



The First Record of the Balkan Endemic Caddisfly *Drusus osogovicus* Kumanski, 1980 (Trichoptera: Limnephilidae) in the Republic of North Macedonia, with DNA barcoding of the species and comments on its ecology and distribution

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Abstract: The Balkan endemic caddisfly species *Drusus osogovicus* Kumanski, 1980, previously known only from the Bulgarian part of the transboundary Osogovo Mountain, is reported from the Republic of North Macedonia for the first time. The species is recorded at four localities in the country: two localities in the North-Macedonian part of Osogovo Mountain and two localities in the Karadak (Skopska Crna Gora) Mountain. In addition to the morphology-based taxonomic analyses of male and female genitalia, the identification is confirmed on the basis of DNA barcoding (partial sequence of mitochondrial cytochrome oxidase I gene). The distribution of the species is restricted to low-temperature (6–9°C) mountain springs, streams and rivers at altitudes of 1380–1608 m. The emergence period of *D. osogovicus* is identified as May – July, suggesting one-year life cycle of this species.

Key words: Drusinae, Balkan Peninsula, freshwater biodiversity, taxonomy, DNA

Introduction

The genus *Drusus* Stephens, 1837 belongs to the subfamily Drusinae Banks, 1916 (WARINGER & GRAF 1997) as a part of the species-rich family Limnephilidae Kolenati, 1848. Many species of this genus represent regional endemics or micro-endemics, each distributed in a single mountain range in Europe and Asia Minor (MALICKY 1979, 1983, DE MOOR & IVANOV 2007, GRAF et al. 2008). The total number of the species within the genus *Drusus* is estimated at about 95 (HICKIN 1967, OLÁH 2010, 2011, MALICKY 2004, OLÁH & KOVÁCS 2013, OLÁH et al. 2015, PREVIŠIĆ et al. 2014,

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IBRAHIMI et al. 2015a, 2016a, VITECEK et al. 2015a, 2015b, 2015c), although some authors (OLÁH et al. 2017) have suggested that this number is higher. Therefore, *Drusus* is a highly diverse genus of the family Limnephilidae. In addition, due to its unique ecological demands, the genus *Drusus* represents one of the most challenging and interesting groups of trichopterans. Many species of it are endemic to high-mountain areas (MARINKOVIĆ-GOSPODNETIĆ 1976, VITECEK et al. 2015a, 2015b, IBRAHIMI et al. 2016d) and inhabit springs, mountain streams and rivers at high altitudes, usually at low water temperatures with small annual variations (KUCINIĆ et al. 2010). These ecological preferences determine their distribution patterns and initiate isolation of mountain populations (MARINKOVIĆ-GOSPODNETIĆ 1976, KUČINIĆ et al. 2014, PREVIŠIĆ et al. 2009) resulting into high rates of speciation (KUMANSKI 1988, MALICKY 2004, 2005, MARINKOVIĆ-GOSPODNETIĆ 1979).

The main knowledge on the caddisfly fauna of North Macedonia has derived from a small number of studies from the previous century and the beginning of this century (RADOVANOVIĆ 1943, BOTOŠANEANU 1960, PAVLOVSKI 1984, 1991, ARSOV 1991, KUMANSKI 1997, KUMANSKI & MALICKY, 1999, VITECEK et al. 2015a, KUČINIĆ et al. 2016, WARINGER et al. 2015). Further information on the diversity of the Trichoptera in this country has been presented by various studies targeting at the examination of the caddisfly diversity of the Balkan Peninsula (OLÁH 2010, 2011, OLÁH et al. 2013a, 2013b, 2014, 2015, 2018, OLÁH & KOVÁCS 2013, 2014, KUČINIĆ et al. 2016, VITECEK et al. 2015b, 2015c). However, a complete and comprehensive study of the North Macedonian caddisfly fauna has not been carried out till present.

Previous faunistic studies have revealed nine species of the genus *Drusus* in North Macedonia: *D. plicatus* Radovanović, 1942, *D. biguttatus* (Pictet, 1834), *D. vernonensis* Malicky, 1989, *D. botosaneanui* Kumanski, 1968, *D. discolor* (Rambur, 1842), *D. discophorus* Radovanović, 1942, *D. macedonicus* Schmid, 1956, *D. krpachi* Kučinić, Graf & Vitecek, 2015 and *D. tenellus* (Klapalek, 1898). Some of them have become the main subject of additional investigations (PREVIŠIĆ et al. 2014, VITECEK et al. 2015b, KUČINIĆ et al. 2016, WARINGER et al. 2015, 2016). In line with the effort to contribute in revealing the diversity of the Macedonian caddisfly fauna, some studies used also molecular methods, since the importance of molecular markers for the identification and delimitation of taxa has increased during

the past two decades (HEBERT et al. 2003a, 2003b, HAJIBABAEI et al. 2005, CYWINSKA et al. 2006, HUBERT et al. 2008, VITECEK et al. 2017). Using molecular approaches, Macedonian samples of *D. botosaneanui* have been involved in the examination of the phylogenetic relationships of *D. balcanicus*, *D. botosaneanui*, *D. serbicus* and *D. tenellus* (see WARINGER et al. 2015). Further studies (WARINGER et al. 2016, KUČINIĆ et al. 2016) have linked larval stages and adults of certain *Drusus* spp. using partial sequence the mitochondrial cytochrome oxidase I gene (mtCOI).

The aims of the present study are to contribute to the knowledge of the caddisfly diversity in the Republic of North Macedonia and to provide information on the distribution of *D. osogovicus* in the country and in the adjacent areas of the Balkan Peninsula. In addition, we comment on the ecological preferences and emergence patterns of the species, confirm the identification based on morphological characters and DNA barcode.

Materials and Methods

Study area

Samples of *D. osogovicus* were collected at four localities in the Republic of North Macedonia (Fig. 1). Two sampling sites (Fig. 2a, b) represent the Duracka River Spring and Duracki Potok (Duracki Stream), the latter being a small mountain stream flowing into the same river. Both localities are situated in the north-eastern part of the country, in the forest zone of the Osogovo Mountain (protected area within the Emerald Network) at 1608 m a.s.l. and 1498 m a.s.l., respectively. The third and fourth sampling sites (Fig. 2c) represent secluded mountain streams in the forest zone at altitudes of 1380 and 1400 m a.s.l. located above Brodec Village and in Tanushevci Village on Karadak Mountain (Skopska Crna Gora) in the northern part of the country. Detailed information about the sampling localities and sex ratio of collected specimens is presented in Table 1. The water temperature at the first two localities on Osogovo Mt. during the studied period was 6°C (Spring of Duracka River) and 9°C (Duracki Potok) while it was 8°C at the sampling localities on Karadak Mt. All four localities are situated in the forest zone of Osogovo Mt. and Karadak Mt., characterised with cold water, sandy bottom accompanied with rocks covered with moss and well-developed riparian vegetation, which correspond with the typical ecological preferences of *Drusus* spp.

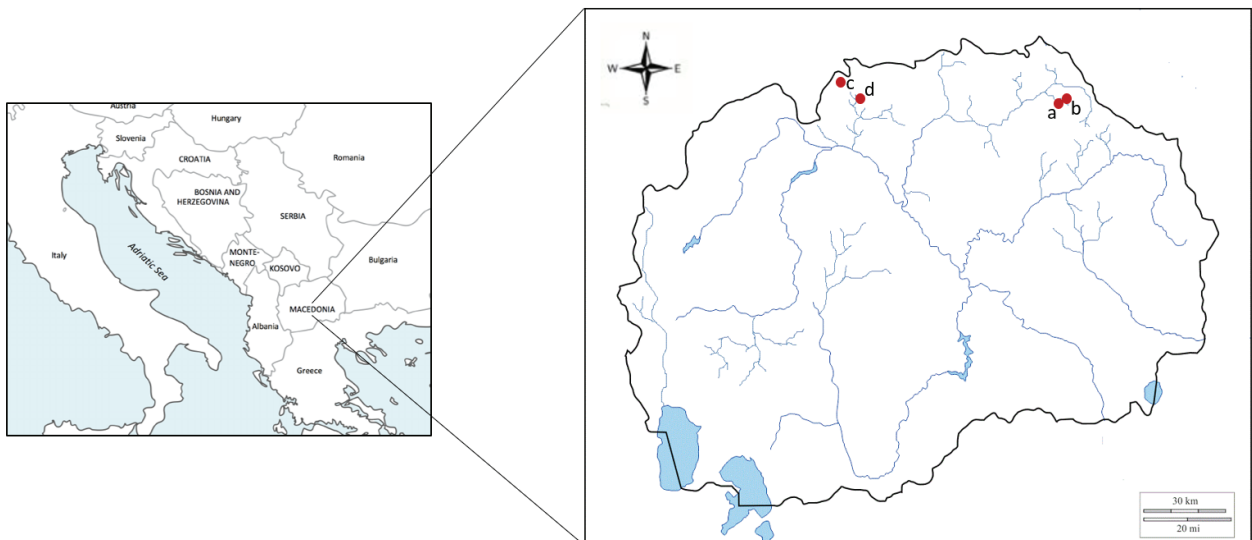


Fig. 1. Map of the sampling sites of *Drusus osogovicus* in the Republic of North Macedonia: **a.** Spring of Duracka River (Osogovo Mt.); **b.** Duracki Stream (Osogovo Mt.); **c.** Mountain stream at Tanushevci Village (Karadak Mt.); **d.** Mountain stream above Brodec Village (Karadak Mt.).

Table 1. Details on the sampling sites and the sex ratio of collected specimens. Abbreviations of names of collectors: AB = Astrit Bilali; HI = Halil Ibrahim; JH = Jelena Hinić; MK = Mladen Kučinić; MM = Milaim Musliu; VSS = Valentina Slavevska-Stamenković.

Sampling site	GPS	Altitude	Date of collection	Water temperature	Collected specimens	Collectors
Osogovo Mt. – Spring of Duracka River	42.1534624 22.3419346	1608 m	19.06.2017	7°C	8 ♂, 4 ♀	HI, JH, MK, VSS
			30.06.2017	6°C	4 ♂, 2 ♀	JH, VSS
			04.05.2018	6°C	1 ♂	JH, VSS
Osogovo Mt. – Duracki Potok	42.1574620 22.3629524	1498 m	04.05.2018	9°C	2 ♂, 1 ♀	JH, VSS
Karadak Mt. – stream in Tanushevci Village	42.23356 21.42733	1380 m	03.06.2017	8°C	1 ♂	AB, MM
			04.06.2017	8°C	1 ♂, 3 ♀	AB, MM
Karadak Mt. – stream above Brodec Village	42.160165 21.448974	1400 m	23.07.2017	8°C	1 ♂	AB, MM

Sampling

Adult caddisflies specimens were collected using ultraviolet (UV) pyramid light trap. The trap was placed on the stream bank after dusk and operated for app. 60 minutes. The sampling was conducted monthly during the period June 2017 – May 2018 at both localities on Osogovo Mt. and in June and July 2017 on Karadak Mt. The collected material was preserved in 96% ethanol and transported to the Laboratory for Invertebrate Zoology, Institute of Biology, Skopje, Republic of North Macedonia as well as to the Laboratory for Zoology, Faculty of Biology, Prishtina, Kosovo.

Taxonomical identification based on morphological characters

Macrophotography and assessment of morphological characteristics of adult specimens were carried out

by Nikon SMZ745 stereomicroscope and Nikon DS-Qi2 camera following KOH-treatment as proposed in MALICKY (2004) using appropriate identification keys (KUMANSKI 1988, MALICKY 2004). The nomenclature follows NIELSEN (1957) (for *Limnephilus flavicornis* Fabricius, 1787) using the simplifying terms “superior appendages” for the lateral processes of segment X (cerci *sensu* SNODGRASS 1935), and “intermediate appendages” for the sclerite and the anterior process of segment X (“paraprocts” *sensu* SNODGRASS 1935). All collected specimens were deposited in the Macedonian National Collection of Invertebrates (MNCI), Skopje (North Macedonia), the Laboratory of Zoology at the Faculty of Natural and Mathematical Sciences in Prishtina (Kosovo) and in the trichopterological collection of M. Kučinić in Zagreb (Croatia).



Fig. 2 a-d. Photographs of the sampling sites of *Drusus osogovicus*: **a.** Spring of Duracka River (Osogovo Mt.); **b.** Duracki Stream (Osogovo Mt.); **c.** Mountain stream at Tanushevci Village (Karadak Mt.); **d.** Mountain stream above Brodec Village (Karadak Mt.).

Molecular analyses

DNA from the specimens from Osogovo Mt. used for barcoding was extracted from leg and thorax muscle tissue using a modified salt-extraction protocol (originally after SUNNUCKS & HALES 1996 and modified by WEISS & LEESE 2016). The Folmer region of the cytochrome c oxidase subunit I (COI) gene was amplified using illustra PuReTaq ready-to-go PCR beads (GE Healthcare UK Limited, Little Chalfont, UK) adding 1 μ L of extracted DNA and 0.5 μ M of each

Folmer primer (HCO2198 and LCO1490; FOLMER et al. 1994), filled up to 25 ml with sterile H₂O. The following PCR settings were used for COI amplification: initial denaturation at 94°C for 2 min, 38 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 30 s and extension at 72°C for 80 s; final extension of 72 °C for 5 min. Samples were purified by adding 0.5 ml of ExoI (20 U/ml) and 1 ml of Fast-AP (1 U/ml, both enzymes Thermo Fisher Scientific, Schwerte,

Germany) to 9 µL PCR product and incubation for 25 min at 37°C followed by an inactivation step at 85°C for 15 min (Table 2). Bidirectional sequencing was conducted by GATC-Biotech (Konstanz, Germany). Sequences were assembled and edited in Geneious 6.1.8 (<http://www.geneious.com>, KEARSE et al. 2012), before comparison with the Barcode of Life (BOLD) database (boldsystems.org). All DNA barcoding protocols were conducted in the Laboratory of Aquatic Ecosystem Research (Faculty of Biology) at the University of Duisburg in Essen (Germany).

Taxonomical identification using DNA barcoding

The DNA barcode sequences obtained in this study were included in similarity search performed using the BOLD Identification Engine (boldsystems.org), which uses all sequences uploaded to BOLD to locate the closest match. Despite using the BOLD Identification Engine, the obtained sequences were additionally blasted in GenBank (blast.ncbi.nlm.nih.gov) in order to compare the results.

Results

During the investigated period, adult males and females of *Drusus osogovicus* (Fig. 3) were collected in

the forest zone of Osogovo Mt. and Karadak (Skopska Crna Gora) Mt. Within the framework of the taxonomic identification workflow, the analyses of morphological features of *D. osogovicus* Kumanski, 1980 were based on the comparison with the rest of the species from the *D. discophorus* species group following literature (KUMANSKI 1980, 1988, MALICKY 2004). The species was recognised firstly by identifying the male specimens. The observation showed that the well-developed superior appendages had distal parts turned inwards and that they were nearly quadrate in caudal view – is one of the main diagnostic characters for this species identification. In addition, the intermediate appendages were placed extremely close to each other (Fig. 3). The female specimens were identified firstly by the dark body coloration and the fusion of the 9th and 10th segment in dorsal view where the wider dimensions of the 9th segment were easily noticeable (Fig. 3).

The comparison of the 630 bp long fragment of mtCOI gene of our specimens with the reference sequences uploaded in BOLD Systems and GenBank showed that the obtained sequence is most similar (99.52%) to a reference sequence of an uploaded record in the BOLD and GenBank database identified as *D. osogovicus*, thus confirming the taxonomical identification based on morphological characters.

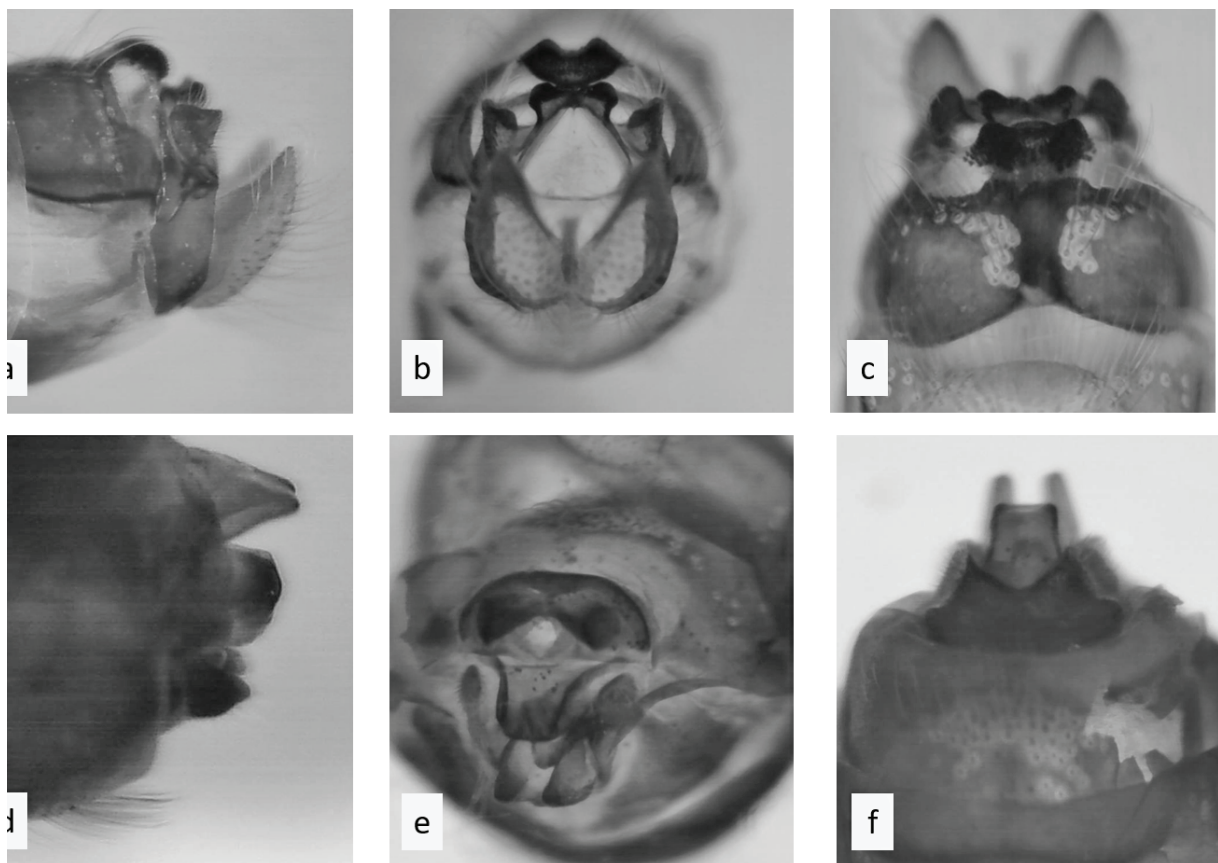


Fig. 3. Male and female genitalia of *Drusus osogovicus*. Male genitalia: a. right lateral view; b. caudal view; c) dorsal view. Female genitalia: d. right lateral view; e. caudal view; f. dorsal view.

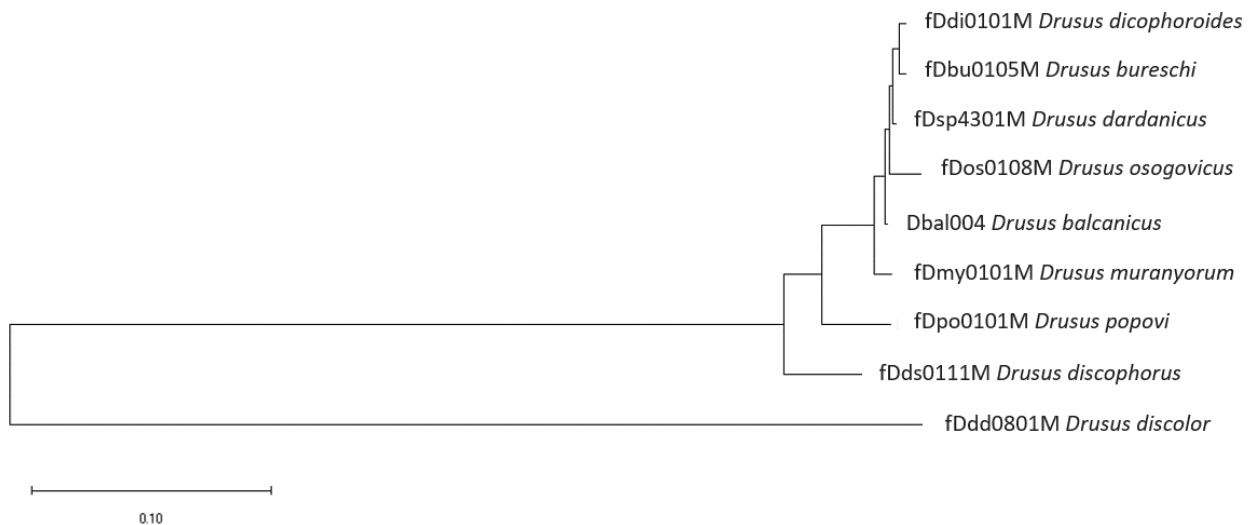


Fig. 4. Neighbour joining phylogenetic tree construction based on mtCOI-5P fragment for testing the affiliation of *Drusus osogovicus* to the *Drusus discophorus* species group.

Discussion

The combination of morphological character analyses and molecular sequence data conducted during the present study clearly indicates that our specimens taxonomically belong to *D. osogovicus*. This endemic caddisfly species belongs to the *D. discophorus* species group (KUMANSKI 1988). According to KUMANSKI (1988), OLÁH (2010) and IBRAHIMI et al. (2015a), this group comprises ten taxa: *D. discophorus* (including *D. discophorus pallidus* Kumanski, 1989 and *D. discophorus rhodopaeus* Kumanski, 1989), *D. bureschi*, *D. balcanicus*, *D. discophoroides*, *D. popovi*, *D. osogovicus*, *D. muranyorum* and *D. dardanicus*. Having in view the high diversity and intricate morphological characters of the subfamily Drusinae make identification of several species challenging (VITECEK et al. 2017), basic differences in male terminalia for rapid delimitation of the species within *D. discophorus* species group are summarized in Table 2. *D. osogovicus* is one of the dark-coloured species in the group. The extremely narrow spinulate zone of the 8th tergite and the turned inwards position of the superior appendages are the main diagnostic features of the male specimens of this species.

The first findings of *D. osogovicus* Kumanski, 1980 at four localities in the Republic of North Macedonia make this species 10th in a row out of *Drusus* spp. found in the country. Since almost all (8) previously recorded species of this genus are known to inhabit the aquatic ecosystems of the Sixth Ecoregion, only *D. discolor* found in larval stage from Zrnovska River (ARSOV 1991) and Bregalnica River (SLAVEVSKA-STAMENKOVIĆ, unpublished data) is known from the Seventh Ecoregion within the ter-

ritory of North Macedonia. Nonetheless, most of the studies targeted at the caddisfly fauna of the western parts while only ARSOV (1991) and KUMANSKI (1997, 2001) were studied the eastern part of the country. The new records reported here add *D. osogovicus* to the list of *Drusus* spp. noted in the Seventh Ecoregion indicating that its freshwater ecosystems are equally important and capable in providing suitable habitats for sustaining healthy populations of rare caddisflies.

Concerning the distribution of *D. osogovicus* on the Balkan Peninsula, the survey of the trichopterozoological literature from several neighbouring to North Macedonia countries – Serbia, Kosovo, Albania and Greece (ŽIVIĆ et al. 2000, 2002, 2006, IBRAHIMI & GASHI 2008, OLÁH 2010, 2011, IBRAHIMI 2011, IBRAHIMI et al. 2012a, 2012b, 2013, 2014, 2015b, 2016a, 2016b, 2016c, 2016d) has shown no records of *D. osogovicus*. Until now, it has also been unknown for the Macedonian fauna despite the studies on caddisfly larvae (PAVLOVSKI 1984), caddisfly adults (RADOVANOVIĆ 1935, 1943, 1953, ARSOV 1991, KUMANSKI 1997, OLÁH 2010, 2011, OLÁH et al. 2013a, 2013b, 2014, 2017, 2018, OLÁH & KOVACS 2013, 2014, VITECEK et al. 2015b, WARINGER et al. 2015, 2016, KUCINIĆ et al. 2016) and aquatic insects in general (SMILJKOV & SLAVEVSKA-STAMENKOVIĆ 2004, KITANOVA et al. 2008, SLAVEVSKA-STAMENKOVIĆ et al. 2010, 2011, 2012; RIMCHESKA et al. 2014, 2015). *D. osogovicus* has been reported only by KUMANSKI (1988, 2001) from Western Bulgaria at two localities on the Bulgarian part of Osogovo Mt. Although the author has performed long-term and precise investigations

Table 2. Diagnostic characters of males of the caddisfly species of the *Drusus discophorus* group (from KUMANSKI 1988, OLÁH 2010, IBRAHIMI et al. 2015a)

Taxon	Body colour	Body dimensions	Forewing length (mm)	Superior appendages	Intermediate appendages	Inferior appendages	Parameres
<i>Drusus discophorus pallidus</i>	Very light coloured	Very small	5.5–9	Rounded, medium big	Dark coloured, in dorsal view wide, in lateral view with oblique distal part	In lateral view elongate, with sharp apices	With subapical spines
<i>Drusus discophorus rhodopaeus</i>	Very dark coloured	Very small	7.5–9.5	Rounded, medium big	Dark coloured, in dorsal view wide, in lateral view with oblique distal part	In lateral view elongate, with sharp apices	With subapical spines
<i>Drusus bureschi</i>	Very light coloured. Brownish-yellow body with brown metatarsa and tergites	Big	11–13	Well-developed, rounded, in lateral view medially recessed	In dorsal view with narrow distal part	Big, well-developed and elongate as in <i>D. discophorus</i>	Equally long as the aedeagus, with one big subapical spine
<i>Drusus balcanicus</i>	Light coloured. Reddish-yellow body with brownish abdomen sclerites	Big	10.5–12.5	Well-developed, rounded	Small, in dorsal view narrower than spinulate zone on the 8th tergite. In lateral view well-noticeable above the superior appendages	Big, well-developed and elongate as in <i>D. discophorus</i>	Shorter than the aedeagus, with one subapical spine
<i>Drusus discophorooides</i>	Very dark coloured	Medium	10–10.5	Poorly developed, small, well sclerotized with long hairs arising	Well-sclerotized, in lateral view vertically placed, in caudal view almost round	Big, well-developed and elongate as in <i>D. discophorus</i>	With subapical spines
<i>Drusus popovi</i>	Light coloured. Brownish-yellow body with darker brown abdomen sclerites	Medium	10.5–11	Dark coloured, short, wide and round in shape	Divergently positioned with widely rounded dorsolateral endings	Well-developed, with protuberance in the middle of the ventral side	Longer than the aedeagus with extremely long distal sharp part
<i>Drusus osogovicus</i>	Smoky brown coloured body with yellowish-brown thorax and legs	Medium	9–12	Well-developed, with distal parts turned inwards. In lateral view rounded. In caudal view nearly quadrate	Dark coloured, well-developed and placed extremely close to each other	Big, well-developed and elongate as in <i>D. discophorus</i>	Longer than aedeagus, with one spine
<i>Drusus murayorum</i>	Dark coloured body with lighter brown legs (except coxa and proximal portion of femur)	Medium	9	Well-developed, sub-quadrate in lateral view	Triangular, spread ventrolaterally	Slightly S-shaped with basoventral heel	Simple, filiform and no longer than the aedeagus
<i>Drusus dardanicus</i>	Dark coloured body	Medium	11–12	In lateral view short, sub-triangular, dorsally straight	In lateral view with rounded tips directed dorsally	Well-developed and elongate as in <i>D. discophorus</i> . In lateral view ventrally slightly constricted	Simple, each with two distinct apical thorn-like spines and dorsal protrusion at their bases

of the caddisfly fauna of almost all water bodies in Bulgaria (KUMANSKI 1972a, 1972b, 1981, 2001), this species has not been detected in any other part of the country. This strongly suggests that the distribution range of *D. osogovicus* in Bulgaria is very small and restricted only to Osogovo Mt. Accordingly, the finding of the species at two further localities in the Macedonian part of the same mountain might be considered expected since these sampling sites are located near the border with Bulgaria. On the other hand, bearing in mind that the Drusinae species in the Balkan Peninsula have highly disjunct and limited distribution (PREVIŠIĆ et al. 2009, 2014, KUČINIĆ et al. 2011, 2014), the presence of *D. osogovicus* in the northern part of the Republic of North Macedonia (Karadak Mt.) might seem a little bit controversial. However, there were no previous studies on the caddisfly fauna of mountain springs and streams of the area between Osogovo and Karadak Mts. Therefore, the possibility for this species to inhabit the whole north-eastern part of the country is yet to be confirmed, although our results clearly show that *D. osogovicus* has a wider distribution range in North Macedonia than in Bulgaria. Similar distribution pattern was noticed in other *Drusus* spp. For example, *D. botosaneanui* Kumanski, 1968 is registered from Lukovo Pole in the western part of North Macedonia (WARINGER et al. 2015) but it also inhabits Osogovo Mt in the north-eastern part of the country (HINIĆ & SLAVEVSKA-STAMENKOVIĆ, unpublished data).

The present results strongly indicate that *D. osogovicus* cannot be treated as Osogovo Mt. endemic as suggested by KUMANSKI (2001). Nevertheless, its presence only in two countries (North Macedonia and Bulgaria) leads to the conclusion that it is a rare, endemic species whose populations might inhabit only the central part of the Balkan Peninsula.

The emergence pattern of *D. osogovicus* has previously been studied by KUMANSKI (1988). According to his results, this species emerges in June–July. This overlaps with our records of collected specimens in May–July, suggesting one-year life cycle of this species. The detection of the adult stage of *D. osogovicus* in May provides more precise information on the species' life cycle indicating its earlier emergence and extended flight period. The successful collection of specimens of *D. osogovicus* during the night makes this dark-coloured species an exception, since they are known to be generally active during the day (KUČINIĆ et al. 2014). Although not quite common, this modified diurnal activity pattern has been previously recorded in other dark-coloured *Drusus* spp. (KUČINIĆ et al. 2016), which points out the complexity of the caddisfly behaviour.

Conclusion

The results of our study provide new data about the caddisfly fauna of the Macedonian part of Osogovo Mt. and considerably expand the knowledge about the distribution of *D. osogovicus* on the Balkan Peninsula. The finding of another endemic and rare species in the Republic of North Macedonia highlights the rich and remarkable biodiversity in this country as well the importance of defining mitigation measures for minimizing the negative anthropogenic impacts towards the habitats of this species. The confirmation of the identification of our findings based on morphological characters by DNA barcoding highlights the importance of the symbiosis between DNA-based analyses and classical taxonomy. Furthermore, it also reveals more information about the caddisflies in North Macedonia, providing a basis for further biodiversity and phylogeographic studies as well as the preparation of the first Macedonian caddisfly checklist.

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