), Vers. 10. The non-parametric Kruskal–Wallis test was used to confirm the presence of differences among the values of the OS markers in the different groups studied. Post hock comparisons between variables were made using Mann-Whitney test.

Results

In this preliminary study the differences of the OS biomarkers were measured both among the selected fish species and among the localities along the Bulgarian Black Sea coast from which they were sampled. The overall results of the analyses are summarized in Table 2. Kruskal–Wallis test indicated significant differences of OS indicators among sampling sites, fish species and organs which were post hock tested by Mann-Whitney statistics.

The first observation in this study was the presence of significant difference between values of the studied OS indicators in the gills and the liver of all fish species. This differences and their statistical significance are summarized in Figure 1. The LPO and SOD were higher in gills of fish from both northern and southern localities. The SOD activity was significantly higher in gills of fish from the southern localities. In contrast to SOD, the activity of CAT was significantly higher in the liver of fish from both northern and southern localities. Despite the individual differences in GSH concentration in the fish species, no significant differences were found between the two organs of the fish from northern and southern localities.

The analysis of the level of the OS indicators in the fish species from different localities (Table 2) showed that round goby (*N. melanostomus*) had the highest LPO level in the liver of individuals from Tulenovo (N1). The highest LPO in gills was observed in greater weever (*T. draco*) individuals from Nessebar bay (S1). In gill high LPO was found in flounder (*P. flesus*) from Shkorpilovtsi (N4) and Sozopol bay (S3), in round goby (*N. melanostomus*) from Tyulenovo (N1) and in greater weever *(T. draco)* from Pomorie bay (S2) and Sozopol bay (S3).

The lowest GSH concentration in liver was observed in round goby from Nessebar bay (S1) and Sozopol bay (S3) and in gill – in round goby from Tyulenovo (N1). The highest GSH values were measured in whiting from Shkorpilovtsi (N4) in both organs liver and gill. SOD activity in red mullet was high in liver and gills from the localities N3, S1 and S2. High values of SOD were observed in gills of greater weever from the southern areas (S1, S2 and S3) and in round goby from Nessebar bay (S1) and in liver of flounder from Tyulenovo (N1), Pomorie bay (S2) and Sozopol bay (S3). The activity of CAT was in general higher in the liver of all fish species analyzed in comparison to gills. The highest values were detected in the liver of whiting from Shorpilovtsi (N4). The lowest values were obtained in gills of round goby from Tyulenovo (N1).

In the studied individuals of European flounder higher LPO was measured in the gills in comparison to liver, as the highest values were measured in fish from Shorpilovtsi (N4) and Sozopol bay (S3), accompanied by relatively low GSH concentrations (Table 2). These observations indicated stronger oxidative stress in these locations. The relatively low LPO in the flounders from Pomorie bay (S2) was probably due to higher SOD activity in liver (the highest one) and gills. The very high activities of SOD and CAT reported in the gill of flounders caught near Tyulenovo (N1) and the high SOD activity in liver were probably responsible for the lower OS, as indicated also by relatively lower LPO level. It could be assumed that the flounders from the Batova bay (N3) were least stressed, judging by the relatively lower levels of LPO, higher GSH concentrations and the lack of antioxidant enzymes induction in both studied organs.

In whiting, higher LPO was found in the gills. The lowest LPO levels (in both organs) were measured in individuals from Shkorpilovtsi, probably due to the antioxidant defense represented by high levels of GSH and SOD activity in both organs and high CAT activity in liver (Table 2). It seemed that the whiting inhabiting the littoral near Tyulenovo (N1) were exposed to higher OS stress, indicated by the higher LPO in liver, accompanied by low GSH content and lower CAT activity.

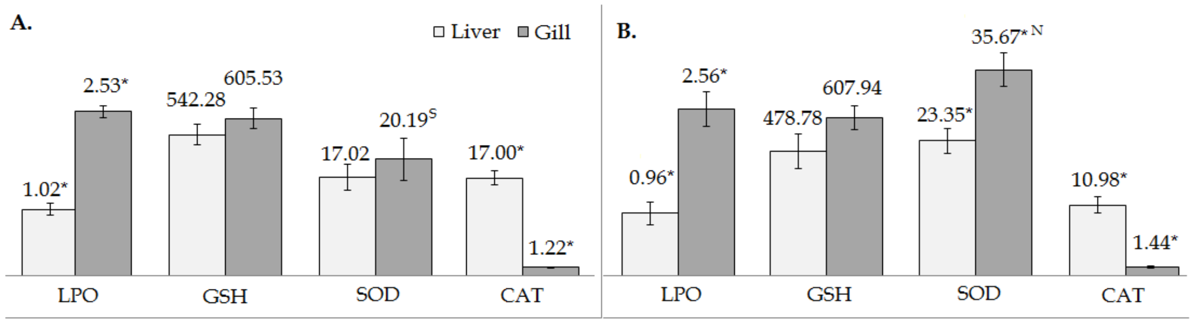
Round goby sampled from Tyulenovo (N1) demonstrated extremely high LPO in liver, accompanied by high activity of SOD compared to the individuals from other localities. The highest LPO level in gills of gobies was also measured in the samples from Tyulenovo (N1), along with the lowest GSH content.

It should be mentioned the low level of GSH in liver of round goby from southern locaties, Nessebar bay (S1) and Sozopol bay (S3), compared to the northern localities. In general, these findings suggested that most probably round goby individuals inhabiting the marine waters near Tyulenovo (N1) were exposed to higher OS stress.

In greater weever the highest LPO both in liver and gills was measured in the specimens sampled from Nessebar bay (S1), together with comparatively lower GSH concentration (Table 2). SOD activities were lowest in the samples from Sozopol Bay (S3) both in liver and gills. Most probably, the marine habitat conditions in Nessebar bay (S1) were more stressful for the greater weever as suggested by the higher LPO in both organs and the low GSH concentration in gills.

In mullet the highest LPO, accompanied by low concentrations of GSH, were found in the liver of the fish from Nessebar bay (S1), suggesting that the mullets in this location were exposed to higher OS stress (Table 2).

In general, the content and activity of the measured OS biomarkers were found to vary not only among fish liver and gills in general, but they varied significantly also among the fish species depending on the different localities they inhabit (Table 3).



(LPO is measured as nmoles MDA/mg protein; GSH is in ng/mg protein; SOD and CAT activities are in U/mg protein)

Fig. 1. Measured OS biomarkers in liver and gills in total fish samples from northern (A) and southern (B) localities (* - difference of indicator between the two organs significant at $p<0.05$; N and S mark significance of difference ($p<0.05$) between indicator from the northern and southern locality correspondingly.

Table 2. Values of the measured OS biomarkers in gills and liver of the studied fish species from different localities of the Black Sea coast (N – northern localities; S – southern localities). *Legend:* *statistical significance of differences at $p<0.05$: N_n or S_n indicate significant differences of the OS indicator in fish species between sites as N=northern sites and S=southern sites; letters (G, L) indicate significance of differences of the OS indicator between organs as G=gill and L=liver.

Organ	**Liver**				**Gills**			
Location/ biomaker	**LPO**	**GSH**	**SOD**	**CAT**	**LPO**	**GSH**	**SOD**	**CAT**
				Platichthys flesus				
N1	*$0.54^{N2,G}$	340.00	$34.49^{N2,N3,N4,S2,G}$	$13.79^{N2,G}$	2.64^{L}	431.35	24.34^{L}	5.73^{L}
Tyulenovo	±0.07	±6.45	±1.45	±1.11	±0.49	±39.26	±1.11	±0.26
N2	$1.04^{N1,N4,S2,G}$	597.09	$2.98^{N1,S2,S3,G}$	$21.48^{N1,N3,N4,S2,S1,G}$	$3.28^{S2,\ L}$	513.87	12.13^{L}	0.77^{L}
Kaliakra cape	±0.21	±77.67	±0.35	±1.56	±0.09	±119.54	±1.01	±0.31
N3	0.61^{G}	694.38	$5.12^{S2,S3,G}$	$14.94^{N2,G}$	2.49^{L}	585.25	12.96^{L}	1.10_{L}
Batova bay	±0.23	±134.82	±1.18	±3.76	±0.40	±106.34	±0.88	±0.11
N4	$0.45^{N2,G}$	343.77	$3.00^{S2,S3,G}$	$14.23^{N2,G}$	$3.62^{S2,L}$	379.12	11.03^{L}	1.76^{L}
Shkorpilovtsi	±0.03	±43.76	±0.77	±1.32	±0.32	±38.20	±0.86	±0.23
S2	0.65	515.05	$47.22^{N1,N2,N3,N4,G}$	$12.75^{N2,\ G}$	$1.74^{N2,N4,S3,L}$	481.11	19.49^{L}	1.03^{L}
Pomorie bay	±0.08	±88.14	±2.47	±1.57	±0.31	±94.64	±5.01	±0.17
S3	0.87^{G}	443.65	$39.63^{N2,N3,N4,G}$	$10.43^{N2,\ G}$	$3.81^{S2,L}$	313.00	17.78^{L}	0.71^{L}
Sozopol bay	±0.25	±142.30	±17.09	±4.78	±0.42	±144.80	±5.14	±0.35
				Merlangius merlangus				
N1	$2.11^{N4,S3}$	734.24^{N2}	27.61^{G}	$17.53^{N4,S3,G}$	2.97^{N4}	$899.11^{N4,L}$	$5.12^{N4,L}$	1.42^{L}
Tyulenovo	±0.08	±41.61	±2.8	±2.10	±0.65	±36.14	±1.18	±0.84
N4	$0.70^{N1,G}$	1082.62^{N1}	30.26^{G}	$30.13^{N1,\ G}$	$1.33^{N1,S3}$	$1673.19^{N1,S3,L}$	$10.45^{N1,L}$	1.18^{L}
Shkorpilovtsi	±0.12	±112.52	±4.10	±4.33	±0.19	±314.48	±0.45	±0.61
S3	$0.92^{N1,G}$	958.53	22.31^{G}	$27.10^{N1,G}$	2.58^{N4}	910.69^{N4}	7.57^{L}	1.38^{L}
Sozopol bay	±0.26	±263.65	±1.92	±0.60	±0.22	±148.07	±1.45	±0.22
				Neogobius melanostomus				
N1	$12.19^{N2,N3,S1,S3,G}$	359.75	$19.36^{N2,N3,S1,S3}$	$6.75^{N3,S3,G}$	$4.18^{N2,N3,S3,L}$	$293.08^{N2,S1,S3}$	27.13^{N2}	$0.55^{N3,L}$
Tyulenovo	±0.94	±63.50	±7.93	±0.63	±0.32	±37.64	±5.16	±0.03

N2	1.68^{N1}	394.30	6.02^{N1}	$5.92^{N3,G}$	1.84^{N1}	$495.33^{N1,S3}$	$1.54^{N1,N3,S1,S3,L}$	$0.82^{N3,L}$
Cape Kaliakra	±0.45	±53.52	±0.54	±2.18	±0.42	±32.83	±0.25	±0.09
N3	1.25^{N1}	518.07	$4.92^{N1,G}$	$23.07^{N1,N2,S1,G}$	2.18^{N1}	427.17^{S3}	$31.69^{N2,L}$	$1.73^{N1,N2,S1,S3,L}$
Batova bay	±0.74	±87.28	±0.92	±1.36	±0.73	±108.66	±1.72	±0.05
S1	2.23^{N1}	$238.19^{N3,G}$	$6.46^{N1,G}$	$4.48^{N3,G}$	2.63^{N1}	$415.67^{N1,S3,L}$	$43.36^{N2,L}$	$0.84^{N3,L}$
Nessebar bay	±0.32	±29.55	±1.76	±1.00	±0.31	±28.87	±3.47	±0.13
S3	$0.93^{N1,S1}$	$287.09^{N3,G}$	$1.98^{N1,G}$	15.58^{G}	$0.85^{N1,N2,N3,S1}$	$929.68^{N1,N2,N3,S1,L}$	$35.49^{N2,L}$	$0.83^{N3,L}$
Sozopol bay	±0.25	±55.74	±0.52	±3.73	±0.16	±199.64	±2.47	±0.37
				Trachinus draco				
S1	$1.08^{S2,G}$	571.21^{G}	7.09^{G}	5.87^{G}	4.96^{L}	$469.13^{S2,S3,L}$	$45.08^{S2,L}$	$2.36^{S2,L}$
Nessebar bay	±0.08	±26.77	±0.75	±0.73	±0.25	±9.52	±4.12	±0.29
S2	$0.47^{S1,G}$	$307.86^{S3,G}$	7.58^{G}	4.75^{G}	3.81^{L}	756.43^{L}	$58.93^{S1,S3,L}$	$1.60^{S1,S3,L}$
Pomorie bay	±0.14	±39.98	±0.45	±0.57	±0.25	±36.72	±1.83	±0.23
S3	$0.72^{S1,G}$	$628.05^{S2,G}$	5.17^{G}	6.98^{G}	3.76^{L}	897.50^{L}	$39.08^{S2,L}$	$2.51^{S2,L}$
Sozopol bay	±0.08	±24.49	±0.66	±0.72	±0.28	±52.57	±1.71	±0.13
				Mullus barbatus				
N3	0.55^{S1}	483.54	44.39	$22.09^{S1,S2,G}$	0.73	357.87^{S2}	51.66^{S2}	1.46^{L}
Batova bay	±0.13	±195.73	±6.49	±4.7	±0.27	±22.25	±6.21	±0.28
S1	0.96^{N3}	390.37	50.05	$11.22^{N3,G}$	0.61	354.62^{S2}	49.99^{S2}	1.21^{L}
Nessebar bay	±0.02	±20.71	±5.02	±0.82	±0.02	±29.37	±2.60	±0.24
S2	0.72	447.28	38.09	$11.18^{N3,G}$	0.74	$504.63^{N3,S1}$	$39.89^{N3,S1}$	1.95^{L}
Pomorie bay	±0.08	±82.98	±10.27	±0.87	±0.10	±34.35	±0.41	±0.09

Table 3. Results of OS biomarker content and activity measured in the different species and localities. *Legend:* *statistical significance of differences at $p<0.05$: N or S indicate significant differences of the OS indicator in fish species among northern (N) sites and southern (S) sites; G (gills) or L (liver) indicate significance of differences of the OS indicator between organs.

	Liver		**Gills**	
	Northern	**Southern**	**Northern**	**Southern**
		Platichthys flesus		
LPO	$0.66±0.28^{G}$	$0.76±0.21^{G}$	$3.01±0.89^{L}$	$2.77±1.09^{L}$
GSH	495.23±176.12	479.34±123.62	477.39±115.48	397.00±148.24
SOD	$6.40±3.35^{G}$	$43.42±2.78^{*G}$	$15.11±5.45^{L}$	$18.64±5.15^{L}$
CAT	$16.12±3.83^{G}$	$11.30±3.66^{G}$	$1.09±0.48^{L}$	$0.87±0.32^{L}$
		Merlangius merlangus		
LPO	1.41±0.71	$0.92±0.20^{G}$	2.16±0.94	$2.58±0.17^{L}$
GSH	908.43±193.75	958.53±124.00	1286.15±447.10	910.69±120.00
SOD	$24.90±3.57^{G}$	$30.26±3.39^{G}$	$7.79±2.81^{L}$	$7.75±1.19^{L}$
CAT	$23.83±7.16^{G}$	$27.10±0.48^{G}$	$1.62±0.76^{L}$	$1.38±0.18^{L}$
		Neogobius melanostomus		
LPO	3.05±1.48	1.59±0.71	2.73±1.16	1.74±0.92
GSH	424.04±97.26	$267.62±50.86^{G}$	405.19±108.75	$672.68±119.59^{L}$
SOD	10.10±4.01*	$4.22±1.30*^{G}$	$20.12±7.32^{S}$	$39.43±5.02^{N,L}$
CAT	$11.92±4.02^{G}$	$10.03±6.19^{G}$	$1.03±0.51^{L}$	$0.84±0.27^{L}$
		Mullus barbatus		
LPO	0.56±0.15	0.84±0.15	0.73±0.31	0.73±0.15
GSH	483.54±113.00	418.83±73.22	357.87±25.70	429.62±89.31
SOD	44.39±7.49	44.07±11.01	51.66±7.17	44.94±5.90
CAT	$22.09±5.43^{*G}$	$11.20±0.93^{*G}$	$1.46±0.32^{L}$	$1.58±0.45^{L}$

Among the studied fish species, a general pattern in the total level of biomarkers in organs was demonstrated, i.e. in flounder - higher LPO and lower CAT activity in gills than in liver; in whiting higher SOD and CAT activities in liver than in gills; in round goby – higher SOD activity in gills than in liver and in contrast lower CAT activity in gills than in liver; in mullet – higher CAT activity in liver than in gills (Table 3). Additionally, significant variations in the OS biomarkers in gills and liver between the fish species from the northern and southern localities were also observed. These variations could have induced the antioxidant enzyme complex. The flounders inhabiting southern localities had higher average SOD activity in liver than those of northern localities; the round goby inhabiting northern localities had higher average SOD activity in liver and lower in gills, and the mullet individuals from the northern localities had a higher average CAT activity in liver.

Discussion

In this preliminary study, biomarkers of oxidative stress in the gills and liver of five marine fish species of the Bulgarian Black Sea part were analyzed as indicators of the stressfulness of the marine environment.

The presence and level of OS in the fish species studied cannot be determined only by the level of a separate marker, rather the interrelation of all markers should be taken into consideration. Both the gills and the liver are considered target organs sensitive to oxidative damage. The gills are the organ in direct contact with the marine environment and the pollutants in it. Thus, gills are exposed to higher concentrations of pollutants than other organs (Heath, 1987). Therefore, our results on the significant differences of the OS markers in fish liver and gills in particular localities are logical and expected. A number of authors note that gills are more sensitive to oxidative damage than the liver and may respond earlier to oxidative challenges induced by pollutants (Ahmad et al., 2004; Guilherme et al., 2012). In general, in the gills of the studied fish the LPO, as a marker for the prooxidant effect of adverse environments, was significantly higher than those in liver. Specifically, we found that the activities of CAT in the gills of the studied fish were significantly lower than those in liver. This finding is consistent with the observations of other authors (Jos et al., 2005; Cazenave et al., 2006; Ballesteros et al., 2009).

Regardless of the variations among the studied fish species our results showed that the fish from the northern localities (Tyulenovo) were more strongly exposed to oxidative stress - the highest LPO in liver of round goby among all tested fish and also high LPO in gills, accompanied by the lowest GSH. Similar pattern of high levels of LPO in liver and gills and low GSH concentrations in whiting from Tulenovo was also present. These findings are in line with the fact that the most northern region of the Bulgarian Black Sea is known to be under the strong influence of contamination by the River Danube inflow (Dineva, 2011). Sewage effluents, even treated, and other pollutants are also known to compromise the health of aquatic organisms (Hébert et al., 2008; Kamel et al., 2012, Yancheva et al., 2020). Localities, exposed to higher coastal inputs of pollutants, due to their proximity to anthropogenic sources, such as the resorts Slanchev Bryag, Nessebar and Sozopol with high touristic flow, were found to cause oxidative stress in the studied fish species from these localities, i.e. flounder (relatively high LPO, low GSH, increased SOD activity), round goby (low GSH, activated SOD) and mullet (low GSH, activated SOD). Changes in the oxidative status of the same or similar fish species caused by anthropogenic contamination in different regions of the Black Sea were reported by other authors (Kovyrshina & Rudneva, 2012, 2016; Sigacheva et al., 2020; Chesnokova et al., 2020; Bozcaamutlu et al., 2020).

Changes in the activity of antioxidant enzymes as biomarkers of the response of organisms to environmental conditions are well accepted in environmental monitoring systems (Winstin & Di Giulio, 1991; Oruc et al., 2004; Jebali et al., 2013; Bozcaarmutlu et al., 2020). Their peculiarity is in the fact that, depending on the duration and strength of the effect, they can be either activated (as an adaptive reaction) or inhibited (Ballesteros et al., 2009). Thus, the activity of antioxidant enzymes changes following a bell-shaped curve and it is difficult to assess the state of organisms by assessment of enzyme activity alone. It is more accurate to observe a set of biomarkers to obtain a more reliable picture of environmental impacts. The SOD and CAT activities in fish liver indicate also the activity of species as the more mobile fish species were found to have higher enzyme activity compared with the low mobile forms (Filho et al., 2007; Rudneva et al., 2010). The higher activity of liver antioxidant enzymes in the more mobile fish species is correlated with the higher oxygen consumption and metabolic rate (Martinez-Alvarez et al., 2005; Filho, 2007). This leads to higher free radical production rates that cause induction of antioxidant defense mechanisms (Zelinski & Portner, 2000). Our results showing higher activity of SOD and CAT in liver of *M. barbatus* and *M. merlangus* are in line with these observations.

Conclusion

The preliminary assessment of the oxidative status of common marine fish species, inhabiting different localities along the Bulgarian Black Sea coast was carried out. Significant variation of the biomarkers of oxidative stress were established which indicated the presence of different effects of anthropogenic pressure and the presence of corresponding response of the studied fish species. All the fish species studied were subjected to different levels of oxidative stress caused by the ecological state of the marine environment in the localities they inhabit. Changes in the oxidative stress biomarkers in fish species from the Bulgarian Black Sea coastal zone can be used in marine monitoring surveys as integral measure of the effects of multiple stressors. Obviously, further studies are needed.

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Losses of Honey Bee Colonies and Risk Factors for their Mortality in Bulgaria During 2020

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Abstract. The study is based on a comparative analysis of the established losses of bee colonies by regions in Bulgaria and on the characterization of the risk factors for available mortality in 2020. Information for different types of forage sources with potential risk for *Apis mellifera* due to pesticide treatment, was presented and analyzed. By using the international standardized COLOSS questionnaire for 2020, members of the National Bee Breeding Association and independent beekeepers, owners of a total of 64 apiaries (over 6,800 bee colonies), located in all regions in Bulgaria, were surveyed. Beekeepers were asked to answer questions about the number of wintering honey bee colonies and how many of them after the winter: were alive but had unsolvable queen problems; were lost through natural disaster and were dead or reduced to a few hundred bees. The survey data show that the highest mortality was found for the North Central region (19%), and the lowest – for the Northwest (1%) and Southwest (2%) regions. Among the reasons for the loss of bee colonies, the leading one is the mortality of honey bees or their significant reduction in the colonies, which is also related to the negative impact of the applied pesticides in the studied areas. In this aspect, the most serious problems were reported in the North Central and Southeast (7%) regions. The presented and analyzed data should be considered when developing activities to protect the honey bee health status in Bulgaria.

Key words: honey bee, *Apis mellifera*, reasons for mortality, forage sources, pesticides.

Introduction

Honey bees are the main pollinators of entomophily vegetation and an important factor for preserving plant diversity in nature. The treatment of agricultural crops with various pesticides is a risk factor for the health and overall vitality of bee colonies. The phenomenon, known today as the Colony Collapse Disorder (CCD), poses a serious challenge for scientists around the world, to analyze the causes of increasing mortality among honey bee populations and to propose measures to protect *Apis mellifera* species. Studies on the problem indicate that the main factors leading to bee losses are *Varroa destructor*, *Nosema* spp., various viruses (mainly *Acute Bee Paralysis Virus* and *Deformed Wing Virus*), pesticides used in agricultural practice and the nutrition of bee colonies (Potts et al., 2010; van Engelsdorp & Meixner, 2010; Gajger et al., 2017; Steinhauer et al., 2018). Among the reasons for the loss of bee colonies are starvation, theft, crude weather conditions, new pathogens and more. Honey bees are often exposed to the simultaneous effects of multiple stressors, leading to a synergistic result that goes beyond the negative effects of each of them individually (Alaux et al., 2010; Johnson et al., 2013; Tosi et al., 2017; van Dooremalen et al., 2018; Straub et al., 2019; Tomljanović et al., 2020). The study by Steinhauer et al. (2021) indicates small but meaningful growth of successful wintering bee colonies through management practices of a higher level. These results show the positive impact of the designed and used programs, as well as the guidelines provided by scientific experts for work in the target groups of beekeepers. Ullah et al. (2021) presented the viral effects on honey bee populations and indicated that areas of the world responsible for supplying frequently consumed food had a high number of bee losses per year. Various environmental factors that negatively affect the viability of honey bees are: intensive farming with long-term use of pesticides, food shortages, habitat loss, new pathogens and pests (Goulson et al., 2015; McMenamin & Genersch, 2015 ; Tantillo et al., 2015; Brutscher et al., 2016). Ullah et al. (2021) express their vision for a unified health approach worldwide in support of beekeeping and its management in the future.

According to the official data of Agrostatistics (February 2020) in Bulgaria there are 13,771 apiaries with 867,561 bee colonies as of October 1, 2019. Of them total, 58,377 bee colonies were destroyed. Statistics show poisoning as the leading cause with the highest percentage of losses - 24,317 bee colonies.

Since 2008, through the European COST action FA0803 (Prevention of honey bee COlony LOSSes), the international organization COLOSS has been established, uniting *Apis mellifera* researchers from all over the world. COLOSS (http://coloss.org) is currently an international non-profit association based in Bern, Switzerland. Its main goal is to improve the welfare of bees globally. COLOSS conducts annual monitoring of the condition of honey bee colonies around the world through its network of researchers. According to a study published in 2020 (Gray et al., 2020) on the situation with honey bees in 35 countries, the losses of bee colonies in Bulgaria are 5.8%.

Among the leading causes of bee losses in Europe and Bulgaria, the negative impact of the use of various pesticides is increasingly mentioned (Tomizawa & Casida, 2003; Elbert et al., 2008; Harper et al., 2009; Gervais et al., 2010; Migdal et al., 2018; Ivanova, 2018; Hayasaka et al., 2019). Nenicotinoids are neurotoxic insecticides that have been reported to have a strong negative effect on the health of honey bees at the population level (Henry et al., 2012; Rundlöf et al., 2015; Tsvetkov et al., 2017). Thiamethoxam and clothianidin because of their chemicals composition have been identified as potential risk agrochemicals to the vitality of the bee colonies (Lundin et al., 2015; Schmuck & Lewis, 2016; Gajger et al., 2017). In Bulgaria, the losses of bee colonies and the complex of reasons for them have been partially studied (Ivanova & Petrov, 2010; Ilieva et al., 2020).

The aim of the present study is to identify, characterize and comparatively analyze the regional differences in the losses of bee colonies and the reasons for them in Bulgaria during the period 2019-2020.

Material and Methods

A national survey among beekeepers in Bulgaria, including a standardized questionnaire for comparability of responses (Van Der Zee et al., 2013), was conducted between April and June 2020 as part of the COLOSS international monitoring survey (Brodschneider et al., 2016, 2018, Gray et al., 2019, 2020). The data were collected by filling in a paper questionnaire and by e-mail. Beekeepers were asked about the number of wintering bee colonies and how many of these colonies were alive after the winter: a) but with unsolvable problems affecting the queen bee; b) were lost due to a natural disaster (fire, storm, flood, etc.); c) have been lost due to mortality or have been reduced to a few hundred bees. The survey was conducted among professional (members of the National Bee Breeding Association) and independent beekeepers, a total of 64 apiary owners with 6897 bee colonies located throughout the country. The questionnaire also gathered information on the presence in the areas of and around the apiaries of orchards and plantations of rapeseed, corn, sunflower, heather and autumn fodder crops, which could be forage sources and a potential risk factor for pesticide poisoning of the honey bees.

Differences between groups compared were analyzed by the Pearson Chi-Square test.

Results

The data from the present study showed that the total percentage of losses of bee colonies in Bulgaria for the period 2019 - 2020 was found to be 12.11%. The largest losses were reported in the North Central region - 18.93%, and the smallest - in the Northwest region - 1.05%. Regarding the three compared criteria - a) unsolvable problems with the queen bee, b) losses due to a natural disaster and c) mortality or reduction of the bee number in the colonies to a few hundred (Gray et al., 2020), the survey data were as follows: 4.57% - by criterion "a"; 1.97% - by criterion "b" - and 5.57% - by criterion "c". The distribution of bee losses by criteria for different regions is presented in Table 1.

Table 1. Distribution of the honey bee losses in Bulgaria by regions according to the studied criteria: a) unsolvable queen problems; b); natural disaster c) death or reduction to a few hundred bees (in %): p < 0.05*; p < 0.01**; p < 0.001***.

Regions	a	b	c	Total losses
2019/2020	4.57%	1.97%	5.57%	12.11%
Northwest	0.0	1.05	0.0	1.05 *
North Central	8.62	3.61	6.70	18.93
Northeast	0.13 *	0.26 *	3.59	3.98 *
Southwest	0.0	0.0	2.36 ***	2.36 *
South central	1.39 **	0.70	2.09 ***	4.17 *
Southeast	0.35 *	0.0	7.35	7.70

It is evident that according to criterion "a", the most significant were the losses in the North Central region - 8.62%. In Northwestern and Southwestern Bulgaria there were no reported losses under this criterion, and in Northeastern, Southeastern and South-Central Bulgaria they were 0.13%, 0.35% and 1.39%, respectively - values statistically significantly lower than the average established by this indicator. According to criterion "b", the most losses were reported again in the North Central region - 3.61%. The losses in the South West and South East regions were zero, and in North West, North East and South-Central Bulgaria the reported bee losses were respectively: 1.05%, 0.26%; and 0.7%. There was a statistically significant difference compared to the average values of this criterion only in terms of losses in the Northeast region. According to criterion "c", the most significant losses of bee colonies were reported for the South East and North Central regions - 7.35% and 6.7%. These values were close to the averages established for our country. In the North East, South

West and South-Central regions the losses according to this criterion were: 3.59%; 2.36% and 2.09%, respectively, the latter two values being statistically significantly lower than the averages established in the course of the study under criterion "c". No losses were reported under this criterion in the North West region (Table 1).

The losses under criterion "c" were significantly related to the alarming trend of mass poisoning of honey bees in some regions of the country due to the widespread use of various pesticides in agricultural practice. In this regard, information was collected on plant resources, which have been also forage sources for honey bees, but at the same time pose a risk to their health due to treatment with pesticides - orchards, oil seed rape, maize, sunflower, autumn forage crops (Table 2).

Table 2. Availability reported by regions for different types of forage sources as potential risk factor for pesticide poisoning of the honey bees.

Forage sources Regions	Orchards	Oil seed rape	Maize	Sunflower	Autumn Forage Crops
Northwest	+			+	+
North Central	+	+	+	+	+
Northeast	+	+	+	+	+
Southwest		+			
South central	+			+	
Southeast	+	+	+	+	

The data from the present study showed that in the North Central and the Northeast regions all of these crops were grown, and in Southeastern Bulgaria - all, except for autumn forage crops. These results, compared with the information on the reported losses of bee colonies by regions, showed that in the South East and the North Central regions most honey bee colonies have died according to criterion "c" (7.35% and 6.7%, respectively). These significant losses in areas with orchards, sunflower, oil seed rape and maize are indicative of the high danger that these crops pose to honey bees.

Discussion

The results obtained in our study could be compared with the official data from the last published monitoring study of COLOSS (Gray et al., 2020). It notes that for all countries in Europe (31 countries out of a total of 35 participants in the survey) the identified losses by criteria were as follows: 4.1% (95% CI 4.0-4.1%) of bee colonies were lost due to problems with the queen bee; 10.7% (95% CI 10.5-10.9%) - due to death after hibernation and 1.9% (95% CI 1.8-2.0%) - due to natural disaster. The average reported loss rate for the countries included in the study was 16.7% (95% CI 16.4-16.9%). For the period 2018 - 2019, the highest percentage of bee losses - 32% - was established for the territory of Slovenia, and the lowest - for Bulgaria - 5.8%. The authors of the study connect the low reported mortality in Bulgaria with the professionalism of the beekeepers included in the survey, who work on a national program for the preservation of the local Bulgarian honey bee. According to Ilieva et al. (2020) in Bulgaria for the period 2017-2018 were reported 2.04%, and for the period 2018-2019 - 5.22% honey bee colony losses. The losses due to problems with the queen bee are of the order of 1.13% for both compared periods, those due to death or reduction to a few hundred bees - 0.78% and 2.88%, respectively for 2017-2018 and 2018-2019, and due to natural disasters - 0.2% and 1.22% for the two compared periods. The significantly lower established values for our country according to the criteria "a", "b" and "c" for the past comparative periods could be explained by the fact that in the surveys at that time mainly professional beekeepers participated. As part of the National Bee Breeding Association (NBBA), they work on a national breeding program aimed at protecting the local Bulgarian honey bee *A. m. rodopica*. In addition to representatives of the NBBA, a number of independent beekeepers took part in the present study, which makes it more objective in characterizing the honey bee colony condition in Bulgaria.

In their study, Gray et al. (2019) notes that intensive feeding with any of the indicated plant sources (orchards, oil seed rape, maize, heather and autumn forage crops) is associated with significantly higher honey bee losses. This is why the authors identify them as potential risk factors for the loss of bee colonies in Europe due to the widespread treatment with pesticides (Gajger et al., 2017; Gray et al., 2019; Tomljanović et al., 2020). In this respect, the situation in Bulgaria is similar - orchards, sunflower plantations and rapeseed areas are available in the habitat areas of the majority of the apiaries included in the study on the territory of the country (Table 2).

Conclusions

Honey bee colony losses in Bulgaria for the period 2019 - 2020 are found to be 12.11%.

The most significant are the losses due to mortality or reduction of honey bees in the colony to a few hundred (5.57%).

Potential risk factors for honey bee vitality could be forage sources such as orchards, sunflowers, oil seed rape, maize and autumn forage crops due to their pesticide treatment.

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Chromosome and Pollen Morphology of Amaranthus hybridus L. and Amaranthus retroflexus L. in Bulgaria

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Abstract. The chromosome and pollen morphology in the Bulgarian populations of *Amaranthus hybridus* L. and *Amaranthus retroflexus* L. was studied. The diploid chromosome number $2n$ = 34 was found. The metacentric type of chromosomes was predominant in all studied populations. In the karyotype of *A. hybridus*, the length of the metaphase chromosome ranged from 0.38 µm to 1.60 µm, and the total haploid length of the chromosome set - from 11.44 µm to 13.50 µm. The length of the chromosomes in *A. retroflexus* ranged from 0.18 µm to 2.27 µm, and the total haploid length of the chromosome set - from 13.01 µm to 23.32 µm. Pollen morphology was examined using a scanning electron microscope (SEM). Pollen was defined as a spherical, pantoporate type. The pollen diameter in *A. hybridus* varied from 18.93 µm to 22.11 µm, and in *A. retroflexus* - from 16.75 µm to 22.21 µm. Differences in the number and diameter of pores have been found between the two species.

Key words: *Amaranthus hybridus* L.; *Amaranthus retroflexus* L.; chromosome number; karyology; pollen morphology; idiograms; Bulgaria.

Introduction

Genus *Amaranthus* L. (Amaranthaceae Juss.) comprises about 65-80 species, spread mainly in tropical, subtropical and warm-moderate areas. Worldwide, some of the species are spread as introduced and naturalized weeds (Mosyakin & Robertson, 1996, 2003; Bojian et al., 2003; Mujica & Jacobsen, 2003; Iamonico, 2016). The representatives of the genus are predominantly edible food plants and a significant part thereof are categorized as agricultural weeds or pseudocereals all over the world (Costea & Halmajan, 1996; Mosyakin & Robertson, 2003). Some species are grown due to their ornamental qualities (Brenner et al., 2000; Jäger et al., 2008; Shukla et al., 2004, 2006, 2010).

In the Bulgarian flora the genus is represented by 13 species (Kovachev, 1966; Assyov & Petrova, 2012; Petrova, 2018). These are spread mainly ruderally, along roads, gardens and agricultural areas, partially in parks and gardens. The present study includes two species - *Amaranthus hybridus* L. and *Amaranthus retroflexus* L.

Up to that moment the Bulgarian populations of both species have not been an object of a detailed study. Chorological data point to their wide spreading all over the country (Assyov & Petrova, 2012). Their habitats are mainly ruderal terrains, orchards and vegetable gardens. They can be found up to 1000 m a.s.l. *A. hybridus* is considered one of the most dangerous weeds in the words. *A.*

retroflexus is a widely spread weed for the Bulgarian flora (Petrova et al., 2013).

The karyological data are scarce and incomplete, partially due to difficult taxonomic differentiation of the species. Worldwide, *Amaranthus* L. comprise a genus exhibiting variation in the chromosome numbers and ploidy level, but interspecies variation is minimum (Pal, 1982). The latest report about the chromosome number of the genus is from the end of the XXth century.

Therefore, the aim of the present study is to investigate the chromosomal and pollen analysis of the species *Amaranthus hybridus* L. and *Amaranthus retroflexus* L. in Bulgaria.

Materials and Methods

The present study includes three populations of each species. The data comprise three floristic regions in the country (Table 1). To establish the species chromosome number and karyotype durable preparation were prepared from the metaphase plates of root tips. All root tips were prepared from seeds collected natural habitats of *A. hybridus* and *A. retroflexus* and germinated under laboratory conditions. The root tips were treated and squashed following the methodology by Grozeva (2007).

The chromosome type was determined in relation to the centromere index I = s / s + l, according to the classification recommended by Grif & Agapova (1986). The karyograms and ideograms were been processed by the Adobe Photoshop 2020 and Karyo Type Win 2018 software. The data were obtained on the basis of three metaphase plates from each population. The interchromosome asymmetry was calculated by means of index A_2 (Zarco, 1986). To determine intrachromosome asymmetry the following indexes were used: general shape stated as percentage - TF% (Huziwara, 1962); percentage of karyotype asymmetry - Ask% (Arano, 1963); symmetry index - Syi (Greilhuber & Speta, 1976); intrachromosome asymmetry A_1 (Zarco, 1986); asymmetry level A (Watanabe et al., 1999); the four categories of Stebbins (1971) - SKS: from A to D according to the arm ratio and the centromere location in the chromosome. Each of the four categories has three subtypes determined according to the largest/smallest chromosomes ration (Table 2).

Table 1. Data about the studied *A. hybridus* and *A. retroflexus* populations in Bulgaria.

Species	Population locality	Coordinates	Altitude (m)	Floristic regions	2n
A. hybridus L.	Elin Pelin	N42^{0}67.047″ E023^{0}59.701″	544	Sofia Region	34
	Pavel banya	N42^{0}35.344″ E025^{0}12.515″	406	East Sredna Gora	34
	Plovdiv	N42^{0}08.086″ E024^{0}47.862″	157	Thracian Plane	34
A. retroflexus L.	Asenovgrad	N42^{0}00.745″ E024^{0}52.317″	238	Thracian Plane	34
	Zvanichevo	N42^{0}11.380″ E024^{0}15.000″	221	Thracian Plane	34
	Plovdiv	N42^{0}08.017″ E024^{0}48.049″	155	Thracian Plane	34

Table 2. Intrachromosomal asymmetry indexes (Stebbins, 1971).

Ratio: largest/smallest chromosomes	Proportion of chromosomes with arm ratio > 2:1			
	0.0	0.01 – 0.5	0.51 – 0.99	1.0
< 2:1	1 A	1 B	1 C	1 D
2:1 – 4:1	2 A	2 B	2 C	2 D
> 4:1	3 A	3 B	3 C	3 D

The morphological characterization of pollen was done by scanning electron microscope (SEM). The study was carried out at the laboratory of the Faculty of Chemistry and Pharmacy at „St. Kliment Ohridski" University of Sofia. The data were obtained from a minimum of ten pollen grains for each studied population. Herbarized plant parts were used. The herbarized materials are mounted on a metal tripod, covered with gold particles in an ionizing chamber and observed under scanning electron microscope (JEOL 5510). The pollen terminology used conforms to Erdthman (1952), Kremp (1965), Walker & Doyle (1975). The following morphological characteristics were determined: 1) Pollen diameter (maximum diameter, µm) - D_1; 2) Polar axis (diameter perpendicular to D_1, µm) - D_2; 3) Distance among three adjacent pores forming a triangle with sides as close as possible to the highest grain focus (µm) - C; 4) C/D_1 ratio; 5) Total number of pores - TNP; 6) Pore diameter (µm) - PD; 7) Pore area (µm^2) - PA; 8) Number of spinules per 100 µm^2 - NS/100 µm^2; 9) Number of spinules in the pores – NSP; 10) Polar shape; 11) Equatorial shape. The statistical analysis was carried out by Microsoft Excel 2010.

Results

Karyology. As a result of the karyological study of the species *A. hybridus* and *A. retroflexus* in Bulgaria diploid chromosome number 2*n* = 34 was established in the six studied populations (Table 1). The karyomorphological data are presented in Table 3. The ideograms of both species are given on Fig. 1.

Two types of chromosomes were reported: meta- and submetacentric. Predominant in both species are the metacentric chromosomes. In the population from Elin Pelin a karyotype was established formed only by metacentric chromosomes – 2*n* = 34m. In the other two *A. hybridus* populations, diploid chromosome number 2*n* = 32m + 2sm was reported for the population from Pavel banya, and 2*n* = 27m + 7sm for the population from Plovdiv. In two of the three studied *A. retroflexus* populations – Asenovgrad and Plovdiv, identical 2*n* = 31m + 3sm karyotype was recorded. In the third population, the one from Zvanichevo, similar data were registered - 2*n* = 33m + 1sm. The chromosome size varies from 0,74 µm for the representatives from Elin Pelin to 1,68 µm for the ones from Zvanichevo. From all studied karyological data the shortest arm is 0,11 µm long and the longest one - 1,38 µm. Both arm values were registered in the same *A. retroflexus* population from Asenovgrad. The total sum of the haploid chromosome length is within small limits - from 11,44 µm to 13,50 µm for the *A. hybridus* populations and from 13,01 µm to 23,32 µm for *A. retrofexus* population. The data are supplemented by the results obtained for the asymmetry index (A_2) given in Table 3.

According to the classification of Stebbins (1971) there are four different types of symmetry. For the *A. hybridus* representative these are: 1B – Elin Pelin, 1C – Pavel banya and 2C – Plovdiv. In *A. retroflexus* the index is of the following type: 1B – Zvanichevo, 1C – Asenovgrad and 2C – Plovdiv. The intrapopulation symmetry shows similar results for the TF% and Ask% indexes. The lowest and the highest Syi value is found in the *A. hybridus* populations and varies: from 72,71 – Plovdiv to 82,72 – Pavel banya. The lowest A_1 and A values are found in the population from Zvanichevo (*A. retroflexus*), and high ones are registered in the population from Plovdiv (*A. hybridus*). Correlation data are given in Table 4.

Pollen morphology

The palynological analysis was made on the basis of ten qualitative and quantitative traits (Table 5, Fig. 2). The data show that all studied populations are characterized by spherical pollen. The pollen is pantoporate, elliptically jagged and covered by numerous spinules.

Morphometric studies showed similar results for diameter D_1 in *A. hybridus* populations: from 20,93 µm – Plovdiv to 21,78 µm – Elin Pelin. Greater range of the values was registered in *A. retroflexus*: from 18,57 µm – Asenovgrad to 22,43 µm – Zvanichevo. The last two populations contain both the smallest and the largest D_2 value in the six studied habitats. The polar axis varies from 14,93 µm to 22 µm. The measured distance between the pores is with similar results, but the highest values were reported in the two populations from Plovdiv. The C/D1 ratio is within the limits from 0,152 to 0,200. The smallest pore diameter was measured in the Plovdiv *A. hybridus* population. The highest value of the diameter – 1,84 µm was measured in both species. The pollen area varies from 2,06 µm to 2,66 µm. The smallest total number of spinules - 279 and number of spinules in the pores – 7,25 were reported in the population from Asenovgrad.

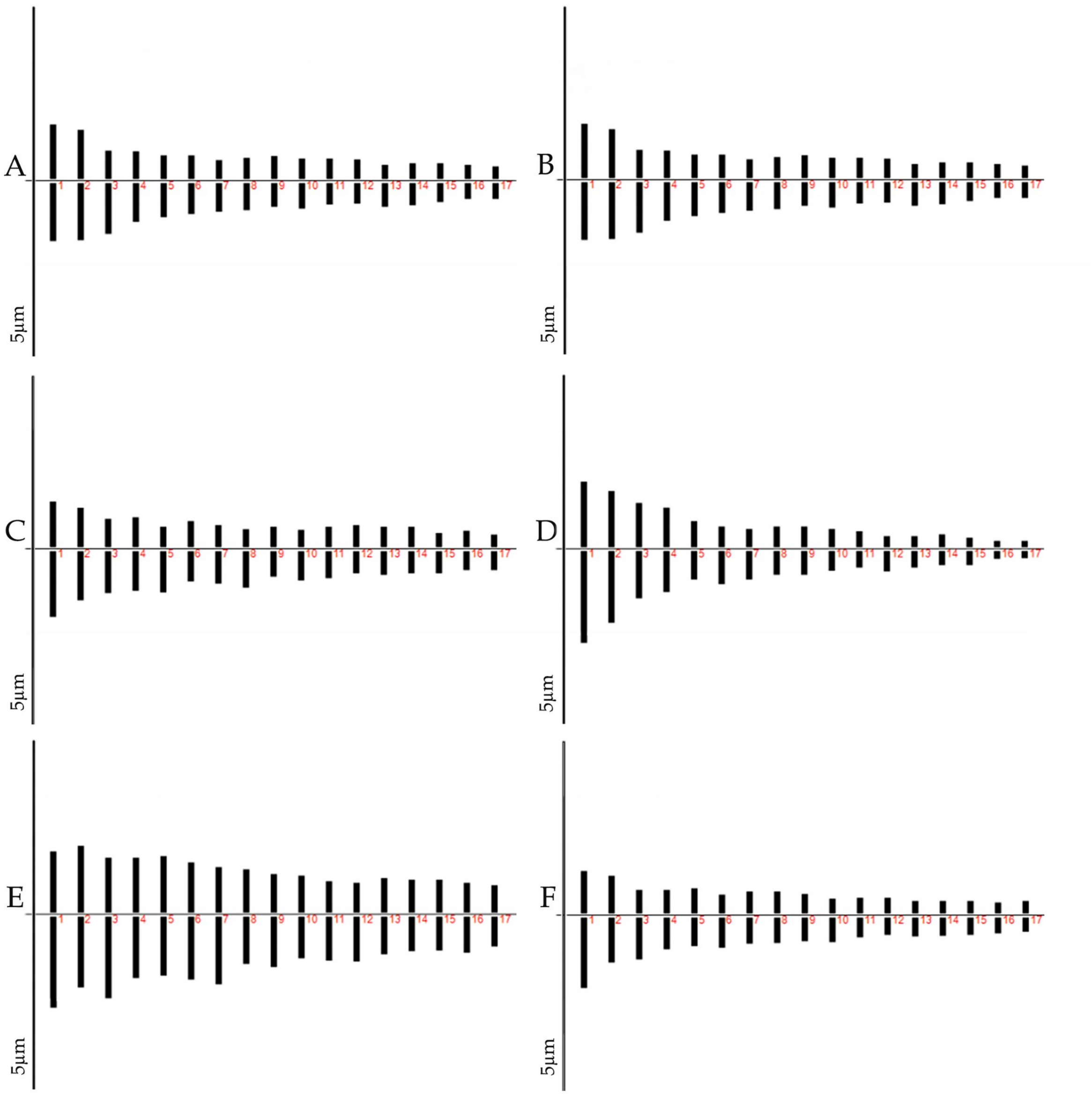

Fig. 1. Idiograms of *Amaranthus hybridus* and *Amranthus retroflexus*, 2*n*=34.
A. hybridus: A) Elin Pelin, B) Pavel banya, C) Plovdiv;
A. retroflexus: D) Asenovgrad, E) Zvanichevo, F) Plovdiv; scale bar 5 µm.

Table 3. Karyomorphometric data for the representatives of *A. hybridus* and *A. retroflexus* in Bulgaria. *Legend:* Chromosome size variation (µm) – short (S) and long (L); total sum of the haploid chromosome length (hcl, µm).

Population	Karyotype formula	S	L	hcl	Inter index	Intrachromosomal index					
					A_2	SKS	TF%	Ask%	Syi	A_1	A
					A. hybridus L.						
Elin Pelin	2*n* = 34m	0.17	0.57	11.44	0.21	1B	44.78	55.22	81.11	0.19	0.11
Pavel banya	2*n* = 32m + 2sm	0.17	0.84	13.00	0.44	1C	45.27	54.73	82.72	0.18	0.10
Plovdiv	2*n* = 27m + 7sm	0.13	1.00	13.50	0.37	2C	42.10	57.90	72.71	0.26	0.15
					A. retroflexus L.						
Asenovgrad	2*n* = 31m + 3sm	0.11	1.38	13.01	0.74	1C	44.34	55.66	79.66	0.19	0.11
Zvanichevo	2*n* = 33m + 1sm	0.39	1.29	23.32	0.31	1B	44.88	55.12	81.43	0.17	0.10
Plovdiv	2*n* = 31m + 3sm	0.16	1.33	13.58	0.47	2C	42.53	57.47	74.01	0.24	0.14

Table 4. Correlations for asymmetry indexes of the studied *Amaranthus* species from Bulgaria. *Legend:* *Correlation is significant at p<0.05.

Indexes	A_2	TF%	Ask%	Syi	A_1	A
A_2	1,000					
TF%	-0,087*	1,000				
Ask%	0,087	-1,000*	1,000			
Syi	-0,093*	1,000	-1,000*	1,000		
A_1	0,021*	-0,976*	0,976	-0,974*	1,000	
A	0,012*	-0,989*	0,989	-0,988*	0,996	1,000

Table 5. Some important palynological characteristics of the studied *Amaranthus* taxa (all measured values are in µm).

Populations	D_1	D_2	C	C/D_1	TNT	PD	PA	NS/100 µm²	NSP	Polar shape	Equatorial shape
					Amaranthus hybridus L.						
Elin Pelin	21.78	20.25	3.37	0.152	34.33	1.74	2.38	336	8.45	circular	elliptic-trucate
Pavel banya	21.57	19.89	3.64	0.167	33.20	1.84	2.66	414	10.00	circular	elliptic-trucate
Plovdiv	20.93	20.30	3.97	0.176	40.00	1.62	2.06	486	9.50	circular	elliptic-trucate
					Amaranthus retroflexus L.						
Asenovgrad	18.57	14.93	3.57	0.190	32.00	1.65	2.14	279	7.25	circular	elliptic-trucate
Zvanichevo	22.43	22.00	3.37	0.160	42.00	1.84	2.65	331	6.00	circular	elliptic-trucate
Plovdiv	21.43	19.00	3.81	0.200	34.66	1.69	2.24	369	8.00	circular	elliptic-trucate

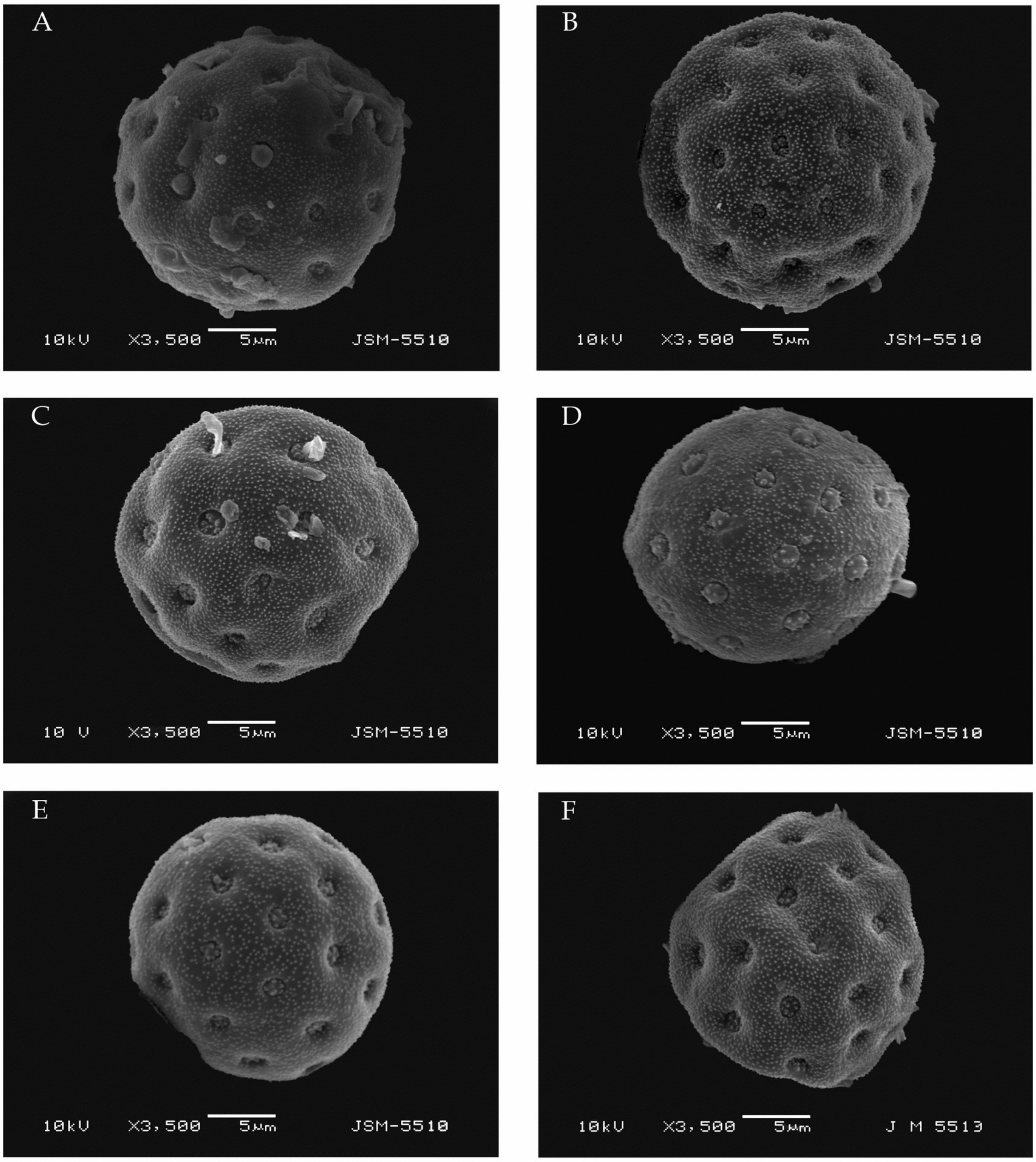

Fig. 2. Pollen microphotography.
Amaranthus hybridus: A) Elin Pelin, B) Pavel banya, C) Plovdiv;
Amaranthus retroflexus: D) Asenovgrad, E) Zvanichevo, F) Plovdiv.

Discussion

Karyology

Amaranthus L. are a genus with great variability in the chromosome number and ploidy level. The genus is tribasic (Prajitha & Thoppil, 2018). Three gametic numbers are reported about it: n = 14, 16, 17 (Greizerstein & Poggio, 1992, 1995, 1997; Carretero, 1985, 1991). Two chromosome numbers have been reported about *A. hybridus*: 2n = 32, 34 (Queirós, 1989; Carretero, 1991). The karyotype contains meta- and submetacentric chromosomes. More frequent is the metacentric type, sometimes with satellites present (Radwan et al., 2014;

Prajitha & Thoppil, 2018). *A. retroflexus*, similarly to *A. hybridus*, is known for the diploid chromosome number 2*n* = 32, 34 (Javurkova, 1980; Dmitrieva et al., 1986; Song et al., 2002; Marhold, 2007; Zykova et al., 2018; Lomonosova, 2018). Metacentric chromosomes predominate in the karyotype, satellites have not been registered (Radwan et al., 2014).

The latest karyological data about genus *Amaranthus* L. in Bulgaria were by Cheshmedziev (1994). Three species were published, one of which is *A. hybridus* with diploid chromosome number 2*n* = 32. The information is for the floristic region of the Thracian plane, more specifically Plovdiv region. There were no data about the chromosome number of *A. retroflexus*.

The base chromosome number for the three studied *A. hybridus* populations is n = 17. The karyotype formulas of the following populations in the country have been established: Elin Pelin - 2*n* = 34m, Pavel banya - 2*n* = 32m + 2sm and Plovdiv - 2*n* = 27m + 7sm. They do not confirm the diploid chromosome number of 2n = 32 established so far, but conform to the taxonomic analyses worldwide. The base chromosome number for the three studied *A. retroflexus* populations is *n* = 17. The karyotype formulas of the following populations in the country have been established: Asenovgrad - 2*n* = 31m + 3sm, Zvanichevo - 2*n* = 33m + 1sm and Plovdiv - 2*n* = 31m + 3sm. The results are new for Bulgaria.

Pollen morphology

In modern systematics pollen morphology is extremely useful for clarifying the systemic relations on inter- and intraspecies level (Iwanami et al., 1988). The currently existing morphological characteristics of pollen give grounds to classify it to *Amaranthus* type with pores type II typical of that type of pollen (Borsch, 1998). Our data largely confirm the already published ones.

The pollen in all six populations has typical spherical shape covered by numerous perforations. It is characterized by small size and a big number of pores. The difference between the species is found in the pollen size, which is bigger in *A. retroflexus* (18,57 – 22,43 μm). The C index is high (3,81 μm, 3,97 μm) in both species. The values have been reported for the same region. That could be due to the same conditions of the environment and could be used as a chorological trait in current studies of the species (Arora & Modi, 2008).

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Genetic Diversity of the Balkan Endemics Moehringia jankae Griseb. ex Janka and Moehringia grisebachii Janka (Caryophyllaceae) from Bulgaria using ISSR markers

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Abstract. Eleven populations of the endangered plant *Moehringia jankae* and twenty-eight of the rare plant *Moehringia grisebachii* were collected across its natural range from Bulgaria. Their genetic diversity was investigated through fifteen selected Inter Simple Sequence Repeats (ISSR) primers. The ISSR primers produced a total of 285 bands, of which 275 were polymorphic and 10 – monomorphic. Capability of the primers was assessed through the high mean values for the polymorphic information content (0.78), effective multiplex ratio (14.73), resolving power (27.90) and marker index (11.36). Based on the obtained mean values of the molecular data the species *M. grisebachii* (effective number of alleles = 1.39, Shannon's information index = 0.38, expected heterozygosity = 0.24, Nei's genetic diversity = 0.25, gene flow= 0.65) demonstrated higher genetic diversity than the species *M. jankae* (effective number of alleles = 1.28, Shannon's information index = 0.26, expected heterozygosity = 0.17, Nei's genetic diversity = 0.23, gene flow = 0.52). These results were supported by Analysis of molecular variance (AMOVA), showing higher variability within populations of *M. jankae* (90%) and *M. grisebachii* (62%), than among populations - 10% and 38%, respectively, and 25% among both species. Neighbor joining and principal coordinate analysis (PCoA) grouped the thirty-nine studied populations by species and region of spread. The data are applicable in conservation programs for protecting and keeping of both species. The registered genetic similarity between the populations of the two species (from Eastern Balkan Range) does not exclude the possibility of hybridization between their natural populations.

Key words: *Moehringia jankae*, *Moehringia grisebachii*, ISSR markers, endemic, genetic diversity.

Introduction

Recent studies of the Bulgarian flora are based, in part, on the problems of conservation of the natural gene fund of plants, the distribution and condition of the Bulgarian and Balkan endemic plant populations, along with the rare and endangered species, and measures for their protection. The Bulgarian flora includes 270 Balkan endemic species belonging to 116 genera and 35 families (Petrova & Vladimirov, 2010). In Bulgaria endemism reflects on the specific and genetic characteristics of the flora. The endemic species, most commonly, have a limited area of distribution, relatively small and

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fragmented populations. Knowing their morphological characteristics, ecological requirements, history, and population structure, in order to protect and preserve them is directly related to knowing their genetic diversity.

According to Martini (1990) the majority of European endemics of genus *Moehringia* were steno endemics, widely distributed in the peripheral mountain ranges around the Mediterranean. *Moehringia jankae* is a protected species according to the Bulgarian legislation (Biological Diversity Act, 2002), it's part of the Red Data Book of Bulgaria (Stoeva, 2015) in the category endangered. The species is protected under the Bern convention (1979) and is included in European Red List of Vascular plants (Bilz et al., 2011) under category Data Deficient (DD). *Moehringia grisebachii* is part of the Red Data Book of Bulgaria in the category endangered (Stoyanov, 2015). Until now the Bulgarian populations of the two endemic species have been the subject of morphological and karyological studies (Zhelyazkova et al., 2019b, 2020a, b; Grozeva et al., 2020).

This study aims to determine the intrapopulation, interpopulation and between species genetic diversity of *Moehringia jankae* and *Moehringia grisebachii* in Bulgaria through ISSR markers.

Due to the higher annealing temperature and the longer sequence of the ISSR primers, they can provide reliable and reproducible bands from RAPD (Nagaoka & Ogihara, 1997; Wolfe et al., 1998; Goulão et al., 2001; Qian et al., 2001). Compared to other markers (RFLP, SSR и AFLP) the prime cost of the analysis is lower (Yang et al., 1996; Wang et al., 2008). ISSR is technically simpler than many other marker systems (Bornet & Branchard, 2001), because it does not require preliminary information about the genome sequence. The ISSR also have disadvantages, as dominant nature, requirement for the high quality of genomic DNA and sometimes have less specificity to the genome (Sarwat, 2012).

Many authors report that high levels of polymorphism, detected with the use of ISSR, confirm that these markers are highly informative for the investigation of genetic parameters of endemic species (Xie et al., 2005; Arzate-Fernández et al., 2005; Cao et al., 2006; Lu et al., 2006; Meloni et al., 2006; Zhang et al., 2006; Xia et al., 2007; Trindade et al., 2012; Zhelyazkova et al., 2019a).

Material and Methods

Plant material

The leaf samples of eleven populations of M*oehringia jankae* and twenty-eight of *Moehringia grisebachii* were included in this study. Plant materials were collected from different parts representing the spread of the species in Bulgaria (Table 1). The Plant samples were identified according to Flora of PR Bulgaria (Kuzmanov & Kožuharov, 1966), Conspectus of the Bulgarian vascular flora (Assyov & Petrova, 2012), Key to the Plants of Bulgaria (Delipavlov & Cheshmedzhiev, 2003) and then were placed in silica gel and stored at -18°C for subsequent DNA extraction.

ISSR assay

Total genomic DNA was extracted using the modified protocols of Plant DNA Preparation Kit (Jena Bioscience). In the study of the genetic diversity of *M. jankae* and *M. grisebachii* were tested 20 ISSR primers. For this study were chosen 15 of them. The selection of these primers was done mainly on the base of literature data from similar studies on the species *M. jankae*, as well as studies on the *Moehringia*, and the family Caryophyllaceae shown on Table 2.

DNA quality and yield have been established by Nano Vue Plus spectrophotometer and Agarose gel (1%) electrophoresis, visualized on Transilluminator (BioImaging System). DNA samples with purity from 1.6 – 1.9 (260/280 nm) were used for PCR amplification.

The PCR amplifications were performed in a total volume of 25 µl, containing 1 µl genomic DNA, 12.5 µl Red Taq DNA Polymerase 2×Master Mix, 1.5 µl Primer (Bioneer) and 10 µl nuclease free ddH2O (Sigma). Amplification was carried out following a protocol by Pourhosseini et al. (2018). PCR was optimized by modification of annealing temperature to specific annealing temperature (sTa°) until maximum results were reached with each separate primer. The recording of ISSR-PCR amplified products was performed through the horizontal electrophoresis, on 1.5% agarose gel with 1×TBE buffer for 50 min at 80 V/cm. Gels were comprised 7 µl of product mixed with 1.5 µl loading buffer and 100+ DNA-ladder (100 – 3000 bp) and then were stained with fluorescent nucleic acid dye GelRed® (Biotium, USA). The presence (1) and absence (0) of bands were recorded with the help of Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel).

Statistical assay

Capability of primers was determined by calculating the following parameters: polymorphic information content (PIC) (Botstein et al., 1980; Roldan-Ruiz et al., 2000), effective multiplex ratio (EMR) (Powell et al., 1996; Nagaraju et al., 2001), marker index (MI) (Varshney et al., 2007) and resolving power (RP) (Prevost & Wilkinson 1999).

GenAlEx (Peakall & Smouse, 2006) ver. 6.5 was used for the parameters: different number of alleles (Na), effective number of alleles (Ne), Shannon's Information Index (I), expected (He) and unbiased (uHe) expected heterozygosity, percentage of polymorphic bands (PPB). Principal coordinate analysis (PCoA) and Analysis of Molecular Variance (AMOVA) were constructed in this statistical package, too.

Mantel test was performed in GenAlEx 6.5 to examine the correlation between geographic (in kilometres) and genetic distance (pairwise GD).

Gene follow (Nm) and Nei's Genetic diversity (H) were calculating with software package PopGene ver. 1.32.

Neighbor joining analysis was conducted using MEGA version 4 (Tamura et al., 2007).

Results

ISSR primers

The fifteen ISSR primers used in this study produced total of 285 bands, of which 275 bands were polymorphic and 10 bands were monomorphic. Eight of all used primers were 100% polymorphic. Minimum (10) and maximum (25) number of bands were obtained with ISSR primers (AC)8G and (AG)8YC, respectively. PIC were in correlation with high and medium polymorphism with values from 0.64 for primer (AG)8YT to 0.90 for (CA)8G, and mean value 0.78. Lowest Rp was recorded for primer (GACA)4 - 15.60, and the highest with primer (AC)8T – 43.41, with mean value 27.9. The mean value of MI was 11.36, it was lowest (8.95) for primer (AC)8G and highest (17.54) for (AC)8T. According to the received results the most effective "marker-primer" system (EMR) was for primer (AC)8T with 20.32, mean value for all primers 14.73 (Table 3). ISSR polymorphism for both species found with the different primers is shown on Fig. 1.

Moehringia jankae

In 11 populations of *Moehringia jankae* the mean frequency of loci was 0.36, ranging between 120 – 2000 bp. Minimum effective number of alleles was 1.13 (ATC6) and maximum 1.46 (GACA4), with average 1.28. The lowest value for Shannon information index was 0.11 and highest 0.42 with primers ATC6 and AG8YC, respectively, and average value 0.26. The value for expected and unbiased expected heterozygosity varied from 0.07 – 0.08 (ATC6) to 0.28 - 0.29 (AG8YC), with average 0.17 - 0.18 (Table 4).

Specific ISSR bands were found in some populations, as follows: with primer ATC6 (150 bp) in population Mj7, with primer CA8RG (550 bp) in population Mj8, with primer ATG6 in populations Mj1 (280 bp) and Mj3 (320 bp), with primer AC8T in population Mj1 (550 bp) and population Mj8 (1400 bp).

Table 1. Location of studied populations of *Moehringia jankae* and *Moehringia grisebachii.*

Moehringia jankae **(Mj), Eastern Balkan Range, Sinite Kamani Natural Park**	**Latitude/Longitude (Altitude, m)**
Mj1 Kaloyanovi kuli area	N 42° 42.755´ E 26° 23.015´(756)
Mj2 Haiduschka pateka area east of Karandila hotel	N 42° 42.704´ E 26° 22.261´(889)
Mj3 Micro dam area	N 42° 42.790´ E 26° 22.612´(972)
Mj4 350 m. south of hotel complex Karandila	N 42° 42.709´ E 26° 22.355´(933)
Mj5 450 m. southwest of hotel complex Karandila	N 42° 42.712´ E 26° 22.252´(908)
Mj6 The rocks south-east of Kamilata area	N 42° 42.593´ E 26° 22.196´(851)
Mj7 The rocks between Karandila and Kamilata area	N 42° 42.726´ E 26° 22.349´(952)
Mj8 The north of Kamilata area	N 42° 42.673´ E 26° 22.217´(866)
Mj9 The rock formation in Kamilata area	N 42° 42.603´ E 26° 22.180´(857)
Mj10 The east of Kamilata area	N 42° 42.647´ E 26° 22.198´(869)
Mj11 rock formations near Karandilska polyana	N 42° 42.873´ E 26° 22.452´(955)
Moehringia grisebachii**, Eastern Balkan Range, Sinite Kamani Natural Park (MgSl)**	
MgSl1 The east of Haiduschka pateka	N 42° 42.785´ E 26°21.349´(921)
MgSl2 The south-east of Karandila hotel	N 42° 42.851´ E 26°22.447´(971)
MgSl3 Kaloyanovi kuli area	N 42° 42.833´ E 26°23.169´(685)
MgSl4 The west of Karandilska polyana	N 42° 42.818´ E 26°22.482´(965)
MgSl5 Gornaka area	N 42° 42.828´ E 26°23.735´(920)
MgSl6 Haiduschka polyana	N 42° 42.290´ E 26°21.655´(641)
MgSl7 The north of Micro dam area	N42° 42.815´ E 26°22.647´(951)
MgSl8 The east of Micro dam area	N 42° 42.818´ E 26°22.482´(975)
MgSl9 The south of Karandilska polyana	N 42° 42.828´ E 26°22.530´(956)
MgSl10 Kamilata area	N 42° 42.595´ E 26°22.181´(838)
MgSl11 Around hotel complex Karandila	N42° 42.871´ E 26°22.447´(938)
MgSl12 High East Rocks - Alpine climbing route	N42° 42.706´ E 26°22.349´(913)
MgSl13 Between Kamilata and hotel Karandila	N42° 43.082´ E 26°22.157´(909)
MgSl14 Bellow hotel complex Karandila	N42° 42.786´ E 26°22.360´(919)
Moehringia grisebachii**, Sredna gora Mts, (MgR)**	
MgR1 Orlite Peak	N42° 28.783´ E 25°06.896´(773)
MgR2 On the path towards Bratan peak	N42° 28.708´ E 25°07.427´(741)
MgR3 Big Rock east of Kara Dere	N42° 29.037´ E 25°05.170´(813)
MgR4 The rock formation Pravite Kamani	N42° 28.935´ E 25°05.290´(738)
MgR5 The northwest of Pravite Kamani	N42°28.845´ E 25°05.206´(602)
MgR6 The north of Chepilskata Cheshma	N 42°29.067´ E 25°07.421´(845)
MgR7 The rocks between Orlite and Popova Turla	N42° 28.794´ E 25°06.975´(786)
MgR8 On the path towards Pravite Kamani	N42° 28.831´ E 25°05.204´(638)
MgR9 The west part of Orlite Peak	N42° 28.783´ E 25°06.896´(773)
MgR10 The west of rock formation Pravite Kamani	N42° 28.929´ E 25°05.271´(725)
MgR11 Little Rock east of Kara Dere	N42° 29.052´ E 25°05.186´(821)
MgP Usoykata area	N42° 29.489´ E 24°48.011´(378)
Moehringia grisebachii**, North-Eastern Bulgaria (MgSh)**	
MgSh1 The Madara rider	N 43°16.631´ E 27°07.181´(293)
MgSh2 The fortress above village Madara	N 43°16.599´ E 27°07.214´(392)

Table 2. Literature sources and sequence for used ISSR markers in the family Caryophyllaceae.

Sequence	Literature sources
AGAGAGAGAGAGAGAGC	Korkmaz & Dogan (2015); Hilooğlu et al.(2016)
AGAGAGAGAGAGAGAGYC	Peng Fu et al.(2008); Kołodziej et al. (2018)
ATCATCATCATCATCATC	Muller et al. (2015); Kołodziej et al. (2018)
GACAGACAGACAGACA	Minuto et al. (2006); Holobiuc et al. (2018); Kołodziej et al. (2018)
CACACACACACACACARG	-
GAGAGAGAGAGAGAGAYG	Minuto et al. (2006); Holobiuc et al. (2018);
ACACACACACACACACG	Minuto et al. (2006); Korkmaz & Dogan (2015); Holobiuc et al. (2018); Kołodziej et al. (2018)
AGAGAGAGAGAGAGAGYT	Fu et al. (2008); Kołodziej et al. (2018)
ATGATGATGATGATGATG	Minuto et al. (2006); Holobiuc et al. (2018); Muller et al. (2015)
GAGAGAGAGAGAGAGAC	Kołodziej et al. (2018); Korkmaz & Dogan (2015)
GAGAGAGAGAGAGAGAT	Holobiuc et al. (2018) Kołodziej et al. (2018)
GTGTGT GTGTGT GTGTYC	Hilooğlu et al.(2016); Kołodziej et al. (2018)
ACACACACACACACACT	Korkmaz & Dogan (2015); Kołodziej et al. (2018);
AGAGAGAGAGAGAGAGG	Fu et al.(2008); Kołodziej et al. (2018)
CACACACACACACACAG	Korkmaz & Dogan (2015); Kołodziej et al. (2018)

Table 3. ISSR primers used for the assessment of the genetic diversity in 39 natural populations of *Moehringia jankae* and *Moehringia grisebachii* and their parameters: Specific annealing temperature (sTa°), Total bands (TB), Polymorphic bands (PB), Monomorphic bands (MB), Effective multiplex ratio (EMR), Polymorphic information content (PIC), Resolving power (Rp), Marker index (MI).

Primer	Sequence	sT_a°	TB	PB	MB	% PB	EMR	PIC	Rp	MI
(AG)8C	AGAGAGAGAGAGAGAGC	52.3	20	20	0	100	10.50	0.88	27.68	9.25
(AG)8YC	AGAGAGAGAGAGAGAGYC	55	25	25	0	100	12.84	0.84	34.72	10.83
(ATC)6	ATCATCATCATCATCATC	49	13	12	1	92.31	15.48	0.72	23.05	11.17
(GACA)4	GACAGACAGACAGACA	52	15	15	0	100	15.87	0.77	15.60	12.17
(CA)8RG	CACACACACACACACARG	56.8	22	22	0	100	14.14	0.82	34.68	11.56
(GA)8YG	GAGAGAGAGAGAGAGAYG	53	22	21	1	95.45	13.86	0.81	30.63	11.19
(AC)8G	ACACACACACACACACG	58	13	10	3	76.92	13.85	0.65	20.36	8.95
(AG)8YT	AGAGAGAGAGAGAGAGYT	55	15	14	1	93.33	18.36	0.64	18.37	11.70
(ATG)6	ATGATGATGATGATGATG	49.3	16	16	0	100	12.75	0.83	28.29	10.54
(GA)8C	GAGAGAGAGAGAGAGAC	50.4	20	19	1	95	14.25	0.76	27.07	10.83
(GA)8T	GAGAGAGAGAGAGAGAT	50	20	19	1	95	18.86	0.67	25.23	12.68
(GT)8YC	GTGTGTGTGTGTGTGTYC	58.3	17	17	0	100	16.94	0.72	26.04	12.20
(AC)8T	ACACACACACACACACT	56.5	24	24	0	100	20.32	0.86	43.41	17.54
(AG)8G	AGAGAGAGAGAGAGAGG	52.4	19	17	2	89.47	12.86	0.77	27.90	9.94
(CA)8G	CACACACACACACACAG	55.3	24	24	0	100	10.17	0.90	34.03	9.84
Total			285	275	10					
	Mean						14.73	0.78	27.9	11.36

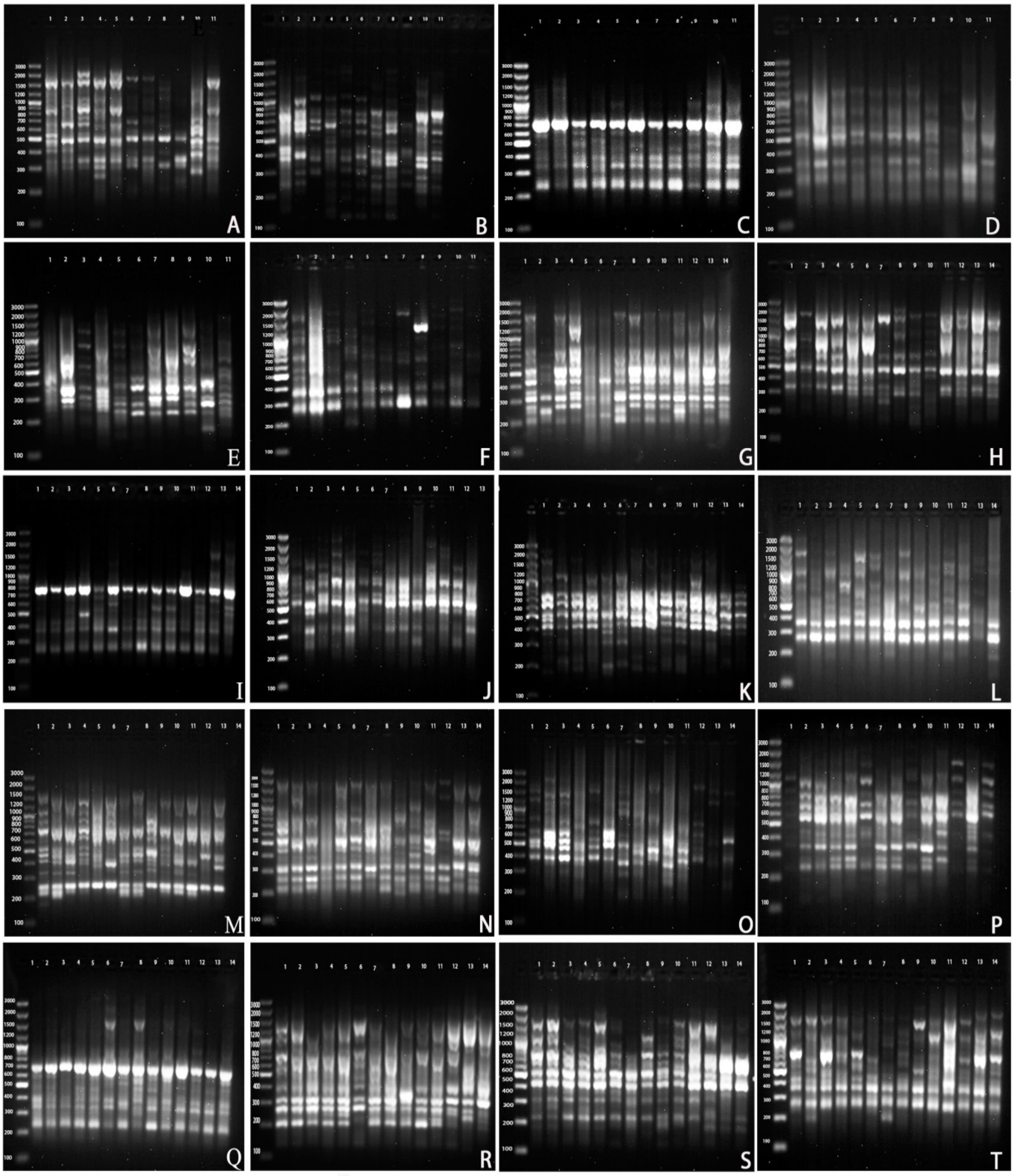

Fig. 1. ISSR genetic diversity in *M. jankae* (Mj1 - Mj11, A - F) and *M. grisebachii* (MgSl1 - MgSl14, G – L and MgR1 - MgR11, MgP - 12, MgSh 13–14, M - T), DNA Marker (Ladder 100 bp+); Primer (AC)8T - A, P; Primer (AG)8YC - B, J; Primer (AC)8G - C, K, Q; Primer (GT)8YC - D, R; Primer (GACA)4 - E; Primer (CA)8RG - F, N, T; Primer (AG)8C - G; Primer (GA)8C - H, O; Primer (CA)8G - I; Primer (GA)8T - L; Primer (AG)8YT - M, S.

Table 4. Genetic diversity in eleven populations of species *M. jankae* based on 15 ISSR markers. *Legend:* * p and q – Allele Frequency, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity.

Primer	Size range,bp	Bands freq.	p	q	Na (mean)	Ne (mean)	I (mean)	He (mean)	uHe (mean)
*AG8C	200 - 1500	0.23	0.15	0.85	1.35	1.24	0.24	0.15	0.16
AG8YC	120 - 1000	0.41	0.27	0.73	1.64	1.47	0.42	0.28	0.29
ATC6	150 - 1200	0.32	0.28	0.72	0.69	1.13	0.11	0.07	0.08
GACA4	180 - 1200	0.47	0.34	0.66	1.60	1.46	0.39	0.26	0.27
CA8RG	200 - 2000	0.34	0.25	0.75	1.27	1.32	0.29	0.19	0.20
GA8YG	120 - 1200	0.32	0.22	0.78	1.38	1.37	0.33	0.22	0.23
AC8G	250 - 1000	0.42	0.38	0.62	0.77	1.14	0.12	0.08	0.08
AG8YT	180 - 700	0.44	0.35	0.65	1.13	1.29	0.25	0.17	0.18
ATG6	200 - 1000	0.26	0.15	0.85	1.50	1.35	0.33	0.21	0.22
GA8C	200 - 1200	0.37	0.31	0.69	1.00	1.21	0.19	0.13	0.13
GA8T	150 - 1300	0.38	0.31	0.69	1.10	1.25	0.22	0.15	0.15
GT8YC	180 - 1200	0.44	0.35	0.65	1.13	1.29	0.25	0.17	0.18
AC8T	250 - 1800	0.33	0.24	0.76	1.63	1.26	0.30	0.18	0.19
AG8G	150 - 1200	0.40	0.33	0.67	0.95	1.25	0.21	0.14	0.15
CA8G	180 - 2000	0.28	0.20	0.80	1.33	1.25	0.27	0.17	0.17
Grand mean		**0.36**	**0.27**	**0.73**	**1.23**	**1.28**	**0.26**	**0.17**	**0.18**
SD					0.05	0.02	0.02	0.01	0.01

For all studied populations of *M. jankae*, Nei's H index (1973) of genetic diversity on the base of 15 ISSR markers was 0.23 ± 0.18. The calculated flow of genes (Nm) between 11 populations of the species was 0.52.

The analysis of molecular variance (AMOVA) shows significant level of intrapopulation diversity in *M. jankae*, with p-value < 0.001, and value Fst = 0,099 (data not shown). The received results for за Ne, I, He and uHe, along with the value for Fst show low to medium level of genetic diversity in the studied populations of the species.

Moehringia grisebachii

In 28 populations of *M. grisebachii* the mean frequency of loci was 0.39, ranging between 120 – 3000 bp. Minimum effective number of alleles was 1.28 (AC8G) and maximum 1.60 (GA8T), with average 1.39. The lowest value for Shannon information index was 0.30 and highest 0.47 with primers AC8G and GA8T, respectively, and average value 0.38. The value for expected and unbiased expected heterozygosity varied from 0.19 (AC8G) to 0.33 (GA8T), with average 0.24 - 0.25 (Table 5).

Specific ISSR bands were found in some populations, as follow: Primer ATC6 has only ISSR band (1500 bp) in population MgR1; Primer CA8RG has only ISSR band (150 bp) in population MgSl14; Primer GA8YG has only ISSR band (450 bp) in population MgSl1; Primer AC8G has only ISSR band (300 bp) in population MgR8; Primer AG8YT has only ISSR band (450 bp) in population MgSl1; Primer GT8YC has only ISSR band (2000 bp) in population MgSl12; Primer AG8G has only ISSR band (900 bp) in population MgSl4.

For all studied populations of *M. grisebachii*, Nei's H index (1973) of genetic diversity on the base of 15 ISSR markers was 0.25±0.17. The calculated flow of genes (Nm) between 28 populations of the species was 0.65.

The analysis of molecular variance (AMOVA) made on the base of 15 ISSR markers and the three main groups of the species MgSl, MgR, and MgSh showed a significant level of intrapopulation diversity in the studied populations of *M. grisebachii*, with p-value (< 0.001), and value Fst = 0,38 (data not shown). The Fst value shows a high level of genetic diversity beween the different groups of the populations of the species.

Between species genetic diversity

On the base of the molecular data generated from 15 ISSR markers in 39 populations, the calculated Nei D (0.115) and Nei I (0.892) for *M. jankae* and *M. grisebachii*, show that the two species are greatly similar to each other (Table 6). The genetic diversity according to the calculated parameters for Ne (1.293), I (0.270), He (0.176) and uHe (0.185), PPB (57.04) is lower for the species *M. jankae,* as is the number of bands (199).

The analysis of molecular variance (AMOVA) made on the base of 15 ISSR markers and a total of 39 populations of the species *M. jankae* and *M. grisebachii* showed significant level of within species diversity with p-value = 0.001, and value Fst = 0,25.

Specific ISSR loci monomorphic for the species *M. jankae* were registered with primers CA8RG (300bp), GA8T (1300 bp) and GT8YC (180 bp). These bands were missing in the species *M. grisebachii* and can be applied in further studies to differentiate between the two species. ISSR specific monomorphic band the species *M. grisebachii* is also registered with primer CA8RG (350 bp). In the populations of *M. grisebachii* in the different locations (MgSl, MgR and MgSh), are also seen ISSR specific band, for the separate groups but they are not shown in the genotype of all studied populations.

The specifics of the locations of the different populations is reflected in the PCoA analysis and the cluster analysis which differentiates the populations not only by species but by location as well, placing them in three separate clusters (Fig. 2 and 3).

The two analysis show that between populations of the two species *M. jankae* and *M. grisebachii* there is greater similarity that between the populations of *M. grisebachii* in the two main areas of the species – Eastern Balkan Range and Sredna gora mountain (Fig. 2, 3).

Mantel test analysis showed no correlation between geographic and genetic distance among *M. jankae* populations (r = 0.323, p = 0.1, Fig. 4a), whereas a significant correlation was found within *M. grisebachii* populations (r = 0.763, p = 0.01, Fig. 4b).

Table 5. Genetic diversity in twenty-eight natural population of species *M. grisebachii* based on 15 ISSR primers. *Legend:* * p and q - Allele Frequency, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity.

Primer	Size range,bp	Bands freq.	p	q	Na (mean)	Ne (mean)	I (mean)	He (mean)	uHe (mean)
AG8C	180 - 1500	0.29	0.17	0.83	2.00	1.32	0.38	0.23	0.23
AG8YC	120 - 1500	0.30	0.18	0.82	1.84	1.39	0.39	0.25	0.25
ATC6	200 - 1500	0.47	0.35	0.65	1.77	1.39	0.38	0.24	0.25
GACA4	180 - 800	0.38	0.24	0.76	1.73	1.46	0.43	0.28	0.29
CA8RG	150 - 2800	0.37	0.24	0.76	1.68	1.44	0.39	0.26	0.26
GA8YG	120 - 2000	0.37	0.24	0.76	1.86	1.38	0.39	0.25	0.25
AC8G	250 - 1500	0.48	0.38	0.62	1.77	1.28	0.30	0.19	0.19
AG8YT	180 - 1500	0.53	0.40	0.60	1.87	1.43	0.41	0.27	0.27
ATG6	200 - 1500	0.35	0.23	0.77	1.63	1.41	0.38	0.25	0.25
GA8C	200 - 1500	0.39	0.26	0.74	1.85	1.44	0.40	0.26	0.27

GA8T	150 - 1500	0.56	0.40	0.60	1.80	1.60	0.47	0.33	0.33
GT8YC	250 - 3000	0.43	0.31	0.69	1.71	1.42	0.37	0.25	0.25
AC8T	200 - 2000	0.25	0.15	0.85	1.58	1.31	0.31	0.20	0.20
AG8G	200 - 1800	0.36	0.25	0.75	1.79	1.32	0.33	0.21	0.21
CA8G	180 - 2000	0.25	0.14	0.86	1.92	1.33	0.36	0.22	0.23
Grand mean		**0.39**	**0.26**	**0.74**	**1.79**	**1.39**	**0.38**	**0.24**	**0.25**

Table 6. Genetic diversity of *M. jankae* and *M. grisebachii* based on 15 ISSR primers. *Legend:* N – number of population, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity, percentage of polymorphic bands (PPB), Nei D and Nei I – Genetic distance and identify, ()-SD.

Species	N	Na	Ne	I	He	uHe	PPB%	Bands	Nei D Mg	Nei I Mj
Mj	11	1.27 (0.05)	1.293 (0.021)	0.270 (0.016)	0.176 (0.011)	0.185 (0.012)	57.04	199	0.115	1.000
Mg	28	1.79 (0.03)	1.393 (0.019)	0.380 (0.014)	0.244 (0.010)	0.248 (0.010)	86.97	261	0.000	0.892

Table 7. Data of AMOVA analysis in studied species. *Legend:* *df – degree of freedom, SS – total sum of square, MS – midle square, Est. Var. – estimated variance.

Source of variation	df	SS	MS	Est. Var.	Variation%
Among the species	1	247.99	247.99	13.173	25
Within the species	37	1487.30	39.93	39.972	75
Total	38	1725.28		53.100	100

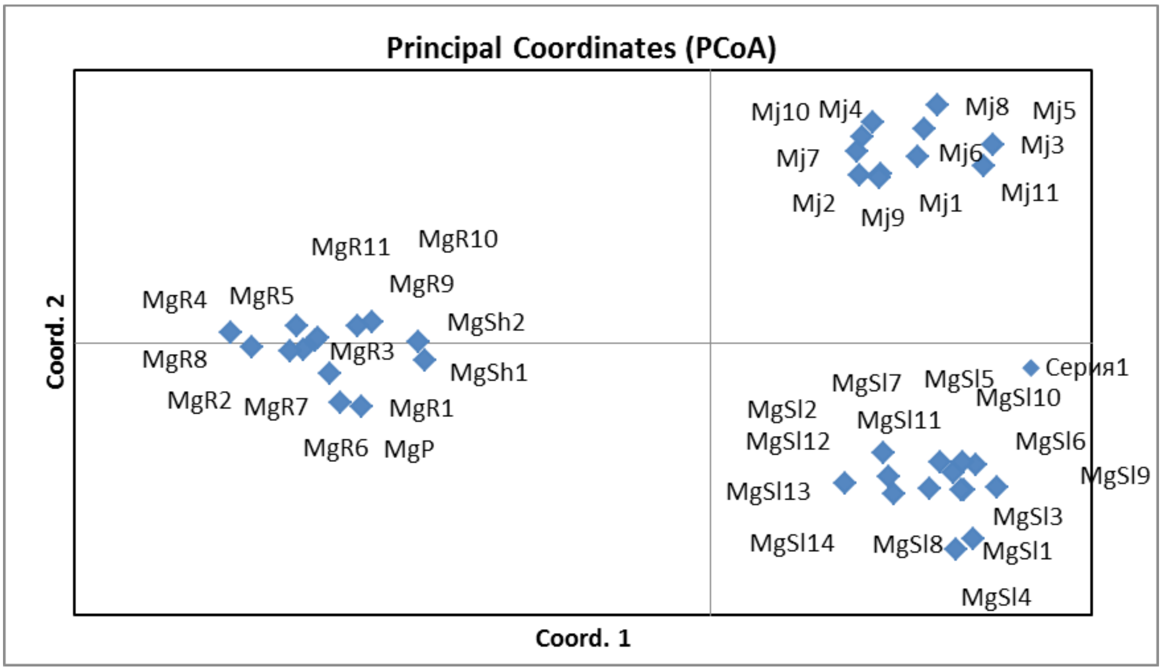

Fig. 2. Two-dimensional plot of PCoA of thirty-nine natural populations of *M. jankae* и *M. grisebachii* based on 15 ISSR primers

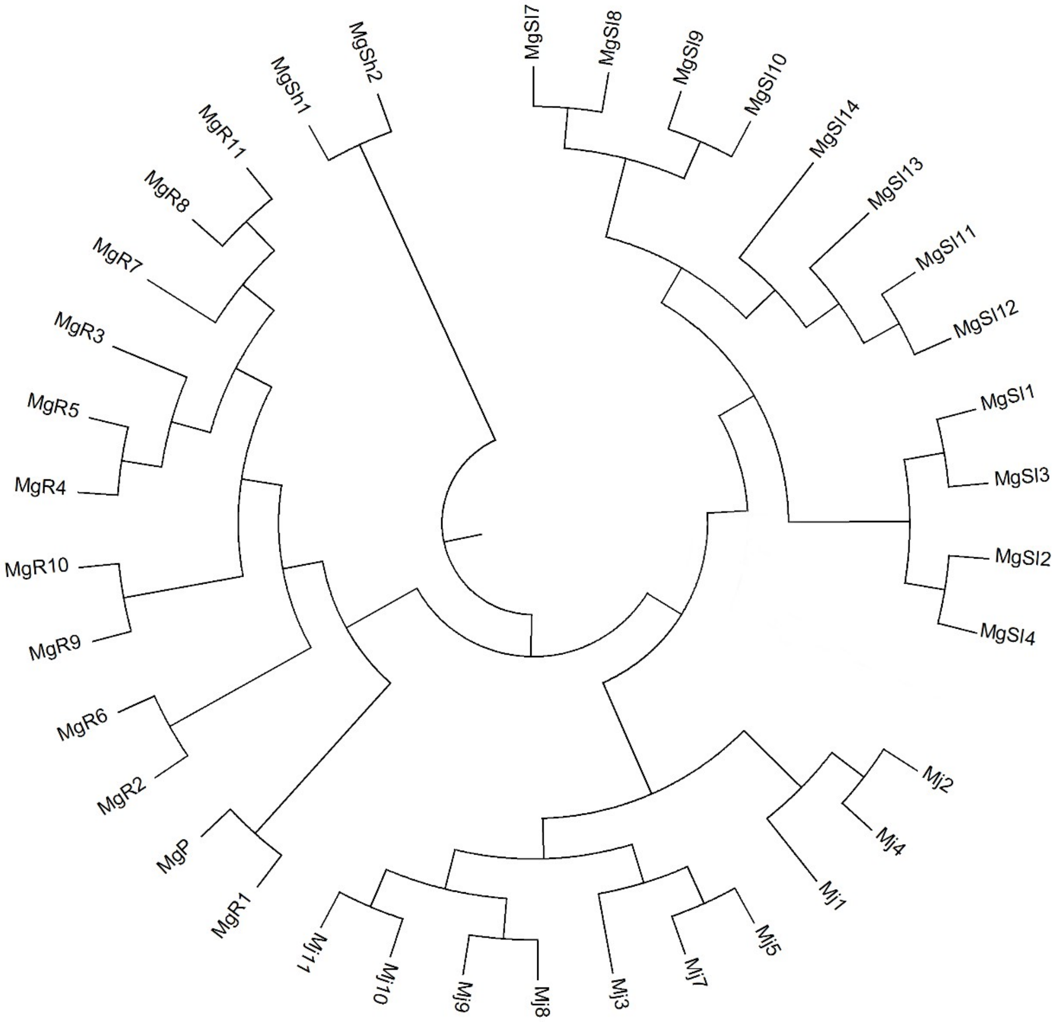

Fig. 3. Neighbor-joining based clustering of genetic diversity of *Moehringia jankae* Griseb. ex Janka and *Moehringia grisebachii* Janka using 15 ISSR primers.

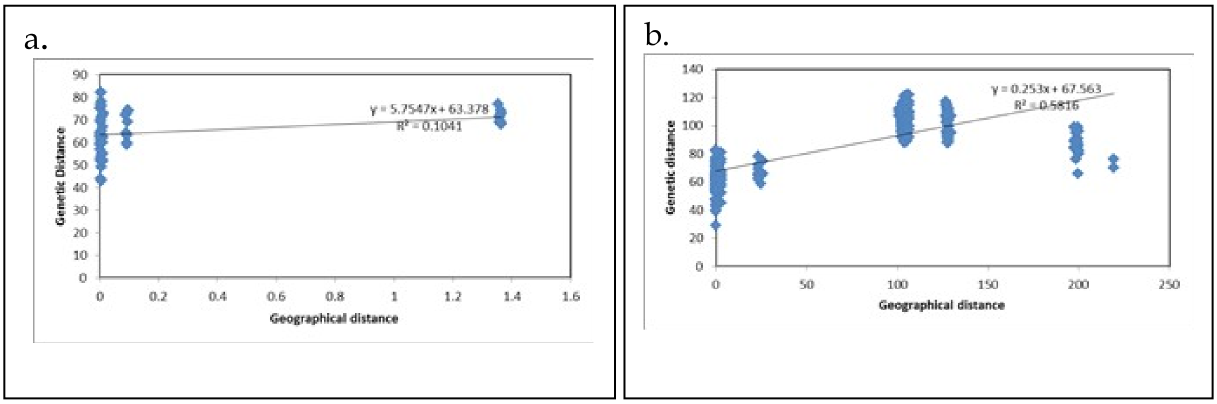

Fig. 4. Correlation between geographic distance (km) and genetic distance (pairwise GD) among 11 populations of *M. jankae* (a) and 28 populations of *M. grisebachii* (b).

Discussion

In this study the intrapopulation genetic diversity is leading in *M. jankae* (91%, Fst = 0.09) as well as in *M. grisebachii* (62%, F_{st} = 0.38). These results are similar to the ones received by Minuto et al. (2006) for *M. lebrunii* (G_{st} = 0.355) and *M. sedoides* (G_{st} = 0.255), whose genetic diversity was also studied with ISSR markers. The species *M. jankae*, similar to the data of Minuto et al. (2006) for the species *M. lebrunii*, has a small area of distribution and a significantly lowered number and size of the populations, which negatively affects the level of interpopulation diversity of the species. The level of genetic diversity inter and intra populations is directly related with the resistance of the species against long term biotic and abiotic changes in the environment (Soulé, 1980). In this study the species *M. grisebachii* shows higher level interpopulations diversity (F_{ST} = 0.38), which is partly due to the bigger area of distribution of the species and possibly better adaptivity. The higher values determined for effective number alleles, Shannon's Information Index, expected and unbiased expected heterozygosity, as well as percentage of polymorphic bands (PPB), in the species *M. grisebachii*, are possibly influenced by the larger number of studied populations (28), but in comparison between the two species are reliable, because there are no data for other distribution areas for the species *M. jankae*. In our previous research (Zhelyazkova et al., 2020a, b; Grozeva et al., 2020) for all studied populations of *M. jankae* and *M. grisebachii* was established diploid chromosome number 2*n*=24 and a karyotype of metacentric and submetacentric chromosomes, with metacentric ones being dominant. The karyological analysis of both species from Eastern Balkan Range doesn't make a definite differentiation between their populations.

The distribution in separate clusters of the total 39 populations according to location and species is seen in the PCoA analysis and the cluster analysis. This confirms the hypothesis that the specific conditions of the location influence the differences between populations, shown through specific loci, for each species as well as each region they inhabit.

Interesting here is that the species *M. jankae* shows more genetic similarity with the populations of *M. grisebachii* on the territory of Eastern Balkan Range, Sliven, than is seen between all populations of *M. grisebachii* in their two main areas of distribution – Sredna gora Mountain and Eastern Balkan Range. In a previous study was found similarity in the changeability of the karyotype of *M. jankae* and *M. grisebachii*, as well, and according to Zhelyazkova et al. (2020a) 5 different karyotypes are repeated in a total of 20 populations of the two species, distributed on the territory of Eastern Balkan Range. Our field researchers (Grozeva et al, 2016) showed that some of the populations of the two species grow close to each other. In subsequent unpublished studies, it was observed that in these populations of *M. grisebachii*, hairing of stems with multicellular non-branched straight hairs varies from weak to abundant hairing. *M. jankae* and *M. grisebachii* can be distinguished on the base of hairing of stem, leaves and flower petioles in *M. grisebachii*, because *M. jankae* is glabrous.

These results and the present results raise the question of a possible hybridization between natural populations of the two species distributed in joint territory. The genetic diversity between *M. jankae* and *M. grisebachii* was confirmed by the calculations Nei genetic distances (Nei D = 0.115) and Nei genetic identity (Nei I = 0.892). On the other side in our results is confirmed their identity as separate species and the level of similarity between then could be due to the level of diversification to the specific habitat. Most representatives from the genus *Moehringia* have specific requirements to the habitats (Fior & Karis, 2007), and this allows for the isolation of the separate populations (Akeroyd & Preston, 1981; Fior & Karis 2007;

Minuto et al., 2006; Lorite et al., 2018). Akeroyd & Preston (1981) reports that most of the colonies for *M. minutiflora* are topographically isolated. This is seen in *M. jankae* and *M. grisebachii* and confirmed by the low flow of genes between populations (Nm = 0.52 и 0.62). Akeroyd (1981) does not have proof for the reproductive level of isolation of the colonies but allows that small flowers and barely visible could lead to a high level of self-pollination. He also supposes that due to the morphology of the species there it is very possible that there is some level of cross pollination between plants from the same colony as they grow with the branches of an individual reaching to those of other individuals. In studies of the morphology of the species *M. grisebachii* Zhelyazkova et al. (2019b) reports for a higher intrapopulation genetic diversity. In a study of the morphology of *M. fontqueri*, *M. glochidisperma* and *M. intricata*, Lorite et al. (2018) reports that *M. glochidisperma* showed differences which according to the authors are contributed by the isolation of the species as endemic for North Morocco (Valdés et al., 2002). In the survival and distribution of species of the genus *Moehringia* which grow on rocks similar to *M. jankae* and *M grisebachii* is considered that the spread of the seeds through ant colonies breaks the isolation of the different populations and is more likely than the spread of flower pollen (Akeroyd & Preston, 1981; Casazza et al., 2008). When the geographical distance between populations increases, the genetic differentiation often increases as well. This is shown by the Mantel test in the present study with statistical significance (p=0.01) in *M. grisebachii*, and no statistical significance in *M. jankae* populations (p=0.1). In a number of studies (Li & Jin, 2008; Sheeja et al., 2009; Ng & Tan, 2015) Inter-simple sequence repeat (ISSR) are not only successfully applied for the study of the genetic structure and diversity in plant species but are a more effective marker than RAPD markers (Fernández et al., 2002; Behera et al., 2008). In our study the effectiveness of ISSR to find polymorphism in 11 populations of *M. jankae* reached 95.24% PB, and 100% PB in 28 populations of *M. grisebachii* and was proven with the recorded high values of the parameters characterising each primer.

Conclusions

The conducted study on the genetic diversity in *M. jankae* and *M. grisebachii* shows that in populations with similar conditions and geographical closeness the individuals have bigger similarity in the genotype. The established greater genetic similarity between the populations of *M. jankae* and *M. grisebachii* from the Eastern Balkan Range than between all studied populations of *M. grisebachii* from Sredna gora Mountain and Eastern Balkan Range does not exclude the possibility of possible hybridization between natural populations of the two species distributed in joint territory. The results confirm that the level of genetic diversity is directly related to the size and area of distribution of the species. The Balkan endemic *M. jankae*, distributed only in Eastern Balkan Range, shows lower level of genetic diversity. In order to protect and conserve this species is necessary the development of in situ and ex situ conservation programmes. Efforts should be aimed to support seed reproduction and increase the number of individuals in the population. The anthropogenic impact must be reduced by limiting access to their habitats.

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Synopsis

"The Boiling Stones": Prospective and Reliable Biodetoxicators

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Abstract. Zeolite is a collective name for minerals and chemical compounds within the group of aluminium silicates. Clinoptilolite is one of the members of the zeolite family and is the most widely used natural zeolite in different studies. Nowadays nature is increasingly exposed to toxic elements, pesticides, and different kind of anthropogenic pollutants. This leads to health problems like decreased immunity, allergies, respiratory disorders, cancer, etc. Zeolites are beneficial in many fields because of their grid structure and chelation-like effect in removing heavy metals. Over the last decades numerous studies with clinoptilolite were performed in both humans and animals. The basis of interest is its biological effects concerns one or more of their physical and chemical properties. In medicine, especially micronized natural zeolite-clinoptilolite is a relatively novel subject of interest. Animal studies demonstrate that zeolites show great promise for environmental protection, capability to detoxication of animal and human organisms, improvement of the nutrition status and immunity of farm animals, separation of various biomolecules and cells, and strong antioxidant activity. There is a significant detoxifying effect after clinoptilolite supplementation in the animal diet because of its ion exchange capacity and effective enterosorption for heavy metals, radionuclides and other inorganic pollutants. Recent findings indicate that clinoptilolite applied orally in laboratory mice for 90 days provides direct detoxification and shows significant and effective reduction of lead accumulation. Further research is needed to explore the effects of a zeolite-clinoptilolite to clarify the role of its detoxification mechanisms and auto-bioregulation in human and animal organisms.

Key words: Zeolite, clinoptilolite, enterosorbent, detoxication, mice.

Zeolite is a mineral occurring naturally in rocks of volcanic origin, formed millennia ago. The volcanic ash was deposited into an alkaline water source and was put under pressure where it reacted and crystallized. Zeolites are usually formed as secondary minerals and form beautiful crystals with pale colors. They are relatively soft and can be crushed and powdered. It is a collective name for minerals and chemical compounds within the group of the hydrated alumino-silicates. Zeolites have large anions containing cavities and channels. They rarely are pure and may contain all of the elements of the periodic table in varying degrees (Hay & Sheppard, 2001).

In prehistoric cultures, various minerals have been widely used and taken as food, as do many animals in nature. There even exists a special term – lithophagy. Surprisingly, when scientists decided to study what kind of stones are licked by animals in the spring, it turned out that these stones were exactly zeolites. Numerous observations prove the existence of lithophagy. Moose, deer and wolves find stones and lick them as an integral part of their vital needs (Panichev, 1989, 2015, 2016). It was thought that animals were seeking salt in nature to compensate their sodium deficiency. It has been found that the stones they select have nothing to do with salt. Studies show that ion exchange processes take place between animals and stones. The organism is released from unnecessary and harmful substances and fills in the missing ones. One of the hypotheses for lithophagy is an instinct for self-healing. Lithophagy is widespread also in monkeys, and even in humans to restore normal exchange in the body (Panichev & Golokhvast,2011; Panichev et al., 2013).

The term "zeolite" was originally introduced in the 18th century (1756) by the Swedish mineralogist Baron Axel Cronstedt. He discovered that when the mineral was heated rapidly a large amount of steam was obtained and named it "zeolite". The name originates from the Greek ζέω + λiθoς. "Zeo", meanis to boil and "lithos" means rock (stone). Zeolites swell when heated strongly and quickly, forming a bubble glass. Zeolite is a name applied to a huge number of extremely porous natural minerals mined in a number of locations worldwide.

For almost two centuries, no one was interested in zeolites and they gained new popularity only at the end of the 20th and in the 21st century.

It was found that the zeolite rock is almost monomineralic and consists of clinoptilolite (CLN). It is the most widely used zeolite. It has been declared the mineral of the 20th century by the World Scientific Organization and became extremely popular due to its exceptional qualities. There are numerous fields of application of natural zeolites, especially clinoptilolite, which can act as medicines (Rodriguez et al., 2006; Pavelic et al., 2001), as concomitant therapy (adjutants) (Auerbach et al. 2003), as a carrier for catalysts and pharmaceuticals (Cerria et al., 2004, Hernandez et al., 2016), in the food industry, agriculture, astronautics, and in different fundamental and applied research concerning animals (Mumpton, 1999; Pavelić et al., 2001).

What makes zeolites so unique?

Zeolites contain water in the spaces between the Al and Si molecules (tetrahedra). This water moves easily in and out of the crystal. Consequently, any molecule similar in size to water or smaller can pass through the zeolites' spaces. Larger molecules, however, cannot. Zeolites are therefore used as molecular sieves or filters to remove molecules of a particular size. Liquids and gases can pass through these spaces or pores. This property is utilized in a wide variety of applications (Breck, 1974).

The structure is the basis of the extraordinary qualities of these minerals. All zeolites have three-dimensional crystal structures enveloping an internal surface available for adsorption because of the same-sized channels with irregular shape and cavities with uniform pores of molecular size, which are uniformly distributed throughout the volume. This structure contributes to the minerals' superior ion exchange and adsorption properties (Petrov & Michalev, 2012). Cations and water molecules are weakly bound to the framework and can be partially or completely removed by ion exchange and dehydration. It is proven that zeolite is multifunctional and has important properties: sorption, molecular sieving, ion exchange and catalytic properties (Vorotnikova et al., 2020). These minerals are microporous, commonly used as adsorbents and catalysts. The most interesting property of zeolites is their open, cage-like honeycomb structure, negatively-charged. Most heavy metals are positively-charged. Like a magnet, zeolite attracts and then traps those heavy metals inside its structure. It's a process called cationic exchange. Zeolites have regular openings with fixed size, which lets small molecules pass straight through but traps larger ones; that's why they're sometimes referred to as molecular sieves.

The properties of zeolite are determined mainly by their structures. Therefore, it is important to know how to explore, utilise and use them for new applications.

What are zeolites used for?

Because of their cage-like structure large adsorption surface and unique physical and chemical properties *zeolites are* important materials with overly broad applications:

- Used as molecular sieves where they can retain molecules that can fit into their molecular cavities; have high cation exchange capacity for heavy metal cations which can be exchanged with other ions;
- Molecules or ions larger than the pore opening of zeolite cannot be adsorbed, smaller molecules or ions can;
- Useful for a variety of environmental remediation processes;

Besides, clinoptilolite effectively removed harmful toxins, many studies have shown that they also have many other vital effects in the body;

- Natural zeolite has diverse biological activities by trapping and slowly releasing valuable nutrients;
- It is used as an Additive in concentrated feed in animal growing facilities to improve feed conversion;
- Zeolite first removes free radicals. Unlike classical antioxidants, clinoptilolites do not neutralize free radicals by transferring an electron to stabilize them, but instead trap the free radicals. Once in the cell, free radicals are inactivated and can thus be safely eliminated from the body;
- Zeolites have a wide range of antiviral properties: first, by attracting and binding viral subparticles, thus preventing viral replication and their elimination from the body, and second, by inhibiting viral proliferation by immune modulation of T-cells;
- Zeolite helps maintain proper pH by removing acid ions and chemicals, which then improves optimal metabolism and immune function;
- Zeolites possess an antitumor effect. They help the elimination of carcinogenic toxins from the body, especially in the category of carcinogens called nitrosamines. They are most common in processed meats, cigarettes and beer, and have been linked to cancers of the pancreas, stomach and colon.

Zeolite deposits in Bulgaria

Alexiev (1968) is recognized as the pioneer discoverer of zeolite raw material and deposits (in quantities amounting to billions of tons) in Bulgaria.

Geological studies have been defined - the largest deposits of zeolites are located in the Eastern Rhodopes - around Kardzhali - " Zhelezni Vrata", the village of Beli Plast, "Beli Bair", "Golo Bardo", "Lyaskovets", etc. presented on Figure 1. The clinoptilolitic rocks in the North Eastern Rhodopes are of potential industrial significance because of their widespread distribution, high clinoptilolite content, considerable thickness of the layers (Djourova et al., 1989; Djourova & Milakovska-Vergilova, 1996).

The oldest deposits are in Australia, but the Bulgarian ones are many times better and amount to 10 billion tons. 60 deposits have been discovered in Russia. Their amounts are only 3.5 billion tons, as many as there are only around the vicinity of Kardzhali.

So far, more than 40 types of natural zeolites are known, the most common of which is clinoptilolite (CLN).

It was found that the zeolite from the deposit near Beli Plast is almost monomineralic and consists practically of pure clinoptilolite. It contains nearly 85 wt. % CLN and opal-CT ~15 wt.%.

Its percentage chemical composition is:

SiO_2 - 62.74%; **Al_2O_3** - 9.68%; **Fe_2O_3** - 0.74%; **CaO** - 6.73%; **MgO** -2.9%; **MnO** - 0.03%; **SO_3** -<0.03%; **TiO_2**- 0.12%; **Na_2O** - 0.29%; **K_2O** - 2.79%; **H_2O** - 5.00%; **3H**- 13.74%

and its chemical formula - $(Ca,Na,K)_{2-3}.Al_3 (Al,Si)2Si_{13}O_{36}.12(H_2O)7O$-7

The range of concentrations of the trace elements reveal very low contents of the consistent elements (as Ni, Cr, Co, Sc, Eu^{2+})

which are often below the limits of detection (Rajnov et al., 1997).

Fig. 1. Indicative map of distribution of natural clinoptilolites in Bulgaria.
Exploited deposits: ❶ - Beli Plast, ❷ - Belia Bair – Zhelezni vrata,
Explored deposits: ❶ - Most, ❷ - Gorna Krepost, ❸ - Lyaskovetz, ❹ – Golobradovo.

In order to better understand the mechanism of binding different toxic elements, it has to be mentioned that zeolites are naturally occurring chelators in removing toxic agents and the prevention of poisoning. Chelators are substances used to separate metal ions from solutions or other mixtures. They have two or more binding sites that bind particular ions and build very stable complexes.

Upon ingestion, a chelator attracts and irreversibly binds heavy metals, chemical elements and free radicals, after which it is excreted through the urinary and digestive tracts. This process is called chelation. The name "chelate" comes from the Greek "chela" - "nail, clippers".

The metal ion is captured by the chelator and thus becomes the central atom of the complex. Thus, the chelate prevents toxicity of some heavy metals (Williams & Halstead, 1982).

One of the most significant advantages of CLN over other chelating agents is its specific affinity for toxic elements and heavy metals. CLN binds first Hg, then, Pb, Cd, As, Al, Ni etc. It is ability to attract and trap them gives it such powerful detoxifying potential.

The pproperties of CLN are essential for the adsorbing characteristics due to the exceptional exchange capacity, as well as the surface area. Thanks to activation technology its sorbing capacities increase. CLN, in general, is known as an environmental decontaminant and has multiple application in the last 20 years.

Studies performed over the last decades show the high potency of zeolites - CLN in diverse medical applications *in vitro* and *in vivo*. In medicine, especially micronized natural zeolite-clinoptilolite is a relatively novel subject of interest.

The unique properties of zeolites were characterized using gravimetric analysis and have been subjected to chemical, microscopic, X-ray fluorescence, powder X-ray diffraction, Fourier transform infrared (FT-IR) spectroscopy, differential thermal analyses and thermophysical evaluation.

Not all types of zeolites are safe to be used. The clinoptilolites from different regions show different behaviour in ion-exchange processes. They possess unique chemistry, specific for each of the species. Because of how they are formed, naturally occurring zeolites are rarely found in pure form. They are often contaminated in varying degrees of metals, quartz or clays. Even CLN natural tuffs contain small quantities of different trace elements, and always are pre-loaded with various cations.

In particular, it has been found that the zeolite erionite is associated with lung cancer and malignant mesothelioma because of containing toxic fibres (Giordani at al., 2017).

Zeolites are often contaminated by other minerals. This makes them extremely important to the type of their application. Theirs porous, honeycomb like molecular structure allows them to accommodate and assist in the exchange of cations such as: Rb^{+}, Na^{+}, Ba^{2+}, Mg^{2+}, Li^{+}, Ag^{+}, Sr^{2+}, Fe^{3+}, K^{+}, Cd^{2+}, Cu^{2+}, Co^{3+}, Cs^{+}, Pb^{2+}, Ca^{2+}, Al^{3+}, NH^{4+}, Zn^{2+}, Hg^{2+}, Cr^{3+} and many others.

But a zeolite that is already full of metals does ***not*** work. It's like a sponge that's already wet; it won't absorb any more water. When using 'un-cleaned' zeolites a high level of Pb, Cd, As and other metals will come out.

For zeolite to trap toxins in the organism, it must first be cleaned of its ***existing*** heavy metals. It has been tested and confirmed and it is the ***only*** way to know what is inside.

The **cage-like structure of zeolites** makes them useful in many ways and sustainable applications for heavy metals detox, alcohol detox, toxins detox, gut health, oxidative damage reduction, mitigation of environmental toxins, detoxication against radioactive materials, counteracting microbial pathogens. In the 1960's, zeolites were given as bio additives to astronauts during long flights.

Zeolites have proven extremely effective at removing radioactive particles from nuclear waste and cleaning up soils contaminated with toxic elements (Lee et al., 2017). During the Chernobyl disaster, specialists secured the site with zeolite powder in order to bind the radioactive isotopes ^{90}Sr, ^{134}Cs and ^{137}Cs, and thus reduce their content and neutralize radiation. Zeolite was effective in removing for the first three months (Voitsekhovitch et al., 1997, Chelishchev, 1995). Following the Fukushima nuclear disaster in Japan in 2011 five types of zeolites were tested (Johan et al 2015). On paddy fields zeolites were spread an attempt to trap any lingering radioactive contaminants (Wakahara et al., 2014).

Natural clinoptilolite has to be activated in order to be applied as a detoxifier.

Bulgarian scientists from the Institute for Applied Research of Mineralogy BAS (Djurova et al., 1989) found that under normal conditions, the CLN charge is pretty weak. This small disadvantage required the need to find a reliable formula to activate it. Popov et al., (1997, 2012), Ivanova et al., (2001) have merit in this respect. Finding an exact formula for activation proved challenging and unexpected difficulties and took about seven years of experimentation, trial and error. Of great importance is the type of CLN and its pre-treatment. The activation is possible after chemical treatment with acid or alkaline solution replacing stabilizing cations in dry or in liquid medium. Another way is to heat the material or to apply mechanical modifications, even micronization in order to increase the surface area, pore size and the adsorption capacity. These treatments allow the removal of impurities (some exchangeable cations located in the channels could be trapped), to enhance the sorption properties, surface area, and porosity (Abdulkerim, 2012; Akimkhan, 2012; Canli & Abali, 2016).

The CLN structure is still stabile after 12 h of heating at 750°C, whereas other zeolites' structures are destroyed after 12 h at 450°C (Ghiara et al., 1999). This structural stability is an essential element for *in vivo* applications. Thus, the specific sorption capacity can be significantly increased and can reach up to 100 mg/g sorption for heavy metals and radioactive substances.

The size of the zeolite surface area is important

1- Zeolite works like a sponge and that allows to soak water. Cutting the sponge in half it becomes double reached the number of cells available to soak up water. If cut again the number of cells will quadrupled. Reducing the size to an average of 0.3 µ gives a much larger surface area and ability to remove toxins.
2- Zeolite particles larger than 0.3 µ are not able to enter the bloodstream than they stay in the colon where the exposure to toxins in some degree limited to removal. For example, strong toxins are usually accumulated in the fatty tissues.
3- The only way to safely remove toxins from the fatty tissues is to ensure that the zeolite can reach them via the blood stream. The way for detoxication is only when zeolite come to physical contact with the toxins or heavy metals for the ion exchange to occur.
4- The larger size of particles remains in colon and help it to cleanse as well.

Are there any side effects from the application of different concentrations of natural zeolite - clinoptilolite *in vivo?*

Blagoeva et al. (1999) provided two-year observations on laboratory mice and rats, which daily consumed food supplemented with 5% CLN. The authors did not establish any effect on basic biological parameters in animals – body weight and survival. No toxic effects were observed on the lungs, heart, spleen and kidneys. After 70 days of oral treatment with food containing 20% CLN there were no visible changes in the digestive tract and no clastogenic effect was observed in the bone marrow cells, nor any changes in peripheral blood parameters.

All conducted experimental studies strongly confirm the complete harmlessness of CLN when fed as a food additive in a concentration of 1 to 6% (Vinichenko, 2011; Gamko, 2012, Phenchenco et al., 2002).

The first comprehensive acute, sub-chronic, and chronic toxicology evaluation of CLN material *in vivo* was performed by Pavelić et al. (2001).

The only zeolite used for medical purposes in animals and humans so far is the natural zeolite - clinoptilolite, because its basic structure is considered to be biologically neutral and non-toxic (Auerbach et al., 2003).

2013 EFSA (European Food Safety Authority) evaluated and demonstrated that non-toxic doses of CLN given to animals have to be up to 10000 mg/kg.

Detoxification **s**tudies performed in the last decades demonstrated a high potency of CLN in diverse medical applications *in vitro* and *in vivo* (Kraljević-Pavelić et al., 2018). They presented the positive effect of CLN due to reversible ion-exchange and adsorption capacity (Mumpton, 1999; Pavelić et al., 2001; Jurkić et al., 2013). This basic CLN characteristic related to elimination of toxic agents, which may be seen as a support to the 'body homeostasis,' could be widely exploited in a severalmedical applications. Ortatatly & Oguz (2001) demonstrated the effects due to the CLN capacity to absorb harmful substances in the gastrointestinal tract.

Animal studies show that CLN demonstrates great promise for environment protection. Its capability for detoxification of animal organism, improvement of the nutrition status and immunity of farm animals, separation of various biomolecules and cells, strong antioxidant activity is remarkable (Pavelic et al., 2001; 2002).

Environment decontamination from different toxins and organic pollution derived from industrial waste is essential for biological ecosystems. Due to its binding capacities, CLN has been generally used in the zootechnical field for water purification and remediation.

Environmental contaminants accumulate throughout the food chain. For this reason, CLN is studied and used for oral supplementation to bind toxic substances such as ammonia or heavy metals in the milieu of the gastro-intestinal tract.

That is why the new term "enterosorbent" was introduced for substances in the digestive tract that bind and neutralize toxins or harmful products (Zhuchkov et al., 2011).

Using the CLN as an enterosorbent contributes to the improvement of livestock and poultry health and increasing their productivity. This process is due to the adsorption of trace elements, creating a certain reserve, which is used by the body evenly for a long time. The concentration of mineral elements in the digestive tract is maintained at the optimum level which improves the overall physiological state of animals. The selective ability of CLN to adsorb NH_4^+ ions in the gastrointestinal tract also contributes to higher productivity. Cations of Mg, Mn, Zn and other elements stimulate the protein synthesis and this leads to increase in the productivity of livestock (Ulitko, 2003; Mysik, 2012; Osinkina, 2012).

Clinoptilolite incorporated into the diet may be effective enough in fighting toxins by direct absorption. CLN has strong chelating ability, which is potential for adsorbing toxic metal cations, especially Pb.

CLN only "passes through" and never goes into the bloodstream. Affinity was proven by dietary administration of 5% CLN in mice, in the presence of amino acids and vitamins. It is very important to know that they will be not absorbed by the CLN material, especially in the smallest particle size.

A lot of *in vivo* studies on the detoxification properties of CLN performed so far have mainly been done on animals and they provide strong evidence on alleviating effects during exposure to different toxicants upon CLN supplementation (Laurino & Palmieri, 2015).

Our research have shown that CLN provides direct detoxifying performance in ICR laboratory mice. For instance, in lead-intoxicated mice, a CLN sorbent KLS-10-MA decreased the lead accumulation in the intestine by more than 70% (Beltcheva et al., 2012, 2015). During the detoxification processes the performed morphological, cell and genetic analysis shows normalization of the physiology of the animals' organism. Prolonged use of CLN has a stimulating effect on the biosynthetic processes (Topashka-Ancheva et al., 2011).

Conclusions

This brief review exemplified the orientation and necessity of research in the field of modern ecotoxicology combined with new approaches s and advanced methodology of exploration.

The efficacy and potential of CLN in medicine seems high. In the organism the possible effects of CLN administration on different micronutrients and trace elements, or on important physiological processes does not affect their homeostasis, but acts rather selectively on heavy-metals and toxicants.

The majority of studies on CLN were done by using different, so-called activated materials to increase either the surface area or to improve the CLN general adsorption or the ion-exchange capacity.

The tested CLN materials proved to be generally safe for (*in vivo*) applications even though each CLN seems to have its own characteristics and demonstrate specific biological effects. That cannot be easy to explain because a great part of mechanisms is not exactly clear and is still not completely understood. The difficulties come from different particle sizes, surface areas, and cation compositions that may induce different biological effects and exert different levels of effectiveness. Biological effects and toxicology data should be carefully evaluated according to the type of CLN used in a particular study or application.

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Citing references that are still "in press" is also considered frown upon, but not forbidden. If possible, please avoid using such references.

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For special symbols (Greek letters, symbols for male and female etc.) use the Symbol list on the Insert menu in Microsoft Word with the following preferable fonts: Symbol, Webdings, Wingdings, Wingdings 2 and Wingdings 3. Degree symbols (°) must be used (from the Symbol list) and not superscript letter "o" or number "0". Multiplication symbols must be used (×) and not small "x" letters. Spaces must be inserted between numbers and units (e.g., 3 kg) and between numbers and mathematical symbols (+, –, ×, =, <, >), but not between numbers and percent symbols (e.g., 45%).

Nomenclature and units. Follow internationally accepted rules and conventions: use the International system of units (SI). If other quantities are mentioned, give their equivalent in SI. Please use a standard format for the units and be uniform in the text, tables and figures. Please choose one of two possible styles: "m/s" or "m s-1". When using "m s-1" leave a space between the symbols.

Italic letters. The Latin genus and species names must be cited completely once in the text and should be typed in *italic*.

Statistics
Mean values should always be accompanied by some measure of variation. If the goal is to describe variation among individuals that contribute to the mean standard deviation (SD) must be used. When the aim is to illustrate the precision of the mean standard errors (SE) should be given. The last paragraph of Materials and Methods section should briefly present the significance test used. Quote when possible the used software. Real p values must be quoted both at significance or non-significance. The use of the sign is acceptable only at low values of p (e.g. p<0.0001).

Ethics
The authors of articles that are based on experiments that caused injuries or death of animals should explain and justify the grounds of the study and state that the scientific results of the study is at least in trade-off with the sufferings caused. In the Materials and Methods section of the manuscript, the authors should explain in detail and as precisely as possible the conditions of maintenance, transport, anaesthesia, and marking of animals. When available, references should be added to justify that the techniques used were not invasive. When alternative non-harming techniques exist, but were not used, the manuscripts may not be considered for publication.

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