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## Contents

- Nikola Bankov  
A new level of confidence: biogeography, morphometry and ecology of enigmatic *Cyclopyxis puteus*  
Thomas, 1960 (Amoebozoa: Arcellinida: Netzeiliidae) 215
- Jozef Oboňa, Eva Čisovská Bazsalovicsová, Alexandru-Mihai Pintilioaie, Viorel Dumitru Gavril,  
Oana Cristiana Vasiliu, Laura-Elena Topală, Peter Manko  
Checklist of Hippoboscidae (Diptera) from Romania 229
- Yurii V. Kornilev, Georgi Popgeorgiev, Dimitar Plachiyski, Angel Dyugmedzhiev, Vladimir Mladenov,  
Kostadin Andonov, Simeon Lukanov, Emiliya Vacheva, Miroslav Slavchev, Borislav Naumov  
Distribution of the grass snake (*Natrix natrix*) and dice snake (*N. tessellata*) in Bulgaria 239

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# A new level of confidence: biogeography, morphometry and ecology of enigmatic *Cyclopyxis puteus* Thomas, 1960 (Amoebozoa: Arcellinida: Netzeiliidae)

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**Abstract:** Testate amoebae *Cyclopyxis puteus* Thomas (1960) has a characteristic morphology, that makes the possibility of overlook or misidentification unlikely. Although this species has been found worldwide, its morphology, biometrics and ecological preferences are poorly understood. In this study, isolated material from Bulgaria was investigated by using light and scanning electron microscopy. Based on morphological and biometrical data, we provide an improved diagnosis of this enigmatic species. A summary of the geographical distribution and ecological preferences is provided.

**Keywords:** Bulgaria, distribution, protists, taxonomy, testate amoebae

## Introduction

Genus *Cyclopyxis* Deflandre, 1929 comprises lobose testate amoebae with shells of agglutinated mineral particles, hemispherical in lateral and circular in ventral view. According to latest taxonomic reviews, it belongs to class Elardia, Order Arcellinida, Suborder Glutinoconcha, Infraorder Sphaerothecina and Family Netzeiliidae (Kang et al., 2019; Kosakyan et al., 2016;). Nowadays, genus *Cyclopyxis* contains approximately 80 species and subspecies (Nicholls, 2005) that can be found in various habitats like soils, leaf litter or mosses. Some of them are widely distributed with high population density in the environment (*Cyclopyxis eurystoma* Deflandre, 1929 and *Cyclopyxis kahli* Deflandre, 1929), while some rare species with restricted or patchy distribution have been described.

One of those rare and enigmatic species is *Cyclopyxis puteus* Thomas, 1960. According to the original description “ce cyclopyxis a l’aspect général et la couleur de *C. kahli* Defl., mais avec une taille bien plus considérable. Une autre particularité réside par la constitution spéciale du pseudostome, qui permettra de toujours le reconnaître facilement.

L’ouverture de ce pseudostome est placée à l’extrémité terminale d’un tube conique fortement invaginé à l’intérieur de la thèque. Ce pseudostome, vaguement circulaire, a un contour légèrement crénelé par la bordure granuleuse. En vue ventrale, le tube du pseudostome, étant large à sa base, dessine un double contour très apparent, car ce tube, observé à l’apic, va en se rétrécissant à l’intérieur de la thèque au niveau du pseudostome. Le revêtement ne présente rien de particulier; il est pierreux à grosses particules sur le dôme. Dimensions: diamètre 145-163 µm; hauteur 110-120 µm; pseudostome 25-35 µm; base du tube 45-50 µm; longueur du tube 28-30 µm.” (this species has the general appearance and colour of *C. kahli*, but with much larger size. Another feature is the special construction of a pseudostome, which allows to recognise it easily. The opening is situated at the terminal end of a strongly invaginated inside the shell conical tube. This roughly circular pseudostome has slightly serrated contours due to the grained edge. In ventral view, the tube of the pseudostome, being wide at the base, forms a very distinct double contour, because it narrows inward into the shell at the level of the pseudostome. The coating does not present anything particular; pieces of quartz, larger on the

dorsal side. Measurements: diameter 145–163  $\mu\text{m}$ ; height 110–120  $\mu\text{m}$ ; pseudostome 25–35  $\mu\text{m}$ ; base of the tube 45–50  $\mu\text{m}$ ; length of the tube 28–30  $\mu\text{m}$ ).

The characteristic morphology and large size of this species makes the possibility of overlook or misidentification unlikely. However, the registered data emphasises the difficulty of defining the distribution patterns. So far, *C. puteus* is reported worldwide but with infrequent findings and low number of specimens. The scarcity of records, on the other hand, leads to a poorly studied morphology, biometry and ecological preferences.

During the investigation of testate amoebae communities from soil mosses and beech's litter from Bulgaria, we found population of *C. puteus* with a high abundance. We carried out detailed light and scanning electron microscopy analyses with additional morphometric examination of the shells. Our primary aims were: 1) to examine the Bulgarian populations of *C. puteus* morphologically and biometrically; 2) to clarify its geographic distribution and ecological preferences.

## Materials and methods

The samples were taken on 8.11.2022 from soil mosses, litter (Ao) and soil humus (Ah – 0–5 cm) of beech forests (*Fagus sylvatica* L.) near Plachkovtsi Village (N 42.79821 E 25.49891, 591 a.s.l.) (Fig. 1). The brown mountain-forest soil is the main soil type within the area. The collected material was washed and examined in petri dish. Isolation was made under stereomicroscope at 100x magnification.

Morphological characters and morphometric variables of 350 shells were studied with optical microscope “Amplival” (Zeiss-Jena) using 40x objective and 10x oculars lens. The light micrographs were taken using an Axio Imager M2-Carl Zeiss compound microscope with a digital camera (ProgRes C7) and specialised software (CapturePro Software 2.8). Helicon Focus 8 software was used for the stacked images. The following morphometric measurements were taken: diameter, depth (= height in the original description), external opening (= base of the tube), internal opening (= pseudostome) and invagination (= length of the tube). For each morphometric variable, we calculated following basic summary statistics: arithmetic mean, median (M), standard deviation (SD), standard error of mean (SE),

coefficient of variation in % (CV), extreme values (Min and Max). Statistical analysis was conducted using the computer program STATISTICA Software, Version 10.0 (StatSoft 2010).

For the comparative analysis of the morphometric data, we used microscopic slides fixed with Canada balsam from two spatially distant locations in Bulgaria: Borisova Gradina area (litter and humus of oak forest (*Quercus* spp.)) and the area close to Mechata Dupka Cave on the path to Treskavets Hut (litter and humus of beech forest (*Fagus sylvatica*)) (unpublished data) (Fig. 1). Additional measurements were taken of single specimens from Eastern Rhodopes (Bulgaria; unpublished data) and North Korea (unpublished data).

Scanning electron microscopy analysis was conducted, as part of the specimens were extracted by using a glass micropipette, washed several times in distilled water, mounted on coverslip and air-dried. The shells were coated evenly with gold in a vacuum coating unit. The micrographs were obtained by using a JEOL JSM-5510, operating at 10 kV.

## Results and discussion

### Biometry

The morphometric characterisations of *C. puteus* according to our studies are given in Table 1. Dimensions of the shell are: diameter 145–190  $\mu\text{m}$ ; depth 93–145  $\mu\text{m}$ ; external opening 42–65  $\mu\text{m}$ ; internal opening 28–44  $\mu\text{m}$  and invagination 39–57  $\mu\text{m}$ . All measured shell parameters are moderately variable (CV between 5.21 and 10.0%). The most stable characters in this population are shell's diameter and depth (5.21 and 7.41, respectively), while internal opening and invagination have maximal values (9.53 and 10, respectively). External opening has an intermediate value of 8.20.

Analysis of the size frequency distribution shows that *C. puteus* is a size-monomorphic species with a relatively well-expressed main-size class of the basic shell characters (Fig. 2). For example, about three-quarters of all measured individuals (73%) have a diameter of shell within the limits of 160–180  $\mu\text{m}$ , whereas only 17% have a diameter less than 160  $\mu\text{m}$  and 10% – more than 180  $\mu\text{m}$  (Fig. 2A). Regarding the shell's depth and diameter of external opening, most of the measured individuals also varied within

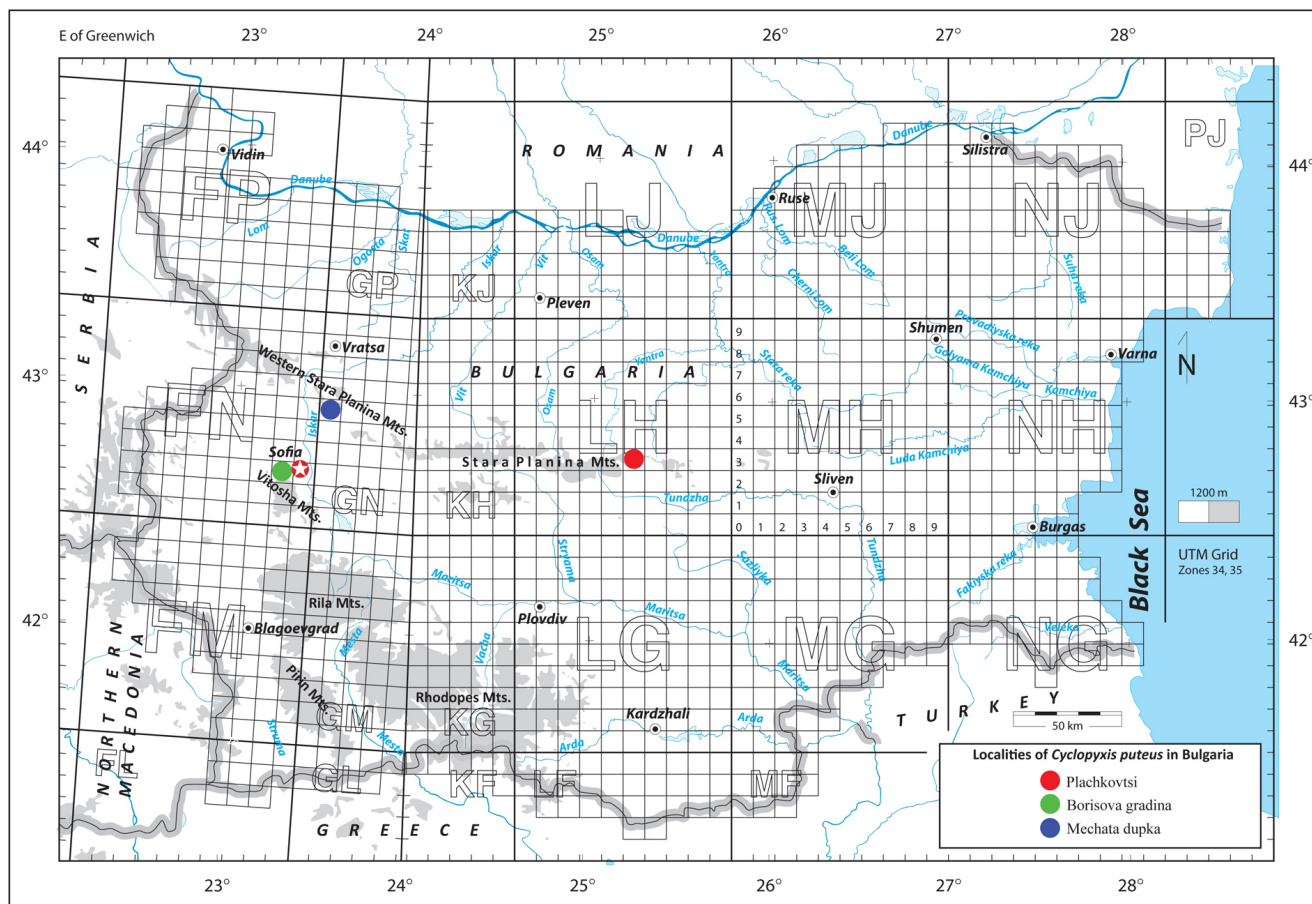


Fig. 1. Localities of *Cyclopyxis puteus* in Bulgaria. The examined population is indicated in red. Populations used as comparative material are marked as follows: green – Borisova Gradina area; blue – Mechata Dupka area.

close limits: depth of shell 105–135  $\mu\text{m}$  (91%) and diameter of external opening 46–64  $\mu\text{m}$  (96%) (Fig. 2 B, C). The presence of a well-expressed main-size class of the basic shell characters and the lack of the subsidiary peaks (bell-shaped curves) indicate a normal distribution. The average values of these characters are respectively  $169.4 \pm 0.47$  (diameter),  $119.8 \pm 0.47$  (depth) and  $55.4 \pm 0.24$  (external opening). They fully correlate with the main-size classes of characters and testify to the monomorphism of the species.

Our results were compared with the morphometric dimensions of the other two populations from Bulgaria (Table 1). Mean values indicate overall larger size dimensions of the Plachkovtsi population. When comparing variability of all characters, analysed populations have moderate coefficient of variation: diameter (between 4.63 and 7.29); depth (6.96–9.08); external opening (7.87–11.75); internal opening (9.41–10.03) and

invagination (8.46–10 respectively for Borisova Gradina and Plachkovtsi; no invagination was measured for Mechata Dupka). The population with the lowest coefficient of variation was that of Borisova Gradina (with an exception of external opening), while Plachkovtsi's population has intermediate position.

Comparative analysis of the size frequency distribution confirms size-monomorphism of *C. puteus*, characterised by a main size class and a small size range. In the Fig. 3 is shown frequency analysis of shell diameter, which confirms larger dimensions of Plachkovtsi population, while “apparent” polymorphism of Mechata Dupka population is due to small number of measurements.

The above mentioned conclusions of comparative analysis are supported by the scatter plot of shell diameter versus shells depth (Fig. 4). All three populations have a linear relationship. Plachkovtsi and Mechata Dupka are characterised with moderate

Table 1. Morphometric characterisations of *C. puteus* from Plachkovtsi, Borisova Gradina and Mechata Dupka.

Characters	Popullation	Mean	M	SD	SE	CV	Min	Max	n
diameter	Plachkovtsi	169.44	170	8.82	0.47	5.21	145	190	350
	Borisova Gradina	156.26	155.7	7.23	0.67	4.63	133	180	117
	Mechata Dupka	153.69	155.7	11.2	1.47	7.29	134	179	58
depth	Plachkovtsi	119.84	120	8.88	0.47	7.41	93	145	350
	Borisova Gradina	103.8	102	7.22	2.28	6.96	96	121	10
	Mechata Dupka	105.13	107.5	9.54	2.88	9.08	94	120	11
external opening	Plachkovtsi	55.48	56	4.55	0.24	8.2	42	65	350
	Borisova Gradina	50.35	50	5.92	0.55	11.75	32	61	114
	Mechata Dupka	48.39	48	3.8	0.56	7.87	42	56	47
internal opening	Plachkovtsi	35.46	36	3.38	0.19	9.53	28	44	329
	Borisova Gradina	33.97	34	3.20	0.37	9.41	27	42	75
	Mechata Dupka	31.04	31	3.11	0.45	10.03	26	39	47
invagination	Plachkovtsi	47.35	47	4.73	0.52	10	39	57	84
	Borisova Gradina	32.44	34	2.74	0.91	8.46	28	36	9
	Mechata Dupka	—	—	—	—	—	—	—	—
depth/ diameter ratio	Plachkovtsi	0.71	0.7	0.048	0.003	6.79	0.55	0.86	350
	Borisova Gradina	0.67	0.66	0.05	0.016	7.44	0.63	0.79	10
	Mechata Dupka	0.7	0.7	0.032	0.01	4.56	0.65	0.77	11
external opening/ diameter ratio	Plachkovtsi	0.33	0.33	0.025	0.001	7.66	0.25	0.40	350
	Borisova Gradina	0.32	0.32	0.035	0.003	10.72	0.24	0.39	114
	Mechata Dupka	0.31	0.32	0.031	0.005	9.88	0.25	0.38	47
invagination/ depth ratio	Plachkovtsi	0.38	0.38	0.037	0.004	9.58	0.28	0.50	84
	Borisova Gradina	0.32	0.31	0.033	0.011	10.42	0.28	0.36	9
	Mechata Dupka	—	—	—	—	—	—	—	—

strength, while Borisova Gradina has almost horizontal slope that indicate a weak strength.

The summarised data of previous biometric records is given in Table 2. The results of the three examined populations correspond well with those given by other authors (Thomas 1960, Golemansky 1962, Bobrov & Mazei 2004, Vincke et al 2006, Todorov & Bankov 2019) and fall within biometrical framework of previous studies. Must be noted that in Bobrov & Mazei 2004 the values of diameter and depth are probably erroneously shifted.

The examination of solitary specimens from the Eastern Rhodopes and North Korea confirmed the above mentioned measurements with an exception of four shells (2 from each locality) with diameter over

200 µm. The measurements are as follows: Eastern Rhodopes: 1) diameter 203 µm; external opening 58 µm; internal opening 34 µm; 2) diameter 214 µm and North Korea: 1) diameter 210 µm; external opening 63 µm; internal opening 42 µm; 2) diameter 229 µm; external opening 75 µm.

#### Redescription

The original description of *C. puteus* (Thomas, 1960) is based on light microscopy with notes on the general appearance and structure of the pseudostome (Fig. 5). The number of measured individuals is not mentioned. The most comprehensive study to

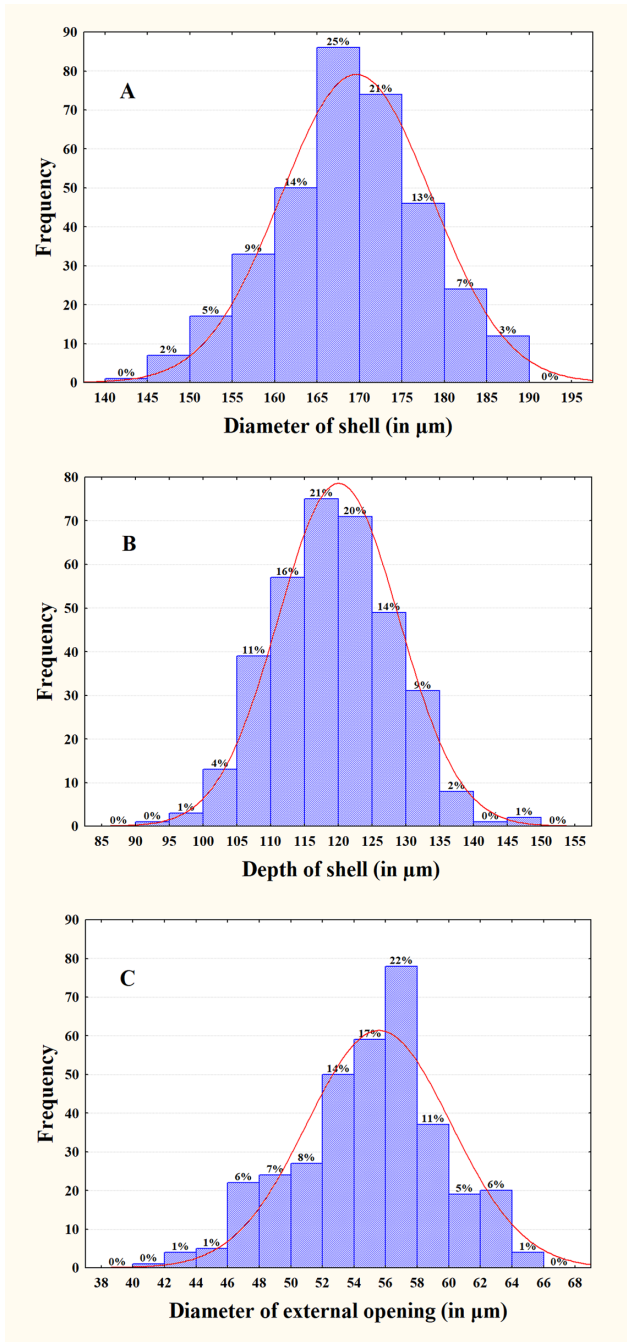


Fig. 2. Histograms showing the size frequency distribution of the shell's diameter (A), depth (B) and external opening (C) in *Cyclopyxis puteus*.

improve this taxonomic characterisation was conducted by scanning electron microscopy and gives first data of species ultrastructure (Todorov & Bankov, 2019). Nevertheless, the number of examined specimens weren't enough to scope the phenotypic plasticity range. In addition, the analysed

data was from microscopic slides, where no information about degree of invagination was collected. The results of this study support and refine previous description. To clarify the description and avoid ambiguous terminology, we refer base of the tube (Thomas, 1960) and diameter of aperture (Todorov & Bankov, 2019) as external opening.

Shell circular in ventral and dorsal views, hemispherical in lateral, yellowish or brownish, composed mainly of quartz particles (larger on the dorsal side) (Fig. 6), embedded in a layer of organic cement with numerous small pores on the ventral and lateral surface (Fig. 7). The outline has a smooth surface. Internal opening circular, central and situated at the terminal end of a strongly invaginated inside the shell conical tube. External opening with larger diameter. Different diameters of external and internal openings, form a very distinct double contour with slightly serrated borders due to the grained edge.

Measurements: diameter 133–190 µm (occasionally up to 229 µm); depth 93–145 µm; external opening 32–65 µm (occasionally up to 75 µm); internal opening 26–46 µm; invagination 28–57 µm; depth/diameter ratio – 0.55–0.86; external opening/diameter ratio – 0.24–0.4; invagination/depth ratio – 0.28–0.5.

#### Geographic distribution

First discovered near Pessac in southwestern France by Thomas (1960), *C. puteus* has been recorded from many localities in Europe, including Belgium (Chardez, 1961; Chardez et al., 1987), Bulgaria (Deltshev et al., 1999; Todorov, 2001; Bankov, 2022; Todorov, unpublished), Czech Republic (Balik, 1994), France (Bonnet, 1972, 1993, Decloitre, 1977), Poland (Golemansky, 1970), Russia (Balik, 1992; Bobrov, 2001; Bobrov & Mazei, 2004; Payne et al., 2020; as fossil record in Bobrov et al., 2003, 2004), Slovakia (Balik, 1997) and in the area between Belgium, North France, Luxemburg and Netherland (no information for a particular country or sample point in Chardez & Lambert, 1981). It has also been reported in South America: Brazil and Paraguay (Bonnet, 1979); French Guiana (Coûteaux & Chardez, 1981) and Peru (Bobrov et al., 2019), Central America: Mexico (Bonnet, 1977a; Bobrov et al., 2013) as well as North America: Canada (Puytorac et al., 1972; Bonnet, 1974; Lousier, 1982; Beyens et al., 1990). Ad-

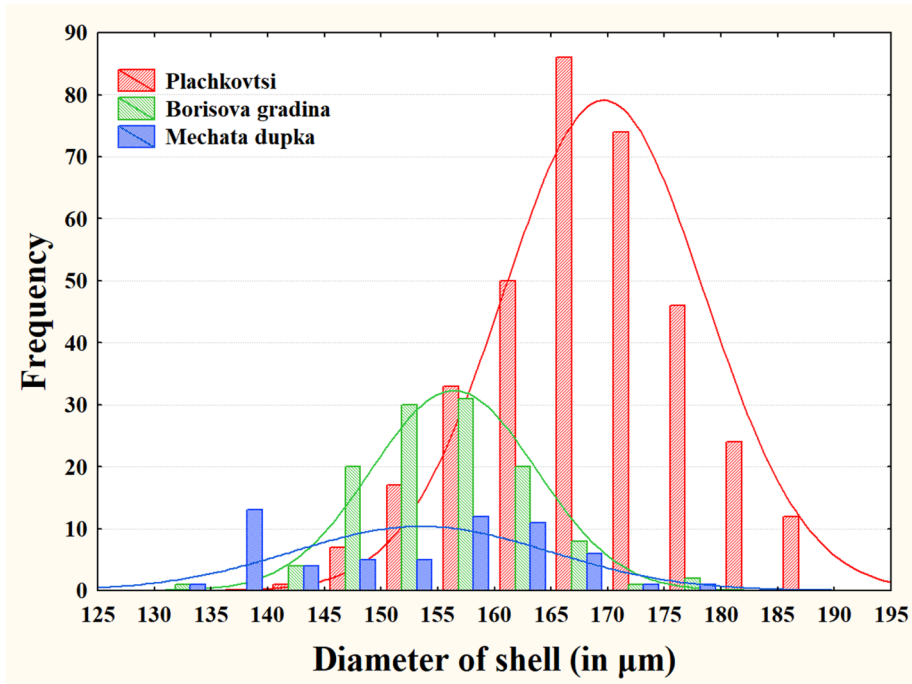


Fig. 3. Histograms showing the diameter frequency distribution of *Cyclopyxis puteus* in three studied populations from Bulgaria.

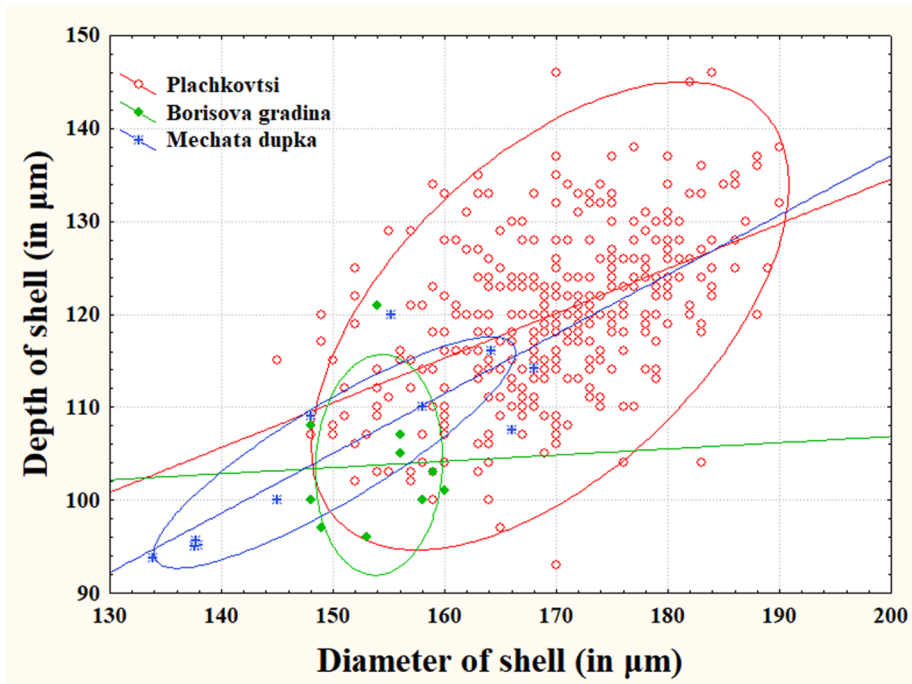


Fig. 4. Scatter plot of shell diameter versus shells depth in *Cyclopyxis puteus*.

ditional evidence for the cosmopolitan dispersion of *C. puteus* is that the species is registered also in Asia: Indonesia (Bonnet, 1992), Nepal (Bonnet, 1977b,

1981), North Korea (Golemansky & Todorov, 1991), Thailand (Bonnet, 1987; Golemansky & Todorov, 2000), Vietnam (Bobrov et al., 2010), Africa: Demo-



Table 2. Measurements of *Cyclopyxis puteus* according to current study and previous biometric records. The number of examined individuals is shown in brackets. Summarised data\* – from Borisova Gradina and Mechata Dupka.

Source autors	Country	Location	Diameter	Depth	External opening	Internal opening	Invagination
Thomas 1960	France	Pessac	145–163	110–120	45–50	25–35	28–30
Golemansky 1962	Guinea	Rég de N'zérékoré area	164	120	—	36	48
Bobrov & Mazei 2004	Russia	Sikhote-Alin Reserve	120–132 (38)	130–180 (46)	—	34–46 (46)	—
Vincke et al 2006		Île de la Possession	195 (1)	—	—	—	—
Todorov & Bankov 2019	Bulgaria	summarised data*	133–179 (64)	94–120 (11)	32–54 (53)	27–38 (15)	—
current study	Bulgaria	Plachkovtsi Village	145–190 (350)	93–145 (350)	42–65 (350)	28–44 (329)	39–57 (84)
unpublished	Bulgaria	Borisova Gradina	133–180 (117)	96–121 (10)	32–61 (114)	27–42 (75)	28–36 (9)
unpublished	Bulgaria	Mechata Dupka	134–179 (58)	94–120 (11)	42–56 (47)	26–39 (47)	—

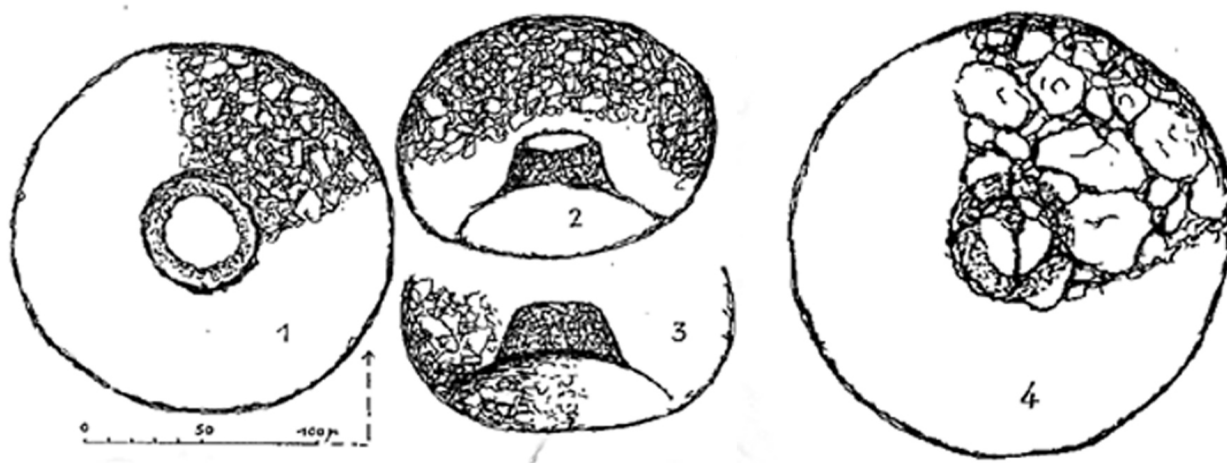


Fig. 5. Original drawings of Thomas 1960, modified: 1) ventral side; 2, 3) lateral side; 4) dorsal side.

cratic Republic of the Congo (Siemensma, 2019–2023); Gabon (Bonnet, 1969); Guinea (Golemansky, 1962); remote sub-Antarctic archipelago Îles Crozet (part of the French Southern and Antarctic Lands, Vincke et al., 2006) and even as part of Antarctic microbiota (Sudzuki, 1979). During the expedition in Guinea, Golemansky (1962) found shells that have the general appearance of *C. puteus*, but with much smaller dimensions (diameter 72 μm; depth 60 μm; pseudostome 18–20 μm; diameter/depth ration – 1.2; diameter/external opening – 4.0). No following de-

scription was made. Subsequently described as a variety – *Cyclopyxis puteus* var. *golemanskyi* Chardez, 1964 (Siemensma, 2019–2023), this morphotype is registered also in Belgium, North France, Luxemburg and Netherland (Chardez & Lambert, 1981) and terrestrial habitats of Antarctica (Sudzuki, 1979).

It must be noted that most of the records are based on single individuals or populations with very low density. This global distribution pattern must be interpreted with caution, given recent molecular studies that reveals species complexes with hidden

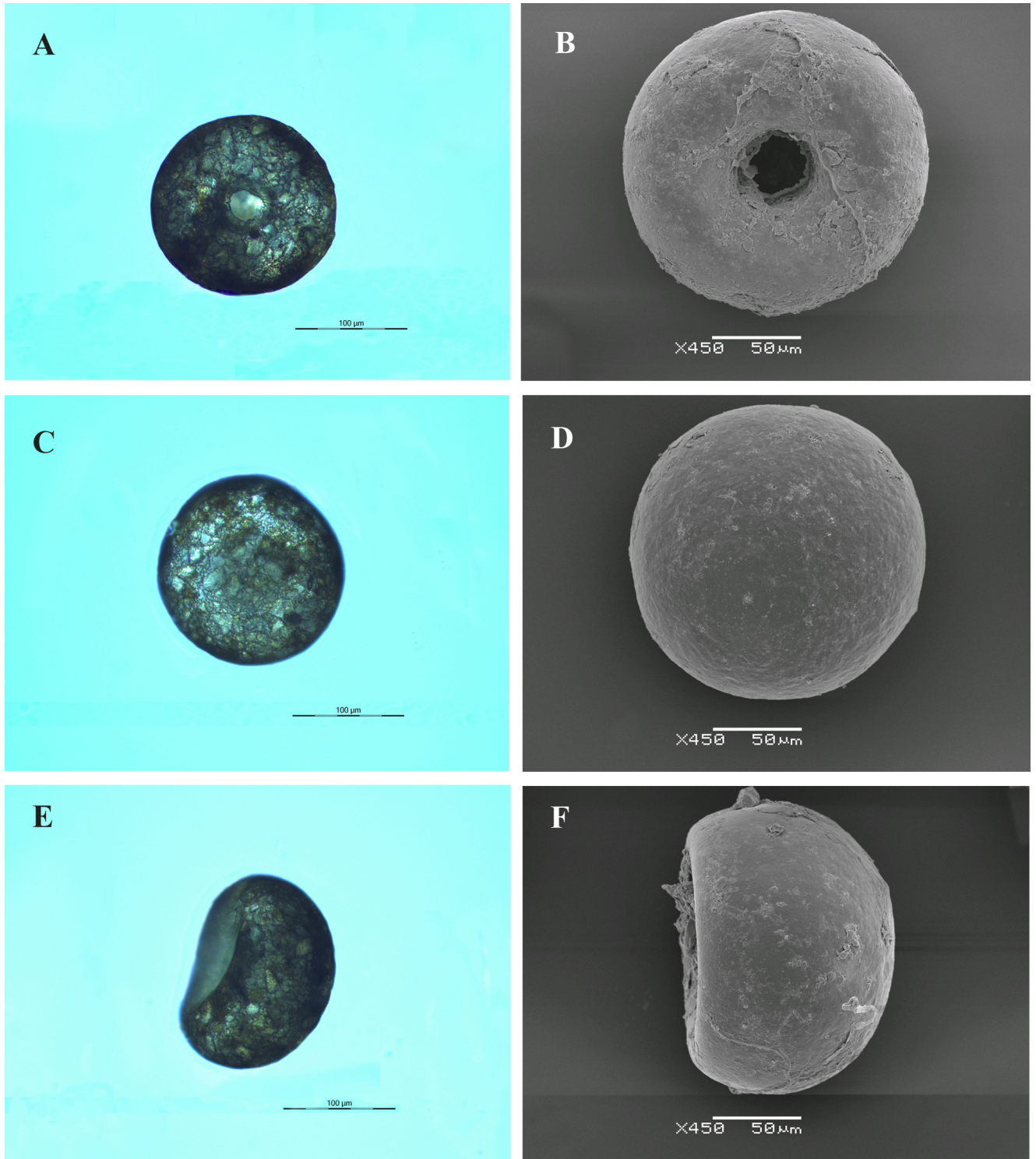


Fig. 6. Light (A, C, E) and scanning electron (B, D, F) photographs of *Cyclopyxis puteus*: (A, B) ventral side; (C, D) dorsal side; (E, F) profile.

(pseudo)cryptic diversity (Kosakyan et al., 2012, 2013, Singer et al., 2015) and different geographical distribution (Heger et al., 2013).

Additional data associated with geographical distribution is available at the Supplementary material 01 – Table A1 [🔗](#).

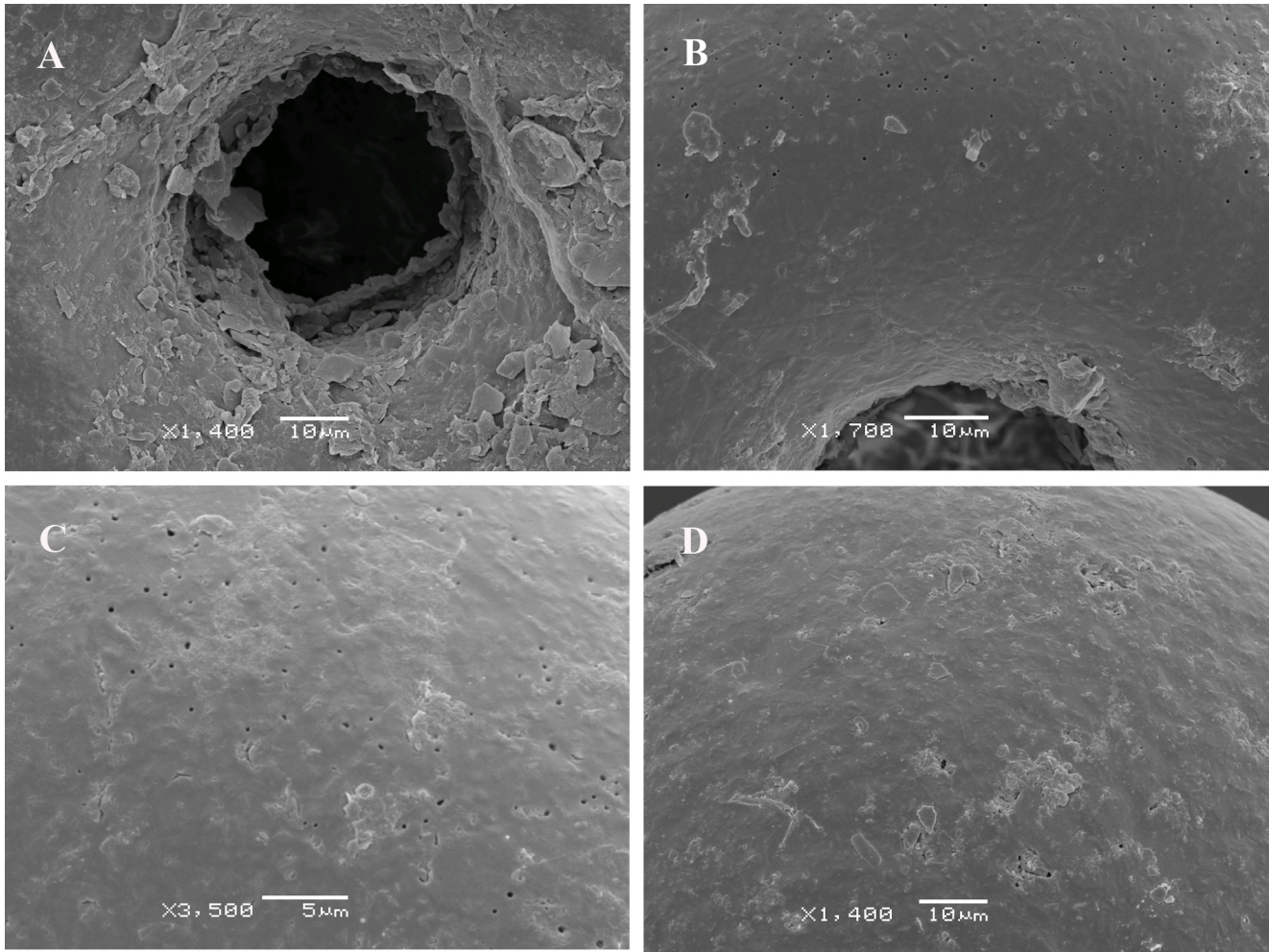


Fig. 7. SEM photographs of the ultrastructure: (A) ventral structure showing double contour; (B) structure of the external opening; (C) ventral side with numerous pores; (D) dorsal side.

## Ecology

The type locality of *C. puteus* is a sandy soil of pine forest. Indeed, subsequent studies confirmed this species as inhabitant of forest litter, soils and mosses (Puytorac et al., 1970; Bonnet, 1977a, b, 1979, 1987, 1992; Chardez & Lambert, 1981, Lousier, 1982; Golemansky & Todorov, 1991, 2000, unpublished data; Balik, 1992, 1994, 1997; Bobrov, 2001; Todorov, 2001, unpublished data; Bobrov & Mazei, 2004; Vincke et al., 2004; Bobrov et al., 2010; 2013; Payne et al., 2020). Records of solitary specimens came from calcimorphic soil on karst (Bonnet, 1992), humic soil without plant cover (Balik, 1992), humus collected from bamboo roots (Bonnet, 1987), flooded savannah (Bonnet, 1997), rhizosphere of epiphytic plants and area with cut off forest, which was subse-

quently burned (Coûteaux & Chardez, 1981). Additional information about its ecological preferences is provided by Bonnet (1974), who registered it in both acidic and carbonated soils in Québec province, Canada. Accidental findings of *C. puteus* in *Sphagnum* mosses (Chardez et al., 1987; Golemansky & Todorov, 1991; Bankov, 2022) and freshwater habitats like a small swamp in rice field (Golemansky & Todorov, 1991) or forest stream (Golemansky & Todorov, 2000) may be due meteorological conditions like wind and/or heavy rain that brought single specimens from adjacent areas. Unfortunately, no differentiation has been made between live and dead specimens in previous studies, therefore the ecological characteristics of the habitats with single specimens or low population densities may not give us additional information about the ecology of the species.

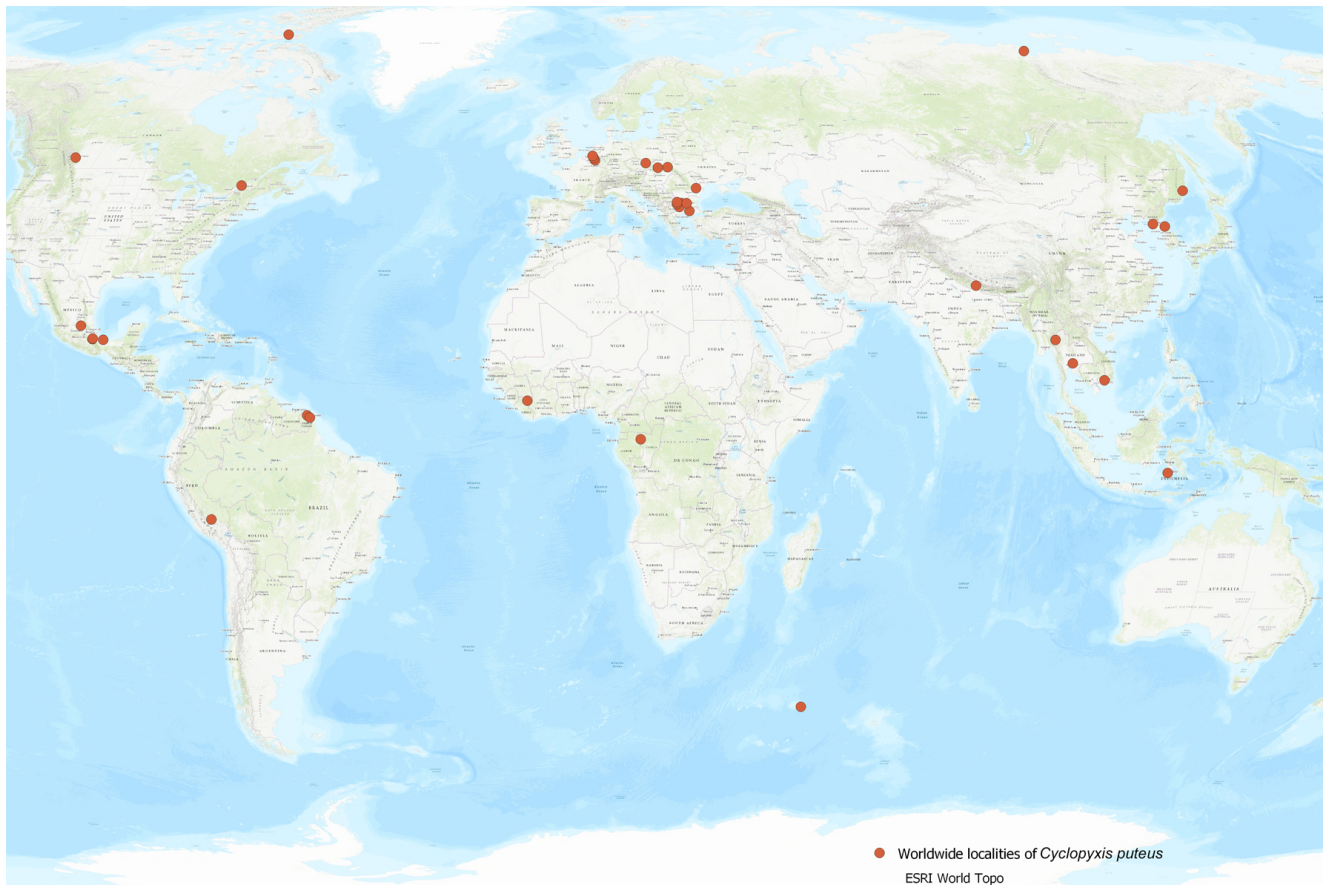


Fig. 8. Worldwide localities of *Cyclopyxis puteus*.

In the studied populations from Bulgaria, the number of living individuals varies between 30–40 %. Samples were taken from beech (Plachkovtsi and Mechata Dupka) and oak (Borisova Gradina) forests, confirming conclusion that *Cyclopyxis puteus* is a soil and litter-dwelling species with occasional findings in peat bogs and freshwater habitats.

The next steps involve additional studies from different geographic areas to establish the phenotypic plasticity and ecological preferences of the species. Genetic characterisation is needed to provide evidences of its cosmopolitan distribution and/or the potential presence of (pseudo)cryptic diversity within *Cyclopyxis puteus*.

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## Supplementary materials

01

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## Research article



## Checklist of Hippoboscidae (Diptera) from Romania

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**Abstract:** The checklist of louse flies or keds from the family Hippoboscidae in Romania with 14 species is given. Among them, six species have been newly recorded from Romania, from Natura 2000 site “Dunele Marine de la Agigea” Natural Reserve; namely: *Icosta minor* (Bigot in Thomson, 1858), *Ornithoica turdi* (Olivier in Latreille, 1812), *Ornithomya chloropus* Bergroth, 1901, *Ornithomya fringillina* Curtis, 1836, *Ornithophila gestroi* (Rondani, 1878), and *Ornithophila metallica* (Schiner, 1864). Out of the total, seven species are autochthonous, while the other seven are probably non-native species, either spreading invasively or only occasionally being imported to Romania or migrating to the country along with their hosts. Three new host-parasite associations have been reported for the first time. Specifically, the eastern olivaceous warbler *Iduna pallida* (Hemprich & Ehrenberg, 1833) represents new host species for *I. minor* and *O. turdi*, while the willow warbler *Phylloscopus trochilus* (Linnaeus, 1758) represents a new host species for *O. gestroi*.

**Keywords:** faunistic, invasive species, keds, literature review, louse flies, parasite-host associations

### Introduction

Flies in the family Hippoboscidae, known as ‘louse flies’ or ‘keds’, parasitising birds or mammals, belong to the Diptera and are a group of obligate parasites (Rahola et al., 2011). Hippoboscids are divided into several tribes, in particular Lipoptenini and Hippoboscini, which exclusively affect mammals, while species of the tribes Olfersiini and Ornithomyini parasitise primarily birds (Reeves & Lloyd, 2019). Thirteen genera, with more than 210 species, have already been described within Hippoboscidae worldwide, out of which 31 species were found in Europe (Pape et al., 2015; Dick, 2018; Nartshuk et al., 2019; Oboňa et al.,

2019b, 2022; Le Guillou & Chapelin-Viscardi, 2022; Yatsuk et al., 2023).

Adults of both sexes are hematophagous insects and are recognised as vectors for numerous infectious agents, including protozoa, bacteria, helminths, and possibly also viruses (e.g. Kosoy et al., 2016; Liu et al., 2016; Buss et al., 2016; Skvarla & Machtinger, 2019; Boularias et al., 2020; Zhao et al., 2020; Bezerra-Santos & Otranto, 2020; Santolíkova et al., 2022; Čisovská Bazsalovicsová et al., 2023; Tiawirisup et al., 2023). While some species exhibit host-specificity, others feed on a wide range of hosts (e.g. Maa, 1969; Ibáñez-Bernal et al., 2015; Mehlhorn, 2016; Veiga et al., 2018).

The primary objective of this work is to provide new faunistic records and summarise the checklist of the family Hippoboscidae of Romania.

## Material and methods

The studied material was collected at two sampling sites in Romania: Agigea Bird Observatory by A.-M. Pintilioaie and L.-E. Topală, except one specimen, collected by V. D. Gavril in Abrud (Constanța County, 44°08'43.4"N 27°58'41.5"E, 60 m a.s.l.).

Agigea Bird Observatory is the first permanent Romanian ringing station (Pintilioaie et al., 2022), being located at Marine Research Station “Prof. Dr. Ioan Borcea” in Agigea, Constanța County, 44°05'11.2"N 28°38'28.2"E, 10 m a.s.l. The mist-nets used to trap, especially Passeriformes for ringing, measure 500 m and are set up in the Natura 2000 site “Dunele Marine de la Agigea” Natural Reserve (permit: ORDIN nr. 1.380 from 8 July 2020). The fly specimens were collected directly from the birds (in this case, the host was mentioned in the paper), or were found inside the bird ringing room (in this case we couldn't assign with certainty the host of the fly).

Collected hippoboscids were placed in the Eppendorf tubes, fixed in ethanol (96 %) and subsequently identified in the laboratory using a determination key by Povolný & Rosický (1955), Theodor & Oldroyd (1964) and Oboňa et al. (2022). The material is deposited in the ethanol collection at the Department of Ecology, Faculty of Humanities and Natural Sciences, University of Prešov, Slovakia.

The primary focus on the hosts is given after Maa (1969); the European distribution follows Petersen (2004).

## Results and discussion

Family Hippoboscidae Samouelle, 1819

Subfamily Hippoboscinae Samouelle, 1819

Tribe Hippoboscini Samouelle, 1819

*Hippobosca equina* Linnaeus, 1758

Published records: Thalhammer (1896), Fleck (1904), Pârvu & Chimișliu (1982), Ursu & Pavel (1993).

Material examined: Abrud, 29.09.2022, 1 ♀, host: horse (*Equus ferus* f. *caballus* Linnaeus, 1758).

Comment: An ectoparasite of livestock (preferably horses and donkeys) and dogs, but has also been reported from humans. Known from Afro-tropical, Australian, Western Palaearctic and Oriental regions (Krištofik, 1998; Soliman et al., 2022; Mašlanko et al., 2022).

*Hippobosca longipennis* Fabricius, 1805

Published record: Ursu & Pavel (1993), Mihalca et al. (2019).

Comment: A rare and non-native species in Europe, distributed in the Mediterranean and Afrotropical regions. It is an ectoparasite of dogs; occasionally it can also occur on other predatory mammals or ungulates (Chalupský, 1980; Oboňa et al., 2016, 2019b; Zerek et al., 2020).

*Melophagus ovinus* (Linnaeus, 1758)

Published record: Ursu & Pavel (1993).

Comment: It is a native ectoparasite of Bovidae, especially sheep (e.g. Chalupský, 1980).

Tribe Lipoptenini Speiser, 1908

*Lipoptena fortisetosa* Maa, 1965

Published records: Pârvu (2005), Lazăr et al. (2017), Salvetti et al. (2020).

Note: According to Salvetti et al. (2020), *L. fortisetosa* from Romania (see Lazăr et al., 2017) was wrongly identified as *L. cervi* (Linnaeus, 1758) on *Capreolus capreolus* (Linnaeus, 1758).

Comment: It is a common invasive and non-native deer ked in Europe (Andreani et al., 2019, 2021; Kurina et al., 2019; Oboňa et al., 2022).

Tribe Olfersiini Maa, 1969

*Crataerina pallida* (Olivier in Latreille, 1811)

Published record: Petersen (2004).

Comment: A common ectoparasite of the bird



Fig. 1. *Icosta minor* (Bigot in Thomson, 1858) ♀, “Dunele Marine de la Agigea” Natural Reserve, 30.06.2022.

species *Apus apus* (Linnaeus, 1758) and *Delichon urbicum* (Linnaeus, 1758) (Krištofik, 1998; Walker & Rotherham, 2010; Petersen et al., 2018).

*Icosta ardeae* (Macquart, 1835)

Published record: Thalhammer (1896; as syn.: *Olfersia ardea* from host *Nycticorax nycticorax* (Linnaeus, 1758); Thalhammer used terms “plumis Nyctiardeae nycticoracis”).

Comment: A not common and non-native species in Europe, widespread in the tropics and subtropics of the Old World (e.g. Chalupský, 1980).

*Icosta minor* (Bigot in Thomson, 1858)

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 30.06.2022, 1 ♀ (Fig. 1), host: *Iduna pallida* (Hemprich & Ehrenberg, 1833); “Dunele Marine de la Agigea” Natural Reserve, 23.08.2022, 1 ♀, host: unknown.



Fig. 2. *Ornithoica turdi* (Olivier in Latreille, 1811) ♀, “Dunele Marine de la Agigea” Natural Reserve, 20.07.2022.

Comment: A relatively small, rare, and non-native species in Europe, distributed in the Afrotropical region and the Mediterranean Basin (Trilar & Krčmar, 2005; Sychra et al., 2020; Jentzsch et al., 2021a). *Iduna pallida* represents a new host species for *Icosta minor*. New for Romania.

*Ornithoica turdi* (Olivier in Latreille, 1811)

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 20.07.2022, 1 ♀ (Fig. 2), host: *Iduna pallida* (Hemprich & Ehrenberg, 1833); “Dunele Marine de la Agigea” Natural Reserve, 8.11.2022, 1 ♀, host: unknown.

Comments: A non-native species in Europe, distributed in the Afrotropical region and southern Palearctic, with a recent increase in records in Europe (Droz & Haenni, 2011; Zittra et al., 2020; Gaponov & Tewelde, 2020; Kock, 2000). *Iduna pallida* represents a new host species for *O. turdi*. New for Romania.



Fig. 3. *Ornithophila gestroi* (Rondani, 1878) ♀, “Dunele Marine de la Agigea” Natural Reserve, 2.05.2022.



Fig. 4. *Ornithophila metallica* (Schiner, 1864) ♀, “Dunele Marine de la Agigea” Natural Reserve, 20.02.2023.

*Ornithophila gestroi* (Rondani, 1878)

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 2.05.2022, 1 ♀ (Fig. 3), host: *Phylloscopus trochilus* (Linnaeus, 1758).

Comment: Not common and non-native (in Europe) parasite species of Falconidae and Accipitridae (Nartshuk & Matyukhin, 2019; Balgooyen et al., 1999; Ganbold et al., 2020; Jentzsch et al., 2021b). *P. trochilus* from the family Phylloscopidae represents a new host species (including the family). New for Romania.

*Ornithophila metallica* (Schiner, 1864)

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 20.02.2023, 1 ♀ (Fig. 4), host: *Parus major* Linnaeus, 1758.

Comment: A non-native polyphagous bird parasites species in Europa, distributed in southern parts of the Palaearctic, Afrotropical, Oriental, and Australian regions (Krištofik, 1998; Nartshuk & Matyukhin, 2019; Lehikoinen et al., 2021; Lee et al., 2022). New for Romania.

*Stenepteryx hirundinis* (Linnaeus, 1758)

Published records: Thalhammer (1896), Petersen (2004).

Comment: A frequent European species, widespread in the Palaearctic region. A common ectoparasite of the bird species *Delichon urbicum*, *Hirundo rustica* Linnaeus, 1758, *Ptyonoprogne rupestris* (Scopoli, 1769), *Riparia riparia* (Linnaeus, 1758), most frequently found in nests (Thalhammer, 1896; Krištofik, 1998; Oboňa et al., 2021).

Tribe Ornithomyini Costa, 1846

*Ornithomya avicularia* (Linnaeus, 1758)

Published records: Thalhammer (1896), Pârvu (2003), Petersen (2004).

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 3.07.2022, 1 ♂, host: *Corvus cornix* Linnaeus, 1758.

Comment: A frequent louse fly species in Europe, widespread in the Palaearctic region. A common ectoparasite of birds from the order Passeriformes and

other orders (Krištofik, 1998; Oboňa et al., 2019a, b, 2021, 2022).

*Ornithomya chloropus* (Bergroth, 1901)

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 18.09.2022, 1 ♀, host: *Anthus trivialis* (Linnaeus, 1758).

Comment: A Palaearctic species distributed in the northern and middle belts of the region. It is an ectoparasite mainly of Passeriformes, but also of species of other bird orders (Povolný & Rosický, 1955; Petersen et al., 2007; Vastveit, 2013; Matyukhin et al., 2021). New for Romania.

*Ornithomya fringillina* Curtis, 1836

Material examined: “Dunele Marine de la Agigea” Natural Reserve, 26.09.2022, 1 ♀, host: *Lanius collurio* Linnaeus, 1758; “Dunele Marine de la Agigea” Natural Reserve, 6.11.2022, 1 ♀, host: *Erithacus rubecula* (Linnaeus, 1758).

Comment: A Palaearctic species distributed in the northern and middle belts of the region. It is an ectoparasite mainly of Passeriformes, but also parasitises species in other bird orders (Krištofik, 1998; Oboňa et al., 2019b; Yoshino & Asakawa, 2020; Tomás et al., 2021; Rekecki & Rajkovic, 2023). New for Romania.

Currently, we have found that 14 species were recorded so far from the country. Out of them, seven species are native to Romania (*H. equina*, *M. ovinus*, *C. pallida*, *S. hirundinis*, *O. avicularia*, *O. chloropus*, and *O. fringillina*). The remaining seven species (*H. longipennis*, *L. fortisetosa*, *I. ardeae*, *I. minor*, *O. turdi*, *O. gestroi*, *O. metallica*) have been probably introduced naturally due to migrating hosts (*I. ardeae*, *I. minor*; *O. turdi*, *O. gestroi*, *O. metallica*), or imported together with domestic or wild animals (e.g. *H. longipennis*, *L. fortisetosa*).

Note: The status of some non-native species is still open to question in Romania (especially in *O. turdi* and *O. gestroi*). The northern limit of their native distribution is likely found in this region. Therefore, it is necessary to know the distribution of these species in more detail.



Fig. 5. The eastern olivaceous warbler *Iduna pallida* (Hemprich & Ehrenberg, 1833) a new host species for *Icosta minor* (Bigot in Thomson, 1858) and *Ornithoica turdi* (Olivier in Latreille, 1812).



Fig. 6. The willow warbler *Phylloscopus trochilus* (Linnaeus, 1758) a new host species for *Ornithophila gestroi* (Rondani, 1878).

*Lipoptena fortisetosa*, in particular, is an aggressive and invasive species that has established a significant population in countries where it has been present for a long time. This species threatens mammals, including humans and even birds (e.g., Oboňa et al., 2019a, b, 2021, 2022).

Furthermore, new host-parasite associations have been documented. The eastern olivaceous warbler *I. pallida* (Fig. 5) represents a new host species for *Icosta minor* and *Ornithoica turdi*, while the willow warbler *P. trochilus* (Fig. 6) represents a new host species for *Ornithophila gestroi*. Interestingly, *O. gestroi* was known only from hosts belonging to the families Falconidae and Accipitridae until now (Jentsch et al., 2021b).

Considering the occurrences of Hippoboscidae in other European countries, it is evident that the list of species in Romania is still incomplete, despite its richness. At least the following three species can be expected to be found in Romania: *Lipoptena cervi* (Linnaeus, 1758), *Ornithomya biloba* Dufour, 1827, *Pseudolynchia canariensis* (Macquart in Webb & Berthelot, 1839), and possibly others.

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## Research article

## Distribution of the grass snake (*Natrix natrix*) and dice snake (*N. tessellata*) in Bulgaria

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**Abstract:** We summarise the distribution of the two species of the genus *Natrix* occurring in Bulgaria, based on records from 147 peer-reviewed publications, grey literature, and data repositories, combined with unpublished data. This is the first extensive mapping for *N. natrix*; records fall in 560 cells of the 10-km MGRS/UTM grid, of which 102 cells (18.2%) were with published information we could not confirm with new data, 175 (31.2%) were with published and confirmed, and 283 (50.5%) were with new localities. For *N. tessellata* we increased the number of cells with records by 64% compared to the 2011 mapping, by identifying 445 cells with localities: 162 cells (36.4%) were previously published and unconfirmed, 152 (34.1%) were published and confirmed, and 131 (29.4%) were with new data. Gross climatic conditions for records with exact locations were assigned following the Köppen-Geiger classification; the distribution for both species does not seem to be highly correlated to climate as they were found in 9 of the 12 Köppen-Geiger classes present, only missing from the 3 classes that are limited to high elevations in Bulgaria and account for less than 1% of the area. The vertical distribution of the observations supports our knowledge that the species are most numerous at lower elevations (92.4% of records were <1000 m above sea level for *N. natrix* and 92.6% were <500 m for *N. tessellata*). Higher elevations and some lowlands remain relatively understudied and future sampling will likely reveal new localities for both species.

**Keywords:** Balkan Peninsula, elevation, mapping, range, Reptilia, Serpentes

## Introduction

The semi-aquatic common grass snake, *Natrix natrix* (Linnaeus, 1758), and the dice snake, *N. tessellata* (Laurenti, 1768), are widely distributed within the Palaearctic across a range of terrestrial and aquatic habitats (lotic, lentic, freshwater to saline; Sillero et al., 2014). They can be easily observed, especially where they attain high local densities (Speybroeck et al., 2016). Aspects of their biology, ecology, and genetics have been extensively researched in Europe (see Gruschwitz et al., 1999; Kabisch, 1999 and references therein; Asztalos et al., 2021).

The two study species are among the most common, abundant and widespread ophidian species in Bulgaria (eastern Balkans) (Stojanov et al., 2011). Some notable publications on their distribution include one of the first national mapping efforts by Buresch & Zonkow (1934), and biodiversity assessments of more limited territories, e.g., Eastern and Western Rhodopes (Petrov et al., 2001, 2006), Natura 2000 protected areas “Ponor” and “Besaparski Ridove” (Popgeorgiev et al., 2010, 2014a, 2014b) and “Oranovski Prolom-Leshko” (Malakova et al., 2018), Vitosha Mountains (Tzankov et al., 2014), Vratschanska Planina Mountains (Naumov et al., 2016), and the Bulgarian part of the Lower Danube River (Popgeorgiev et al., 2019). Multiple other publications provide limited reports. Naumov et al. (2011) published a current state of the knowledge on the distribution of *N. tessellata* in Bulgaria, but this was prior to an increase in the number of active field researchers and a much improved and centralised data collection through the means of the SmartBirds.org system. No recent and exhaustive national mapping of *N. natrix* has been made.


However, the perceived abundance and commonness of the study species often lead to field observations about them being unrecorded, even by herpetologists. Thus, the genus *Natrix* remains understudied and under-reported in Bulgaria, including aspects of the distribution and ecological requirements. Therefore, the main aim of this study was to combine published and unpublished observations to produce a comprehensive and up-to-date database on the distribution of the two *Natrix* species in Bulgaria. Our goals were to 1) update the distribution of *N. natrix* and *N. tessellata* on a 10×10 km UTM grid, 2) evaluate their vertical distribution, and 3) evaluate their occurrence within the Köppen-Geiger climatic classes.

## Materials and methods

Bulgaria covers ca. 111000 km<sup>2</sup>, containing 970 whole and 287 partial cells of the 10×10 km Military Grid Reference System grid (MGRS; spatially identical with UTM). It encompasses diverse eco-physiographic conditions and habitats. Elevation ranges from 0 to 2925 m a.s.l., separated into five hypsometric belts: lowlands (0–200 m, 31.4% of the territory), hills (200–600 m, 41.0%), low mountains (600–1000 m, 15.3%), mountains of average height (1000–1600 m, 9.8%) and high mountains (>1600 m, 2.5%) (Simeonov & Totzev, 1997). The climate is dominated by Mediterranean, oceanic, and continental influences, with 12 Köppen-Geiger climate classes identified (Beck et al., 2018).

In Bulgaria the study species reach 114 cm for *N. tessellata* and 163 cm for *N. natrix* (Naumov et al., 2020). *Natrix natrix* has a ubiquitous distribution, predominantly at lower elevations, but reaching up to ~2000 m (Buresch & Zonkow, 1934; Naumov & Tomović, 2005); no quantified vertical distribution has been published. *Natrix tessellata* is also widespread, with ~85% of the known localities in Bulgaria occurring below 500 m, and only two observations recorded above 1100 m – the highest being at 1420 m from Rila Mountains (Naumov et al., 2011; Tzankov et al., 2011). Both species inhabit diverse fresh waters (e.g., streams and river courses, temporary and permanent ponds, spills, natural and artificial lakes, reservoirs, marshes, canals, etc.), as well as brackish ones, such as river mouths at the Black Sea (Stojanov et al., 2011).

For analyses, text data were stored and manipulated with a spreadsheet software (LibreOffice Calc v. 6.4, The Document Foundation, Germany). Spatial data were manipulated, visualised, and analysed using QGIS (v. 3.26, the Open Source Geospatial Foundation, USA). Maps were made using ArcGIS 10.3.1 (ESRI, Redlands, CA, USA).

We combined locality data from the following sources, accessed in March 2023: 1) Personal observations of the authors and colleagues, either requested directly or kindly provided with permission from the users of the field data collection system <https://SmartBirds.org> , comprising a mobile application (SmartBirds Pro) and a web-based interface; 2) Over 1000 publications (including dissertations, reports, and other grey literature) with information on the Bulgarian herpetofauna, spanning

from 1892–2022; 3) Data from GBIF.org, that aggregates a number of sources including iNaturalist, specialised Facebook groups, etc.; 4) Collection data from the Zoological Research Museum Alexander Koenig, Germany (ZFMK), and the California Academy of Sciences, USA (CAS). Records from the open data repositories (GBIF, ZFMK, CAS) were considered as “published”.

We collated the records in a database, where a record usually represents an observation of one or more snakes of the same species in the same place at a given time. We carried out a quality assessment of the location accuracy, species identification, and other available details for each record manually, and removed the dubious data. We also manually identified and removed duplicate records (e.g., observations in SmartBirds.org also available in a publication; identical records from GBIF.org and CAS; observations with the same coordinates and time entered twice by two observers). Locality data that remained were thus assigned into one of three categories: ‘exact locality’ (accuracy within 50 m), ‘approximate’ (accuracy 51–250 m in GBIF, or only a UTM-grid identifiable), or ‘unclear’ (not used in spatial analyses; the accuracy was less than 250 m, or the locality could not be identified unambiguously, e.g., “Kresna gorge”).

Records with exact geographic coordinates were either obtained by using handheld GPS units (including mobile phones) during the observation or were digitised at a later date using georeferenced aerial/satellite images and detailed locality description. Using QGIS, observations with exact coordinates were assigned an elevation based on a Digital Elevation Model (DEM) with a pixel size of 40 m, a 10-km UTM cell, and the Köppen-Geiger climatic class (available from Beck et al., 2018; due to missing data in the original raster from the Black Sea Coast, some observations were manually assigned one of the two classes present locally).

From published data lacking exact geographic coordinates but with a locality description, we considered as a separate record every original description of a location where one or more individuals were found and which was different (e.g., date, count, observer) from other descriptions in the publication. When names of settlements or geographic objects were given as reference points for the locations, we used digital and paper maps to assign these as best as possible to a UTM 10×10 km grid using the MGRS naming

of cells (UTM zone 35N, datum WGS 1984). When possible, we used unpublished data from the original observers to clarify the locality. Some presence records were already provided as cells in the 10-, 5-, 2-, or 1-km UTM/MGRS grid; few records had precise coordinates, especially before ~2010, when handheld GPS units became more widely available and used locally. We also assigned, if possible, an elevation (“exact”, or within a 100- and 500-m band) using maps.

The raw data varied greatly in quality and available details as they have been collected by multiple people, either in a non-systematic way or systematically, but within a limited geographic area and usually within a short time. Also, areas of special interest to herpetologists and tourists (e.g., the Black Sea Coast, Struma River Valley) and those near the major cities tended to be overrepresented. Although this biases the data, our field experience is that the data is partially reflective of the real situation (e.g., in terms of vertical distribution). We considered each record as a single individual and disregarded counts if provided. We have not removed records based on proximity to other records.

## Results and discussion

We analysed 4368 records in total, 2398 of *N. natrix* (‘exact locality’: 1627, ‘approximate locality’: 693, ‘unclear locality’: 79) and 1970 of *N. tessellata* (1129, 774, and 67, respectively). We identified 147 publications, containing 964 records for *N. natrix* and 986 for *N. tessellata*; previously unpublished records were 1434 and 984, respectively. We managed to assign the year of observation to a decade for 3686 records, with ~55% of those being from 2011–2020, and ~18% post-2021 (Table 1).

The increase in the amount of data positively correlates to an increased search effort by more experts, more unified and easily accessible data collection, and an increased number of publications, and is unlikely to signify increases in the range or population densities overall.


Altogether, we managed to place observations of *N. natrix* into 560 10-km UTM cells, based on 2320 records (Fig. 1; [Supplementary material 01](#) ) . Of these, 102 cells (18.2%) were previously published and we did not find unpublished data for, 175 (31.2%) were published and confirmed with unpublished data,

Table 1. Distribution of records with identified years of observations for *Natrix natrix* and *N. tessellata* in Bulgaria, per decade.

Decade	<i>N. natrix</i>		<i>N. tessellata</i>		Total	
	#	%	#	%	#	%
1881–1890	1	0.0	–	0.0	1	0.0
1891–1900	3	0.1	–	0.0	3	0.1
1901–1910	3	0.1	–	0.0	3	0.1
1911–1920	3	0.1	2	0.1	5	0.1
1921–1930	39	1.9	27	1.7	66	1.8
1931–1940	11	0.5	19	1.2	30	0.8
1941–1950	–	–	–	–	–	–
1951–1960	17	0.8	6	0.4	23	0.6
1961–1970	40	1.9	39	2.5	79	2.1
1971–1980	22	1.0	18	1.1	40	1.1
1981–1990	31	1.5	25	1.6	56	1.5
1991–2000	106	5.0	100	6.3	206	5.6
2001–2010	288	13.7	212	13.4	500	13.6
2011–2020	1171	55.8	849	53.5	2020	54.8
2021–	365	17.4	289	18.2	654	17.7
<b>Total</b>	<b>2100</b>	<b>100.0</b>	<b>1586</b>	<b>100.0</b>	<b>3686</b>	<b>100.0</b>

and 283 (50.5%) were new. For *N. tessellata*, we identified 445 UTM cells, based on 1903 records; of these, 162 cells (36.4%) were published, 152 (34.1%) were confirmed, and 131 (29.4%) were new (Fig. 2; [Supplementary material 01](#) [↗](#)).

We stipulate that continued and more intensive sampling in areas currently lacking reports of the species (especially at lower elevations) would yield new localities. Several such regions with limited data for both species exist that seem potentially suitable based on expert knowledge and Maxent models of their potentially suitable habitats (Kornilev et al., in press): the Danubian Plain, the central and eastern Thracian Lowland, and the Ludogorie and Dobrudzha Regions. For example, Naumov et al. (2011) hypothesised that the lack of records for *N. tessellata* from Ludogorie and Dobrudzha Regions (square NJ; data for NJ08, NJ18, NJ80, NJ90; see op. cit. fig. 1 with distribution data and fig. 2 for names of major geographic objects referred to in the text) was most likely due to lack of sampling; here we provide data from two previously unpublished cells (NJ20, NJ44), supporting the need for additional sampling to reveal

new localities in this part of the country. This is further reinforced when considering that the herpetofaunal data in SmartBirds.org for the area is lacking for a number of common species expected to be found there. Additionally, here we update the known distribution for both species from the vicinity of the Danube River, updating the recent mapping effort along the river (Popgeorgiev et al., 2019). The geographic scope of the previous publication was limited to 10 km from the Danube, and most of the data were from projects targeted at a few protected areas and focusing on the river itself. We hypothesise that the species are distributed all along the Danube and its tributaries, supported by the potential distribution models (Kornilev et al., in press).

Overall, most observations of *Natrix* spp. were made close to big cities (e.g., Sofia, Plovdiv, Burgas), close to roads, and around herpetologically popular sites and ones where specific studies were made (e.g., the Struma River Valley, the Eastern Rhodopes, and the Black Sea Coast) and within Natura 2000 sites where specific surveys were made as part of scientific/conservation projects.

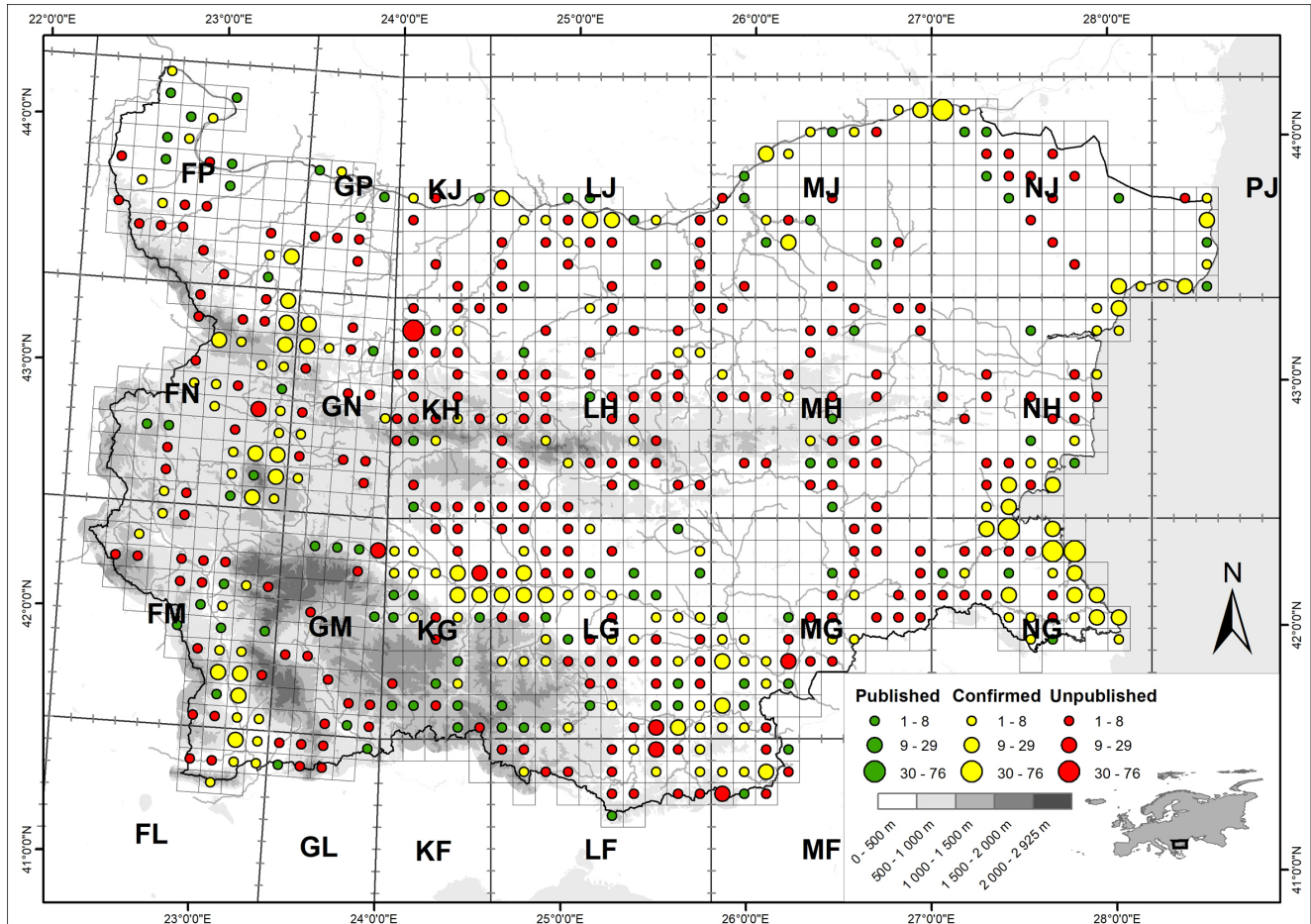


Fig. 1. Distribution of *Natrix natrix* in Bulgaria, based on a 10-km MGRS grid. The colour and size of the circles denote the source of the data (published/confirmed/unpublished) and the number of records per cell, respectively.

Improving our knowledge on the distribution of common species can aid in identifying areas that are likely undersampled and can thus help obtaining further data on rare species. To collect a large database with observations, it is recommended to use modern methods for recording, managing, and sharing data, such as GBIF or SmartBirds.org. Since 2016, when SmartBirds.org became operational, over 33000 records have been submitted to it. Still, a way to fill knowledge gaps in distribution and minimise some of the collection bias is to increase the amount of data obtained through non-professional biologists. One such underutilised data source in Bulgaria is citizen science – which can be really helpful in obtaining distribution data, especially from locations outside of protected territories that usually are not sampled professionally. Online social networks, that could be used for citizen science, can provide new and interesting data (e.g., Naumov et al., 2020a).

In Bulgaria, both species were found predominantly at lower elevations, especially at 0–100 m, with observations rapidly decreasing with the increase of elevation (Fig. 3). *Natrix tessellata* is mainly observed below 500 m a.s.l. (over 92% of the records), closely matching the 85% reported in Naumov et al. (2011). *Natrix natrix* reaches higher elevations and multiple records are found up to 1500 m; only 16 records exist above that. For both species, the highest elevations continue being previously published records. For *N. natrix*, it is the observation in Naumov & Tomović (2005), reported there at around 2100 m; however, updated precise coordinates by BN and subsequent elevation estimation puts this record at ~2030 m. For *N. tessellata*, the highest observation was at 1420 m in the Rila Mountains (Tzankov et al., 2011). Six additional records exist above 1000 m: up to 1100 m on the SW slopes of the Pirin Mountains (Beschkov, 1961), 1034, 1055, 1056,

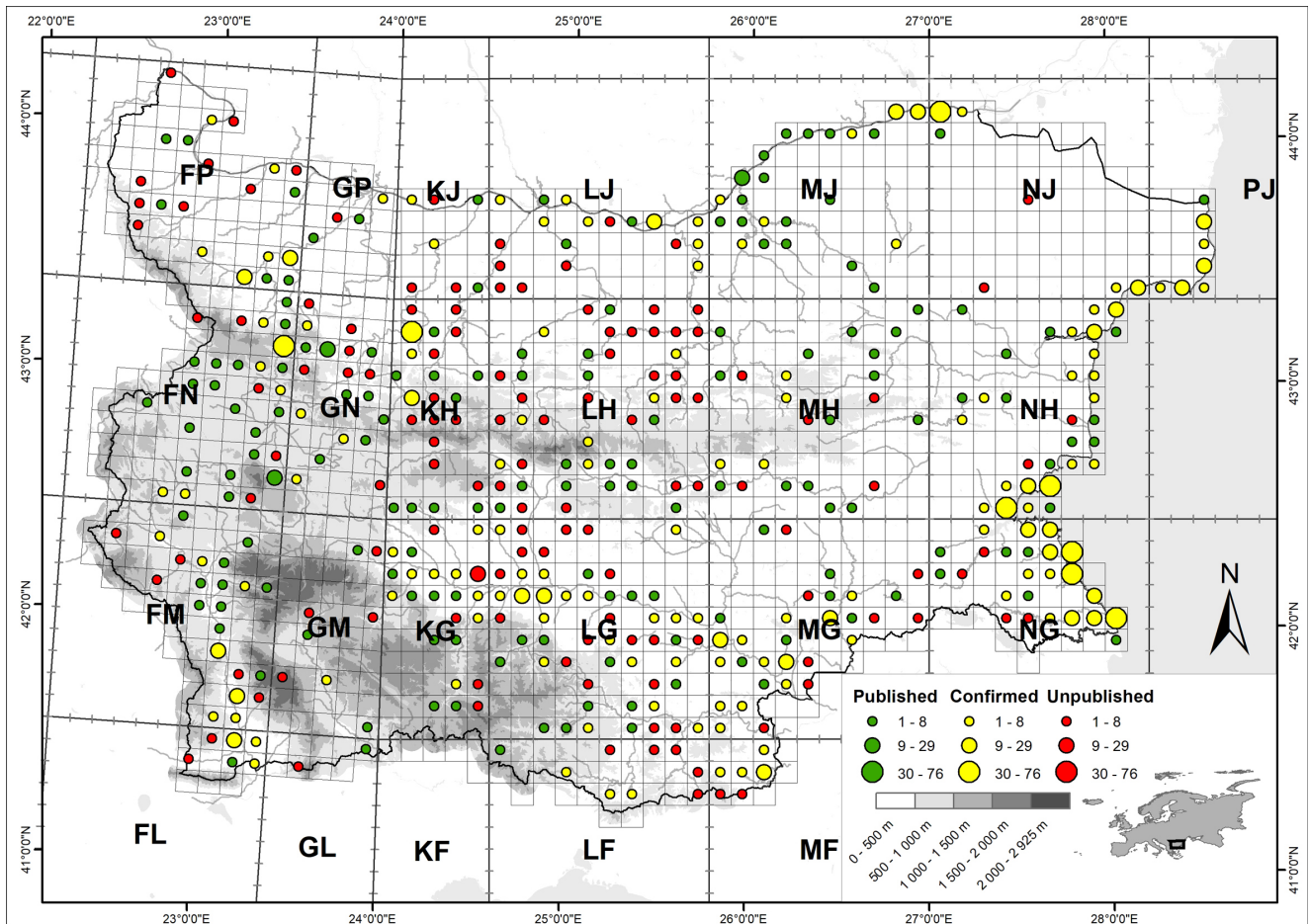


Fig. 2. Distribution of *Natrix tessellata* in Bulgaria, based on a 10-km MGRS grid. The colour and size of the circles denote the source of the data (published/confirmed/unpublished) and the number of records per cell, respectively.

and 1090 m on the SW slopes of the Vitosha Mountains (N42.4975°, E23.2143°; N42.4970°, E23.2176°; N42.4972°, E23.2190°; N42.4964°, E23.2293°; observed in 2015–2017 by AD), and 1128 m in the Western Rhodopes (N41.8436°, E24.8866°; observed in 2019 by MS).

Out of the 12 available Köppen-Geiger climatic classes in Bulgaria, 1627 *N. natrix* and 1129 *N. tessellata* records were attributed to territories belonging to all but three classes with very little areas, which also correspond to high elevations (Dsc, ET, Dsa) (Table 2; Fig. 4). Generally, the classes with larger area account for higher proportions of the records. Some clear exceptions exist. BSk represents only 7.4%, while harbouring 16.8% and 33.7% of the records of *N. natrix* and *N. tessellata*. These records are clustered along the Struma River valley in the SW and along the northern Black Sea Coast. Both of these areas are also highly popular herpetological spots and

generally support high-density populations at many locations, which leads to detection bias.

Although the climatic preferences of both species remain understudied, environmental niche models for Bulgaria revealed limited impacts of temperature on their potential distribution (Kornilev et al., in press); although elevation explained 20–24% for both models and precipitation variables contributed ~15%; these likely relate to the presence of water bodies. Furthermore, the northernmost populations of the dice snake are likely the result of recent colonisation during the Holocene climatic optimum, demonstrating geologically rapid response to climatic changes (Marosi et al., 2012). Their extensive ranges further support that both species are environmental generalists that could thrive in diverse climatic zones.

Knowledge of the distribution of *Natrix* species is important not only for their long-term survival, but because of their ecological role. The diet of *N. natrix*



Distribution of the grass snake (*Natrix natrix*) and dice snake (*N. tessellata*) in Bulgaria

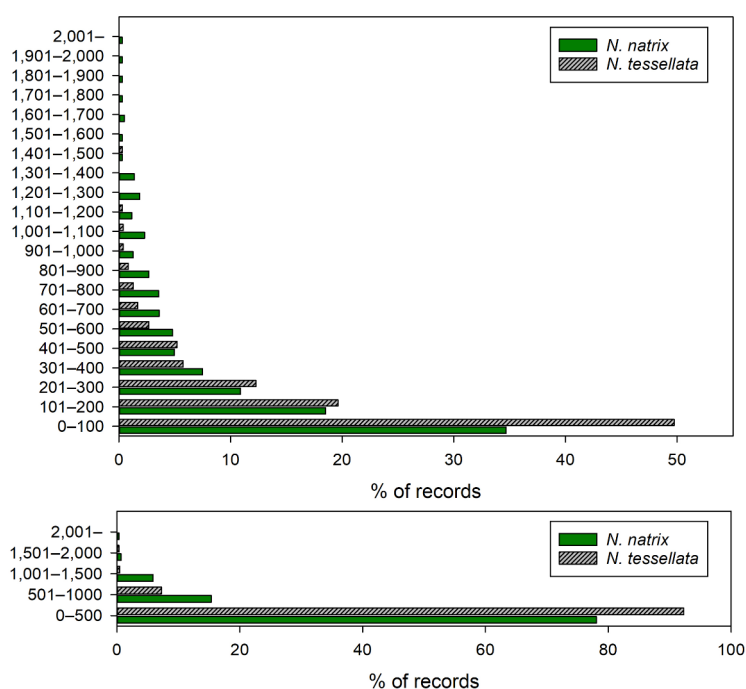


Fig. 3. Altitudinal distribution in metres of *Natrix natrix* and *N. tessellata* in Bulgaria, based on a) 1831 and 1305 records attributable to a 100-m band (top); and b) 2180 and 1710 records, respectively, attributable to a 500-m elevation band (bottom). For visualisation purposes, percents less than 0.3% were increased to 0.3%.

Table 2. Percent distribution of records of *Natrix natrix* (N = 1627 records) and *N. tessellata* (N = 1129) within the available territory of each Köppen-Geiger (K–G) climatic class in Bulgaria (BG, %).

K–G class		BG	<i>N. natrix</i>	<i>N. tessellata</i>
Dfb	Cold, no dry season, warm summer	34.6	23.7	12.7
Dfa	Cold, no dry season, hot summer	28.7	18.4	21.7
Cfa	Temperate, no dry season, hot summer	23.3	33.8	28.1
BSk	Arid, steppe, cold	7.4	16.8	33.7
Csa	Temperate, dry summer, hot summer	2.7	6.0	3.2
Dfc	Cold, no dry season, cold summer	1.0	0.4	0.0
Dsb	Cold, dry summer, warm summer	0.7	0.3	0.2
Cfb	Temperate, no dry season, warm summer	0.7	0.2	0.2
Dsc	Cold, dry summer, cold summer	0.4	0.0	0.0
ET	Polar, tundra	0.3	0.0	0.0
Csb	Temperate, dry summer, warm summer	0.2	0.4	0.3
Dsa	Cold, dry summer, hot summer	0.0	0.0	0.0

on the Balkans is diverse, with adults feeding predominantly on amphibians and fish, while *N. tessellata* consumes mostly fish (Šukalo et al., 2014;

Speybroeck et al., 2016). Locally, both species can reach potentially high densities – for example, up to 5800 *N. tessellata*/~18 ha island (Ajtić et al., 2013).

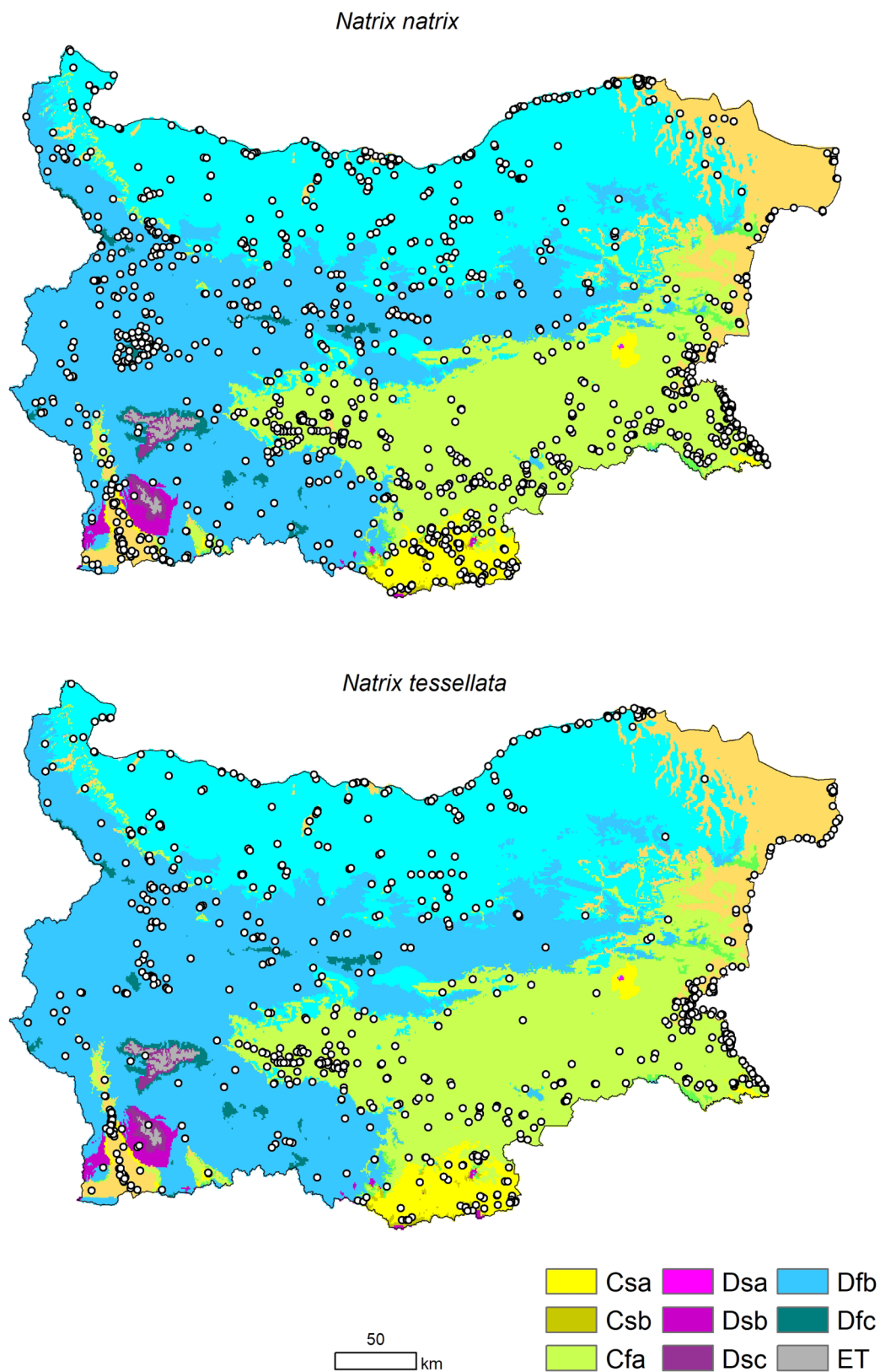


Fig. 4. Indicative distribution of *Natrix natrix* (top; N = 1627) and *N. tessellata* (bottom; N = 1129) with exact locality within the Köppen-Geiger climatic classes (see Table 2 for class' descriptions) in Bulgaria.

On the other hand, the snakes are prey for a number of avian and mammalian predators, some of which might be protected. Therefore, coupled with their ectothermic biology, this makes *Natrix* an important component of food webs and provide an ecological service by aiding transfer of energy from aquatic to terrestrial environments.

In conclusion, the new data on the distribution of the two species, confirm our understanding that in Bulgaria the ranges of *N. natrix* and *N. tessellata* are continuous and largely overlap. A major limiting factor seems to be elevation, which generally correlates with local climate conditions. A more detailed comparison between the distribution of the two species could be obtained as a result of specific studies on their habitat preferences and requirements.

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## Supplementary materials

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