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First Russian record of *Erpobdella monostriata*: DNA barcoding and geographical distribution

(Annelida, Hirudinida, Erpobdellidae)

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New information on the range of *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890) was obtained. Results of our study show that this leech occurs not only in Central Europe but reaches European Russia. However, *E. monostriata* has not been recorded from the vast interspace between its localities in Central Europe and a dystrophic lake in the Voronezh Oblast in Russia, the easternmost spot of its range known so far. The taxonomic identity of the leeches was confirmed both by a morphological examination and a molecular phylogenetic analysis based on COI sequences. Genetic differentiation between western and eastern samples of the species points towards exploring more specimens and markers. If not disjunct, the present geographical gap of records may be explained by low population sizes and the scarcity of suitable habitats; recent climate changes might cause population expansions in the east.

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Introduction

Erpobdellid leeches are common members of freshwater benthic communities. They are macrophagous predators of aquatic invertebrates originating from hematophagous ancestors (Siddall 2002). Some of the species in this group have been used in ecological studies and water quality assessment (Lukin 1976, Schenková et al. 2007, Nesemann & Moog 2002). The classical systematics of erpobdellids was based on the annulation of segments. In the molecular phylogenetic era, the importance of annulation has been reconsidered and the genus *Erpobdella* sensu stricto has been found non-monophyletic (Siddall 2002). Further phylogenetic analyses and taxonomic revisions are needed to reveal the evolutionary history of erpobdellids and resolve taxonomic uncertainties.

The taxonomy of a number of common erpobdellid species has been confused or neglected. The taxonomic identity of *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890) was confused for a long time since its formal description. This taxon was initially described as *Nephelis octoculata* var. *monostriata* Lindenfeld & Pietruszynski, 1890. Then the preoccupied epithet "*monostriata*" was employed again to designate another leech taxon, *Herpobdella* *atomaria* var. *monostriata* Gedroyć, 1916, which is currently recognized as a synonym of the correct designation of this species, *Erpobdella vilnensis* (Liskiewicz, 1925) (Agapow & Bielecki 1992, Nesemann & Neubert 1999).

In his seminal monographs, Lukin (1962, 1976) mentioned only "*Erpobdella monostriata*" (Gedroyć, 1916) for the Carpathians and adjacent areas and did not record *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890) for Ukraine and the former USSR. Recently Utevsky et al. (2012) have recorded *Erpobdella vilnensis* (Liskiewicz, 1925) from the Ukrainian Carpathians and south-western Ukraine and by doing so fixed the nomenclatural problem of confusing *E. vilnensis* with *E. monostriata* at the putative eastern border of the range of the former. At the same time, the geographical distribution of *E. monostriata* is still poorly studied. It has been unclear how far its range extends to the east. Until recently *Erpobdella monostriata* was not found in the former Soviet Union (Lukin 1962, 1976) and newly independent countries of Eastern Europe. This research addresses the information gap in our knowledge of the geographical distribution of *E. monostriata.*

Material and methods

Sample collection

Samples of *E. monostriata* were collected during the summer season of 2011 and on 16th June, 2013 from a small forest lake Ugolnoe in the Usman Pine Forest in the Voronezh Oblast, Russia near the village of Maklok (51°48'40.20"N, 39°24'23.58"E), relaxed in 10 % ethanol, fixed and preserved in 96 % ethanol. Altogether, 37 individuals were studied by using morphological and molecular methods. A newly sequenced specimen of *Erpobdella vilnensis* from the Mykolayiv Oblast, Ukraine (see Utevsky et al. 2012) was also used for the analysis. The specimens are stored in the collection of invertebrates of the Department of Zoology and Animal Ecology, V. N. Karazin Kharkiv National University.

Measurements

Measurements were made with an eyepiece micrometer. Arithmetical means of the body length and width and their standard errors were calculated. The measurements were based on 32 specimens.

Mapping

Geographical coordinates were assigned to the new and previous localities where *E. monostriata* was found. Agapow & Bielecki (1992) and later publications were considered. In the field, the geographical position was determined using a global positioning system (GPS) device. In other cases, localities were identified according to the description in the literature. The Google Earth (version 4.3) online application (http://earth.google.com/) was used. The record points were plotted on a map using QGIS 2.0.

DNA extraction, amplification and sequencing

Small pieces (approx. 5 × 2 × 1 mm) of skin and muscle tissue were cut from the lateral part of the body. Care was taken not to reach the digestive system, which often contains remnants of unknown prey species. Genomic DNA was isolated using the GENE ELUTE Mammalian Genomic DNA minprep kit from Sigma-Aldrich (Steinheim, Germany).

The mitochondrial cytochrome oxidase subunit one (COI) fragment was amplified using LSO and HCO primers described in Folmer et al. (1994). PCR was performed by applying 35 cycles of 1 min at 94 °C, 1 min at 46 °C, and 2.5 min at 72 °C, following a 4 min denaturation step at 94 °C. In order to purify the PCR products two enzymes, Exonuclease I and Shrimp alkaline phosphatase (SAP) (Fermentas, Thermo Fisher Scientific, USA), were used. Exonuclease I (0.2 µl) and SAP (1 µl) were added to 10 µl of the PCR product. After that, the mixture was incubated for 45 min at 37 °C and followed by a 15 min incubation at 80 °C. The purified products were sequenced in both directions with amplification primers under BIG DYE Terminator cycling conditions, purified by ethanol precipitation, and run on an Applied Biosystems 3730xl sequencer by Macrogen (Seoul, Korea). Sequence chromatograms were edited and assembled with the help of ChromasPro 1.32 (Technelysium Pty., Queensland, Australia). The correctness of COI sequences was verified at the amino acid level. The obtained sequences were assigned GenBank Accession Numbers. Some COI sequences belonging to other erpobdellid species from previous studies were also used for inferring a phylogenetic tree (Table 1).

Phylogenetic analysis

The COI sequences were unambiguously aligned using ClustalW associated with BioEdit v7.1.7 (Hall 1999). The length of the aligned COI sequences was 660 bp. The best-fit models for each partition, the first, second and third codon positions of COI, were identified with the Bayesian information criterion (Schwarz 1978) using KAKUSAN4 (Tanabe 2011): GTR with proportion of invariant sites (+I), HKY85+I and HKY85 with gamma distribution (+G) respectively.

Phylogenetic relationships were determined by Bayesian inference using MrBayes v3.1.2. (Ronquist & Huelsenbeck 2003). Two arhynchobdellid species *Hirudo orientalis* Utevsky et Trontelj, 2005 (Hirudiniformes) and *Orobdella jimai* Oka, 1895 (Erpobdelliformes) were used as outgroup taxa to reveal the phylogenetic relationships between erpobdellid taxa. Searches were performed in two parallel runs with four chains each for five million generations, sampled every 100th generation. After discarding the first 25 % of the sampled trees, final topologies were consented following the 50 %

majority rule. Numbers of base differences per site (*p*-distances) and their standard errors were calculated using MEGA5 (Tamura et al. 2011).

Habitat

The basin of the Ugolnoe Lake is a former riverbed of a left-bank tributary of the Voronezh River, ceased to exist during the formation of the estuary of the Usman River during an activation of the Voronezh neotectonic uplift (Khlyzova et al. 2007). The name "ugolnoe" comes from a facility that was located here in the late 19th and early 20th centuries to produce birch charcoal. According to hydrochemical characteristics determined by Zhivotova & Koroteeva (2002), the lake should be classified as a dystrophic (humic) water.

Forest wildfires of 2010 had a significant impact on the hydrochemical and hydrobiological regime of waters of the Usman Pine Forest, including the Ugolnoe Lake. It is known that after wildfires the absolute and relative (compared to nitrogen) content of organic carbon and a number of ions increased in surface waters due to the accumulation of ash and fly ash from the catchment area; the balance of biogeochemical fluxes changes due to dramatic alterations in vegetation and soil permeability (Bitner et al. 2001, Meyer et al. 1995, Strauss & Lamberti 2000).

Results

Morphology

External characters (Fig. 1) of the specimens examined are in agreement with the previous description of *E. monostriata* by Nesemann & Neubert (1999). The leeches are small, the average total length including suckers is 15.13 ± 0.56 mm (range 8.50–21.00 mm). The maximum body width is 2.16 ± 0.12 mm (range 1.50–5.20 mm). The body surface is smooth, papillae are lacking. A mid-body segment consists of five annuli equal in length. The gonopores are separated by four annuli. The coloration of living leeches is light brownish. All preserved specimens have a mid-dorsal dark longitudinal stripe located along

Table 1. Collection sites and sequence accession data for the leech species analysed.

Species	Collection site	GenBank accession No.	Reference
Erpobdella monostriata (Lindenfeld & Pietruszynski, 1890)	Voronezh Oblast, Russia	KP300764	This study
Erpobdella monostriata (Lindenfeld & Pietruszynski, 1890)	Mecklenburg-Western Pomerania, Lake Neustädter See, Germany	HM246601	Trajanovski et al. 2010
Erpobdella monostriata (Lindenfeld & Pietruszynski, 1890)	Magdeburg, Germany	DQ009665	Pfeiffer et al. 2005
Erpobdella nigricollis (Brandes, 1899)	Kassel-Wehlheiden, Germany	DQ009664	Pfeiffer et al. 2005
Erpobdella nigricollis (Brandes, 1899)	Mecklenburg-Western Pomerania, Lake Neustädter See, Germany	HM246603	Trajanovski et al. 2010
Erpobdella testacea (Savigny, 1820)	France	AF116027	Apakupakul et al. 1999
Erpobdella vilnensis (Liskiewicz, 1925)	A stream flowing to the Southern Bug, Mykolayiv Oblast, Ukraine	KP300763	This study, Utevsky et al. 2012
Erpobdella vilnesis (Liskiewicz, 1925)	Wörlitzer Park, Germany	DQ009663	Pfeiffer et al. 2005
Erpobdella vilnensis (Liskiewicz, 1925)	Mecklenburg-Western Pomerania, Germany	HM246585	Trajanovski et al. 2010
Erpobdella octoculata (Linnaeus, 1758)	Uzbekistan	HQ336344	Oceguera-Figueroa et al. 2011
Erpobdella octoculata (Linnaeus, 1758)	Lake Ohrid, Macedonia	HM246555	Trajanovski et al. 2010
Erpobdella octoculata (Linnaeus, 1758)	Lake Prespa, Macedonia	HM246599	Trajanovski et al. 2010
Dina ohridana Sket, 1968	Lake Ohrid, Albania/Macedonia,	HM246594	Trajanovski et al. 2010
Dina lepinja Sket & Šapkarev, 1986	Macedonia, Lake Ohrid	HM246539	Trajanovski et al. 2010
Dina lineata (O. F. Müller, 1774)	Mecklenburg-Western Pomerania, Germany	HM246611	Trajanovski et al. 2010
Trocheta haskonis Grosser, 2000	Wörlitzer Park, Germany	DQ009668	Pfeiffer et al. 2005
Orobdella jimai Oka, 1895	Nagano, Japan	AB679674	Nakano 2012
Hirudo orientalis Utevsky & Trontelj, 2005	Azerbaijan	JN104645	Trontelj & Utevsky 2012

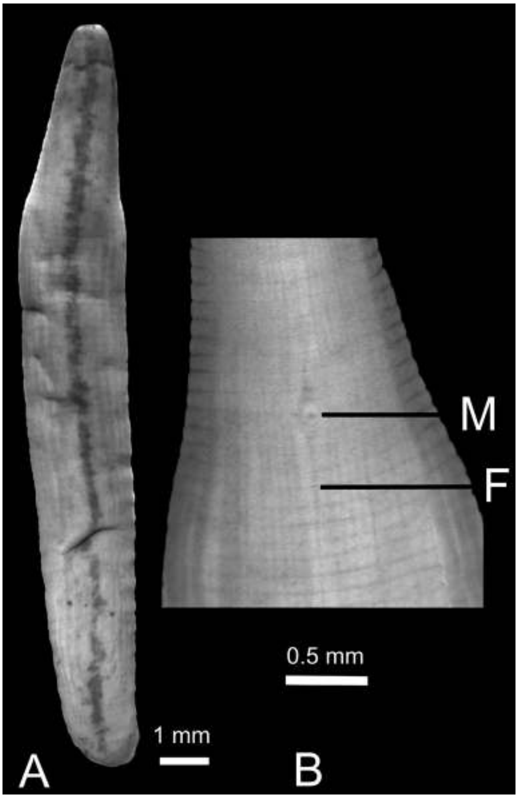

Fig. 1. *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890). **A.** Entire specimen with a characteristic mid-dorsal dark longitudinal stripe. **B.** Clitellum, ventral view, with two gonopores separated by four annuli: M, male gonopore; F, female gonopore.

the entire body without interruption. The stripe is blurred and associated with clouds of dark pigment in many specimens. In most of living leeches, the stripe is indistinct or lacking whereas this distinguishing feature emerges in specimens fixed and preserved in strong ethanol.

Barcoding and phylogeny

The resulting Bayesian tree (Fig. 2) indicates that the sample of the leech specimens collected in Russia joined a clade with two other samples of *E. monostriata* from Germany. The posterior probability of the monophyletic group is 1.00. The *p*-distances between the Russian sample and two sequences HM246601 and DQ009665 are 0.015 ± 0.005 and 0.018 ± 0.006 respectively. The *p*-distance between the two samples originating from Germany is as low as 0.004 ± 0.003. The mean *p*-distance within *E. monostriata* was 0.014 ± 0.005 (Table 2). The distances between the

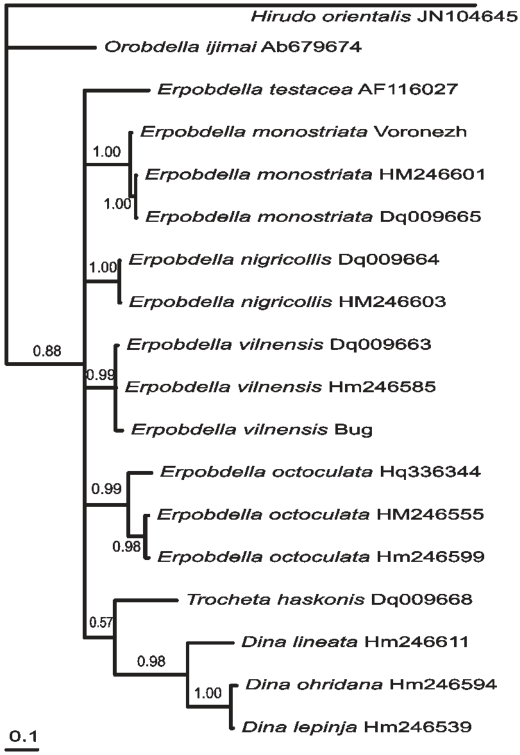

Fig. 2. Phylogenetic relationships between major groups of Palaearctic erpobdellids obtained by Bayesian inference and based on COI sequences. Posterior probabilities are shown for clades. The tree is rooted at *Hirudo orientalis*.

Erpobdella species are shown in Table 3. The sample of *E. vilnensis* collected in south-western Ukraine (Utevsky et al. 2012) joined a well-supported clade with its conspecifics.

Geographical distribution

Altogether 64 localities of *E. monostriata* were considered. Its range reaches as far to the west as 06°22'07.82" in the Netherlands. The easternmost locality is 39°24'23.58", a new record from the Russian Federation. The northern frontier of the explored

Table 2. The number of base differences per site from averaging over all sequence pairs within each *Erpobdella* species (π) and its standard error (based on *p*-distances).

Species	π
E. monostriata	0.014 ± 0.005
E. nigricollis	0.002 ± 0.001
E. vilnensis	0.018 ± 0.006
E. octoculata	0.057 ± 0.022

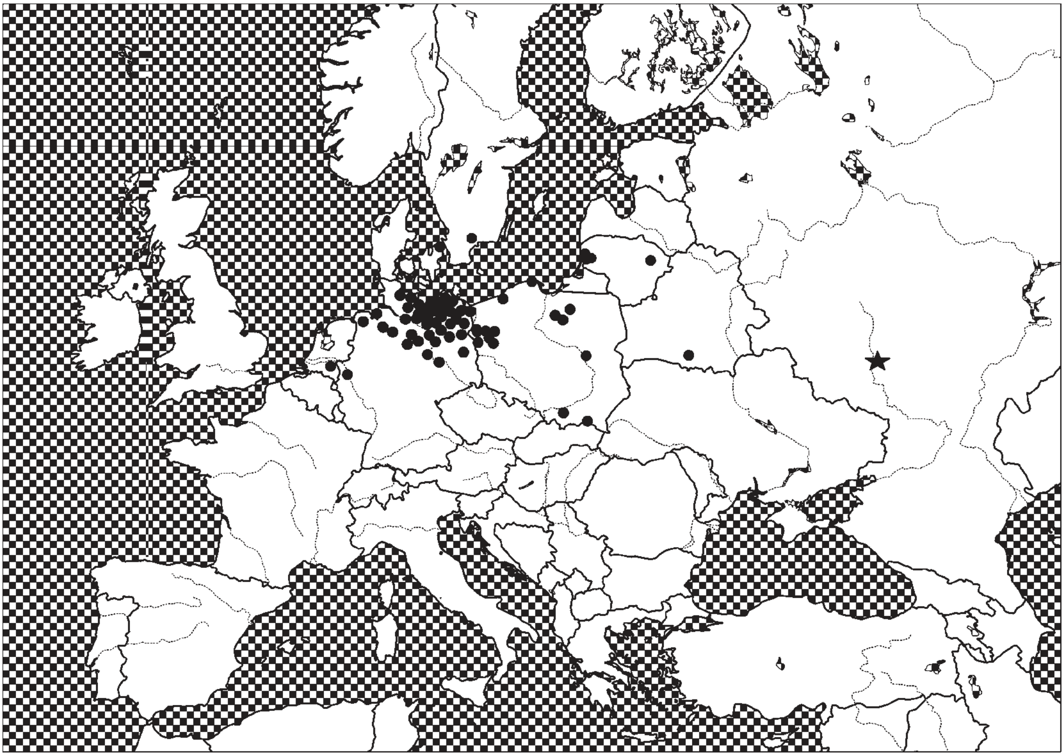

Fig. 3. Geographical distribution of *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890). Previous records are mapped with circles and the new record from Russia is represented by a star.

range of *E. monostriata* is 56°04'08.73" in Sweden (Fig. 3). The southern frontier of the range is still unclear (see below). Reliable records of *E. monostriata* were found for eight European countries: the Netherlands (Haaren et al. 2004), Germany (Grosser 1996, 2003, Jueg 1998, 2013, Jueg et al. 2007, Haesloop 2002, Pfeiffer et al. 2005, Westendorff et al. 2008, Trajanovski et al. 2010, Enting & Arndt-Dietrich 2012), Denmark (Trontelj et al. 1996), Sweden (Uwe Jueg, personal communication), Poland (Agapow & Bielecki 1992, Fleituch 2003, Bielecki et al. 2004, 2011 a, b, Szczęsny 2005, Nabodnik et al. 2004, Agapow et al. 2006, Agapow & Nabodnik 2006, Agapow et al. 2008, Raczyńska & Chojnacki 2009, Koperski 2010, Koszalka 2012), Lithuania (Uwe Jueg, personal communication), Belarus (Haaren et al. 2004) and Russia (this research).

Discussion

Both morphological and molecular analyses showed that the leeches sampled in the Ugolnoe Lake, Voronezh Oblast, Russia should be assigned to *Erpobdella monostriata* (Lindenfeld & Pietruszynski, 1890). The leeches possess a distinguishing feature of the spe-

Table 3. The number of base differences per site from averaging over all sequence pairs between *Erpobdella* species and their standard errors (based on *p*-distances).

Species	1	2	3	4
1 *E. monostriata*				
2 *E. nigricollis*	0.096 ± 0.012			
3 *E. testacea*	0.131 ± 0.013	0.116 ± 0.012		
4 *E. vilnensis*	0.117 ± 0.013	0.107 ± 0.013	0.132 ± 0.013	
5 *E. octoculata*	0.139 ± 0.013	0.117 ± 0.012	0.124 ± 0.012	0.123 ± 0.012

cies, the mid-dorsal dark longitudinal stripe located along the entire body. That coloration pattern has never been found in other *Erpobdella* species. The COI tree recovers a well-supported clade of *Erpobdella monostriata.* The eastern specimen differs by less than 2 % from GenBank sequences of two western *E. monostriata*, but the latter form a strongly supported clade, suggesting geographic structure. Being more than eight times higher than within species differences of *E. monostriata*, the *p*-distance between *E. monostriata* and other *Erpobdella* species ranges from 0.096 to 0.139. This can be considered as reliable evidence of the species status of the *E. monostriata* clade (see Birky 2013). Our analyses also corroborated species-level genetic differences between *E. monostriata* and *E. vilnensis* that went through a long period of nomenclatural confusions (see Agapow & Bielecki 1992). Further analyses dealing with more samples and diverse molecular markers are necessary to reveal accurate intraspecific relationships within *E. monostriata* and its place among other erpobdellid taxa.

Recent studies suggest that ranges of a number of Western Palaearctic leech species extend more easterly then considered before. For example, *Hirudo verbana* Carena, 1820 occurs in Uzbekistan (Utevsky et al. 2010) and *E. vilnensis* has been recorded from south-western Ukraine (Utevsky et al. 2012) and Kyrgyzstan (Jueg et al. 2013). Our research, in turn, indicates that *E. monostriata*, recorded mainly from Central Europe, reaches the Don basin in Russia in the eastern portion of its known range. Records from the Czech Republic, Slovakia, Hungary and Greece (Fauna Europaea 2013) and Spain (Camargo 1992) should be substantiated. According to Vladimír Košel's personal communication, *E. monostriata* does not occur in Slovakia and the Czech Republic. The southernmost recent record has been made in Bulgaria (Moskova & Uzunov 2011). The species was also recorded from the Upper Tisza (Zakarpattia, Ukraine) (Afanasyev et al. 2013). The two latter records were made from mountain rivers that are not typical habitats of *E. monostriata*, which prefers lowland lakes and less often rivers (Agapow & Bielecki 1992, Jueg 2013). Moreover, the records are not based on morphological examinations. Therefore, the southern frontier of the species range remains unclear. *Erpobdella monostriata* has not been found in the vast interspace between its localities in Central Europe and the new locality in the Voronezh Oblast (Fig. 3). It is still unknown whether *E. monostriata* occurs in Ukraine but we may hypothesize that the species could be either overlooked or confused with related erprobdellid leeches between its eastern and western localities recorded in the previous studies. This species might be recorded as *Erpobdella testacea* by Lukin (1929) for the Kharkiv Oblast of north-eastern Ukraine.

There are no impermeable geographical barriers in Eastern Europe that could disrupt the range of *E. monostriata*. However, our analysis revealed a substantial genetic differentiation between western and eastern populations of the species. These differences can be caused by isolation by distance or distinct postglacial colonization histories that have been already reported for freshwater leeches in the Western Palaearctic (Trontelj & Utevsky 2012).

The lack of Eastern European records may be also explained by low population sizes and the scarcity of suitable habitats. According to previous records, in 2000 only one individual of this species designated as "Erpobdellidae sp." was found in the Sinyutino bog situated in the Usman Pine Forest (Prokin & Silina 2007). It might be supposed that recent climate changes, i. e. exceptionally warm and dry springs and summers in 2010 and 2011 (All-Russian Research Institute of Hydrometeorological Information 2013) and forest wildfires (see above) have favoured a population expansion in the Ugolnoe Lake and after that *E. monostriata* could be found during an ecological survey. Moreover, we cannot suppose any invasive pathways and vectors for this species' range expansion. We can further speculate that the environmental changes are not favourable for *E. monostriata* in the long term as this leech prefers colder boreal freshwaters of Europe.

This research suggests that *E. monostriata* occurs from the Netherlands to the European Russian Federation. Its range may extend even more easterly where favourable habitats and climate conditions occur.

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