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Article

First molecularly substantiated records of the pond snail *Ampullaceana fontinalis* in the Siverskyi Donets River Basin, Ukraine (Gastropoda: Lymnaeidae)

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Abstract
Ampullaceana fontinalis (Studer, 1820) has not been considered as a separate species for a long time. Here we present new records of *A. fontinalis* for tributaries of the Siverskyi Donets River in Donetsk and Luhansk regions of Ukraine based on sequences of the cytochrome *c* oxidase gene and morphological characters. The newly generated sequences joined the clade of *A. fontinalis* and entered different subclades within it. The genetic diversity observed in the study area suggests separate evolutionary histories of geographically proximal populations of *A. fontinalis.* Our phylogenetic analysis confirmed species-level monophyletic groups of *Ampullaceana ampla* (Hartmann, 1821), *Ampullaceana dipkunensis* (Gundrizer & Starobogatov 1979), *Ampullaceana balthica* (Linnaeus, 1758), *Ampullaceana lagotis* (Schrank, 1803) and *Ampullaceana fontinalis* (Studer, 1820). The low statistical support of the latter may be attributed to the single-gene phylogeny and may be enhanced by analyzing more loci and samples.

Key words: mollusks, COI, gene, Donetsk, Luhansk, phylogenetic tree, DNA, molecular analysis, sequences.

Introduction

The pond snails (Lymnaeidae) have posed an issue of taxonomic uncertainties for a long time. Based on a recent revision of the Old-World *Radix*-like pond snails, its subfamily Amphipepleinae Pini, 1877 was acknowledged with 10 genera in it (Aksenova et al. 2018). The genus *Ampullaceana* Servain, 1881 includes eight species of this subfamily. Two of them, *A. relicta* (Polinski, 1929) and one unnamed species (*Ampullaceana* sp.), are endemics to Albania and Macedonia (Ohrid and Prespa Lakes). Information about *A. intermedia* (Lamarck, 1822) comes from France and Spain only (Aksenova et al. 2018). The species identity and distribution range of *A. dipkunensis* are still uncertain. Its type locality is situated in northern central Siberia (Kruglov & Starobogatov 1993b), from where it was described (Gundrizer & Starobogatov 1979). According to recent records, its range covers the European north of Russia and Eastern Europe (Poland) (Aksenova et al. 2018). The following three species of the genus *Ampullaceana*, *A. ampla*, *A. balthica* and *A.*

lagotis, are widely distributed in the Northern Palearctic – Europe, Western and Eastern Siberia, and Central Asia (Bank & Neubert 2017; Aksenova et al. 2018; Vinarski et al. 2017; Vinarski et al. 2020; Vinarski & Glöer 2007; Andreeva et al. 2010; Schniebs et al. 2011; Glöer 2002 2019; Welter-Schultes 2012). They have independent species statuses and are accepted as "good species" by both "Eastern European" (Andreeva et al. 2010; Vinarski & Kantor 2016) and "Western European" schools of malacology (Glöer 2002, 2019; Schniebs et al. 2011; Schniebs et al. 2015; Welter-Schultes 2012).

This cannot be said for another, the eighth, species of the genus, *A. fontinalis*. Its species status is accepted mainly by the members of the East European school (Kruglov & Starobogatov 1983, 1993b; Stadnichenko 2004; Khokhutkin et al. 2009; Andreeva et al. 2010; Aksenova et al. 2018). The studies of Western European malacologists do not consider this binomen as an independent species (or even a subspecies) (Hubendick 1951; Jackiewicz 1998; Glöer 2002; Welter-Schultes 2012; Bank & Neubert 2017). Recently, however, molecular genetic evidence that favours its species rank has emerged. Specimens identified as *Lymnaea (Peregriana) fontinalis* sensu Kruglov & Starobogatov (1993) were shown to be distinct from *A. lagotis* and form the separate sister clade of species rank (Aksenova et al. 2018). The type locality of *A. fontinalis* is situated in Switzerland, from where it was described (Studer 1820). The presence of the species there has been confirmed molecularly, as has its distribution east, north-east and south of its type locality up to the northern parts of Western Siberia, Tajikistan and Turkey (Aksenova et al. 2018). Ukraine as a country of the species distribution was not mentioned in that study, although the range described by Aksenova et al. (2018) completely covers the country's territory. This is probably due to the fact that no molecular genetic data (apart from a few sequences from the Crimean Peninsula) confirming the presence of *A. fontinalis* in Ukraine as a separate species (rather than as a part of more inclusive species-level taxa) have been obtained from here. For accurate taxonomic identification and molecular confirmation of the occurrence of the species in eastern Ukraine (Siverskyi Donets River Basin), samples morphologically resembling pond snails of the genus *Ampullaceana* were subjected to phylogenetic studies using sequences of cytochrome *c* oxidase subunit I gene (COI), which was performed for the first time for Ukraine.

Materials and methods

Sample collection

From the river, shoots of aquatic plants were removed; they were examined and the discovered mollusks were collected. Specimens of *A. fontinalis* were sampled in the following locations: 1) 19/05/2019, Donetsk region, Kramatorsk, First Belenkaya River, coordinates: 48º46′23″ N, 37°35′31″ E (one sample, PS3); 2) 06/09/2020, Luhansk region, Severodonetsk, Borovaya River, coordinates: 48º58′07″ N, 38°27′53″ E (two samples – PS42 & PS43). The latter habitat can be seen in Fig. 1. The mollusks were passed through a series of relaxation stages, increasing the concentration of ethanol to 10%, finally fixed in 96% ethanol, and then stored at –25°C in 96% ethanol for molecular genetic analysis. Mollusk specimens (except PS3) together with shells (vouchers) are stored in the collection of invertebrates of the Department of Zoology and Animal Ecology of V. N. Karazin Kharkiv National University.

Morphological examination

Morphological study

The specimens were identified as *Lymnaea* (*Peregriana*) *fontinalis* sensu Kruglov & Starobogatov (1993) based on the lectotype illustration (Forcart 1957; taf. 5, fig. 27; Naturhistorisches Museum der Burgergemeinde Bern, Switzerland).

Photo imaging of the PS42 and PS43 samples was carried out using a Sony L 65V camera with the Focus Stacking function with a step size of 0.2 mm. The resulting layered images were combined into a single image using the Combine ZP Image Stacking Software by Alan Hadley (Fig. 2).

Measurements of the shells were carried out using a caliper. The following parameters of external morphology were measured: shell height (SH), shell width (SW), aperture height (AH), aperture width (AW), curl height (CH), last whorl height without aperture (LHA), last whorl width without aperture (LWA). We examined the structure of the male reproductive system of the mollusks to substantiate its affiliation to *Lymnaea* (*Peregriana*) *fontinalis* sensu Kruglov & Starobogatov (1993) (Kruglov 2005; Vinarski et al. 2020).

Figure 1. Habitat of *Ampullaceana fontinalis* in the Borova River, Luhansk region, Ukraine.

Figure 2. Voucher of PS43 specimen, *Ampullaceana fontinalis*, from the Borova River, Luhansk region, Ukraine.

DNA extraction, amplification and sequencing

The molecular analysis was carried out in the Laboratory of Molecular Biology and Phylogenetics of the Department of Zoology and Animal Ecology, Faculty of Biology, V. N. Karazin Kharkiv National University in May 2019 (sample PS3) and in September 2020 (samples PS42 and PS43). For DNA extraction, small pieces of tissue were taken from the samples. Genomic DNA was isolated using a GeneElute Mammalian Genomic DNA Minprep Kit. A fragment of the mitochondrial COI gene was amplified using the following primers (Folmer et al. 1994): LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward) and HCO2198, 5'-

TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse). PCR was carried out under the following amplification conditions: 94°C for 3 minutes; 5 cycles of 0:30 minutes at 94°C, 1:30 minutes at 45°C, 1:00 minutes at 72°C; 35 cycles of 0:30 minutes at 94°C, 1:30 minutes at 51°C, 1:00 minutes at 72°C; 72°C for 5 min. Amplification products were purified using an enzymatic method using exonuclease I and FASTup phosphatase. The cleaned PCR products were then sequenced in both directions by Macrogen Inc. (the Netherlands) using the same primers as at the amplification stage. The chromatograms of sequences were processed in ChromasPro 1.32 (Technelysium Pty., Queensland, Australia). The length of the newly generated COI sequences was 661–683 b.p.

The resulting sequences were deposited in GenBank (accession numbers are OP115749 for the PS3 sample, OP115750 for the PS42 sample and OP115751 for the PS43 sample).

Phylogenetic analysis

The 100 COI gene sequences closest to the PS42 sample were extracted from GenBank using a BLAST search. The percentage of identity varied from 90.18 to 98.93% in this dataset. In addition, COI sequences of other species of *Ampullaceana*, *A. lagotis*, *A. balthica*, *A. ampla* and others, were included in the analysis (Aguilar, Ogburn & Hines, unpublished; Aksenova et el., 2016; Aksenova et al., 2017; Aksenova et al., 2018; Albrecht et al., 2004; Albrecht et al., 2008; Albrecht et al., 2014; Dewaard, unpublished; Feldmeyer et al., 2015; Grabner et al., 2014; Hebert et al., unpublished; Klussmann-Kolb et al., 2008; Leerhoei, unpublished; Mirfendereski et al., 2021; Nasibi et el., unpublished; Paltser et al., unpublished; Pantoja et al., 2021; Patel et al., 2015; Pfenninger et el., 2003; Pfenninger, Cordellier & Streit, 2006; Pienkowska et al., 2015; Schniebs et al., 2018; Schniebs et el., unpublished; Vinarski et al., unpublished a; Vinarski et al., unpublished b; Wethington & Guralnick, unpublished; Young et al., 2021) (see Table 1).

Table 1. List of sequences of the cytochrome c oxidase subunit I gene (COI) used in this phylogenetic studies.

Locality (country, region/state, name)	Voucher/isolate code	GenBank Accession Nos.	Source
***Ampullaceana ampla* (Hartmann, 1821)**			
Russia, Kaliningrad region	Mlym651	MH190044	Aksenova et al., 2018
***Ampullaceana dipkunensis* (Gundrizer & Starobogatov 1979)**			
Russia, Arkhangelsk region	Mlym12	MH189854	Aksenova et al., 2018
	IEPN399/3	MH190050	
***Ampullaceana fontinalis* (Studer, 1820)**			
Russia, Krasnodar Terr.	Mlym9	MH189853	Aksenova et al., 2018
Russia, Republic of Karelia	Mlym88	MH189903	Aksenova et al., 2018
	Mlym99	MH189909	
Ukraine, Republic of Crimea	Mlym91	MH189905	Aksenova et al., 2018
	Mlym92	MH189906	
Tajikistan	Mlym131/1	MH189933	Aksenova et al., 2018
Russia, Moscow region	Mlym143/1	MH189955	Aksenova et al., 2018
	Mlym143/2	MH189956	
	Mlym143/3	MH189957	
Russia, Arkhangelsk region	IEPN490	MH190051	Aksenova et al., 2018
Ukraine, Donetsk region	PS3	OP115749	This paper
Ukraine, Luhansk region	PS42	OP115750	This paper
	PS43	OP115751	
***Ampullaceana lagotis* (Schrank, 1803)**			
Russia, Nenets Autonomous Okrug	IEPN-G142	KM067619	Aksenova et al., 2018
	IEPN-G219	KM067621	
	IEPN-G138.1	KM067625	
	IEPN-G143.1	KM067627	
	IEPN-G143.4	KM067630	
	Mlym48	MH189884	

	Mlym56	MH189892	
Russia, Kirov region	IEPN-G225.4	KM067645	Aksenova et al., 2018
	IEPN-G225.6	KM067646	
Russia, Arkhangelsk region	Mlym18	MH189859	Aksenova et al., 2018
Russia, Tyumen region	Mlym82	MH189901	Aksenova et al., 2018
Tajikistan	Mlym136/1	MH189939	Aksenova et al., 2018
	Mlym137/2	MH189942	
	Mlym141/1	MH189952	
	Mlym145/2	MH189959	
	Mlym146/2	MH189962	
	Mlym148/3	MH189969	
Russia, Yamalo-Nenets Autonomous Okrug	Mlym156/3	MH189995	Aksenova et al., 2018
***Ampullaceana* sp.**			
Iran	Iran snail 1	MW435323	Mirfendereski et al., 2021
	Iran snail 2	MW435324	
	Iran snail 3	MW435325	
	Iran snail 4	MW435326	
	Iran snail 5	MW435327	
	Iran snail 6	MW435328	
	Iran snail 7	MW435329	
	Iran snail 8	MW435330	
	Iran snail 10	MW435331	
***Lymnaea stagnalis* (L., 1758)**			
No data	SMF 325458	EF489390	Klussmann-Kolb et al., 2008
Germany, Thuringia	22	EU818795	Albrecht et al., 2008
No data	MTD Moll 53108	FR797865	Vinarski et al., unpublished a
	MTD Moll 53094	FR797868	
No data	SNSD Moll 49239	HG932243	Vinarski et al., unpublished b
	SNSD Moll 49864	HG932244	
	SNSD Moll 53080	HG932246	
	SNSD Moll 53485	HG932251	
	SNSD Moll S3436	HG932257	
	SNSD Moll S5300	HG932258	
	SNSD Moll S5305	HG932260	
	SNSD Moll S5784	HG932261	
No data	DNA5	KP070784	Pienkowska et al., 2015
***Peregriana peregra* (O. F. Müller, 1774)**			
No data	DM400	MT862403	Leerhoei, unpublished
***Physa heterostropha* (Say, 1817)** accepted as ***Physella acuta* (Draparnaud, 1805)** (World Register…, 2022)			
USA, Pennsylvania	paP9	AY651193	Wethington & Guralnick, unpublished
***Physella acuta* (Draparnaud, 1805)**			
Egypt	no data	KF412768	Grabner et al., 2014
Greece, Central Macedonia	UGSB 12309	KF737936	Albrecht et al., 2013
Canada, Ontario	BIOUG00776-F07	KM611944	Hebert et al., unpublished
Iran, Kerman	IRPAC_008	KT280435	Nasibi et el., unpublished
Iran, Bardsir	IRPAC_079	KT280436	Nasibi et el., unpublished
Canada, Ontario	BIOUG07961 G09	MF545164	Dewaard, unpublished
USA, Maryland	USNM:IZ:1463181	MH087555	Aguilar, Ogburn & Hines, unpublished
	SERCINVERT1750	MK308256	

Iceland, Reykjavik	19ANH	MZ396244	Pantoja et al., 2021
USA	SRP016	OK510594	Young et al., 2021
No data	SRP018	OK510596	Young et al., 2021
***Planorbarius corneus* (L., 1758)**			
No data	No data	AY282590	Albrecht et al., 2004
No data	No data	AY350524	Pfenninger et el., 2003
No data	No data	DQ980188	Pfenninger, Cordellier & Streit, 2006
No data	MTD Moll 52556	FR797857	Vinarski et al., unpublished a
	MTD Moll 52557	FR797858	
***Radix auricularia* (L., 1758)**			
No data	SNSD Moll 53087	HG932217	Vinarski et al., unpublished b
No data	ZUE	KP098540	Feldmeyer et el., 2015
France	AGL4	KP242322	Patel et el., 2015
	BGC12	KP242342	
	BGC3	KP242348	
	BGC4	KP242349	
	LYC2	KP242690	
Spain	SLM2	KP242771	Patel et el., 2015
	VNV13	KP242865	
	ZUE1	KP242882	
Russia, Arkhangelsk region	Mlym-58	KU950448	Aksenova et el., 2016
No data	SNSD:Moll:53086	LT623597	Schniebs et el., 2018
Russia, Leningrad region	Mlym60	MH189895	Aksenova et el., 2018
Ukraine, Republic of Crimea	Mlym90	MH189904	Aksenova et el., 2018
***Radix balthica* (Linnaeus, 1758)** accepted as ***Ampullaceana balthica* (Linnaeus, 1758)**			
France	BLL11	KP242357	Patel et al., 2015
	BSC1	KP242367	
	BSC10	KP242368	
	BSC11	KP242369	
	BSC3	KP242372	
	CMT1	KP242441	
	CMT2	KP242442	
	CMT4	KP242444	
	CMT5	KP242445	
	CPG1	KP242449	
	CPG6	KP242465	
	DUN2	KP242511	
	FEL1	KP242558	
	FEL14	KP242563	
	FEL16	KP242565	
	ISG10	KP242615	
	ISG12	KP242616	
	ISG13	KP242617	
	ISG2	KP242624	
	ISG7	KP242630	
	JRC10	KP242634	
	JRC12	KP242636	
	JRC14	KP242638	
	JRC2	KP242639	
	JRC7	KP242644	
	LMY2	KP242678	

	LMY9	KP242683	
	PYO10	KP242752	
	PYO14	KP242756	
	PYO2	KP242757	
	PYO6	KP242761	
	PYO9	KP242764	
Spain	DAS11	KP242471	Patel et al., 2015
	DAS18	KP242478	
	GNL1	KP242585	
	GNL10	KP242586	
	GNL11	KP242587	
	GNL3	KP242590	
	HEN1	KP242606	
	HEN6	KP242611	
	LES3	KP242664	
	LES4	KP242665	
	LES5	KP242666	
	SSB10	KP242777	
	SSB11	KP242778	
	SSB12	KP242779	
	SSB8	KP242786	
***Radix lagotis* (Schrank, 1803)** accepted as ***Ampullaceana lagotis* (Schrank, 1803)** (World Register…, 2022)			
No data	SNSD:Moll:52815	LT623601	Schniebs et al., 2018
	SNSD:Moll:53239	LT623602	
	SNSD:Moll:S3770	LT623603	
***Radix* sp.**			
Turkey, Isparta	CA-2008	EU818802	Albrecht et al., 2008
No data	MOTU3	KP098538	Feldmeyer et al., 2015
***Radix zazurnensis* (Mozley, 1934)** accepted as ***Kamtschaticana kamtschatica* (Middendorff, 1850)** (World Register…, 2022)			
Russia	IEPN-G0225.4	KF918624	Paltser et al., unpublished
	IEPN-G0142	KF918625	
	IEPN-G0137	KF918626	
	IEPN-G0138.1	KF918627	
	IEPN-G0143.1	KF918628	
	IEPN-G0143.4	KF918629	
Russia, Irkutsk region	MlymB-108.1	KT867320	Aksenova et al., 2017
***Stagnicola palustris* (Müller, 1774)**			
No data	MTD Moll 48715	FR797869	Vinarski et al., unpublished a
	MTD Moll 53095	FR797871	
	MTD Moll 53096	FR797872	
No data	SNSD Moll S5141	HG932232	Vinarski et al., unpublished b
No data	Sp1	KP070773	Pienkowska et al., 2015
	Sp2	KP070774	
	Sp3	KP070775	
	EZ	KP070776	
	Sp39	KP070777	
No data	SNSD Moll S5141	LN515547	Schniebs et el., unpublished
No data	SNSD:Moll:S5798	LT623589	Schniebs et el., 2018
Russia, Nenets Autonomous Okrug	Mlym52	MH189888	Aksenova et el., 2018

More distant species of the family Lymnaeidae Rafinesque, 1815 were analysed as outgroups that included *Lymnaea stagnalis* (L., 1758) (13 sequences), *Radix auricularia* (L., 1758) (14 sequences) and *Stagnicola palustris* (Müller, 1774) (12 sequences). Moreover, 12 sequences of *Physella acuta* (Draparnaud, 1805) from the family Physidae Fitzinger, 1833 and 5 sequences of *Planorbarius corneus* (L., 1758) from the family Planorbidae Rafinesque, 1815 were included in the final dataset. All of the taxa, according to current taxonomy (World Register…, 2022), belong to the superfamily Lymnaeoidea, superorder Hygrophila, subclass Heterobranchia, class Gastropoda. Both the sequences obtained from our samples (PS3, PS42 & PS43) and all GenBank data were used to build a phylogenetic tree (Fig. 3).

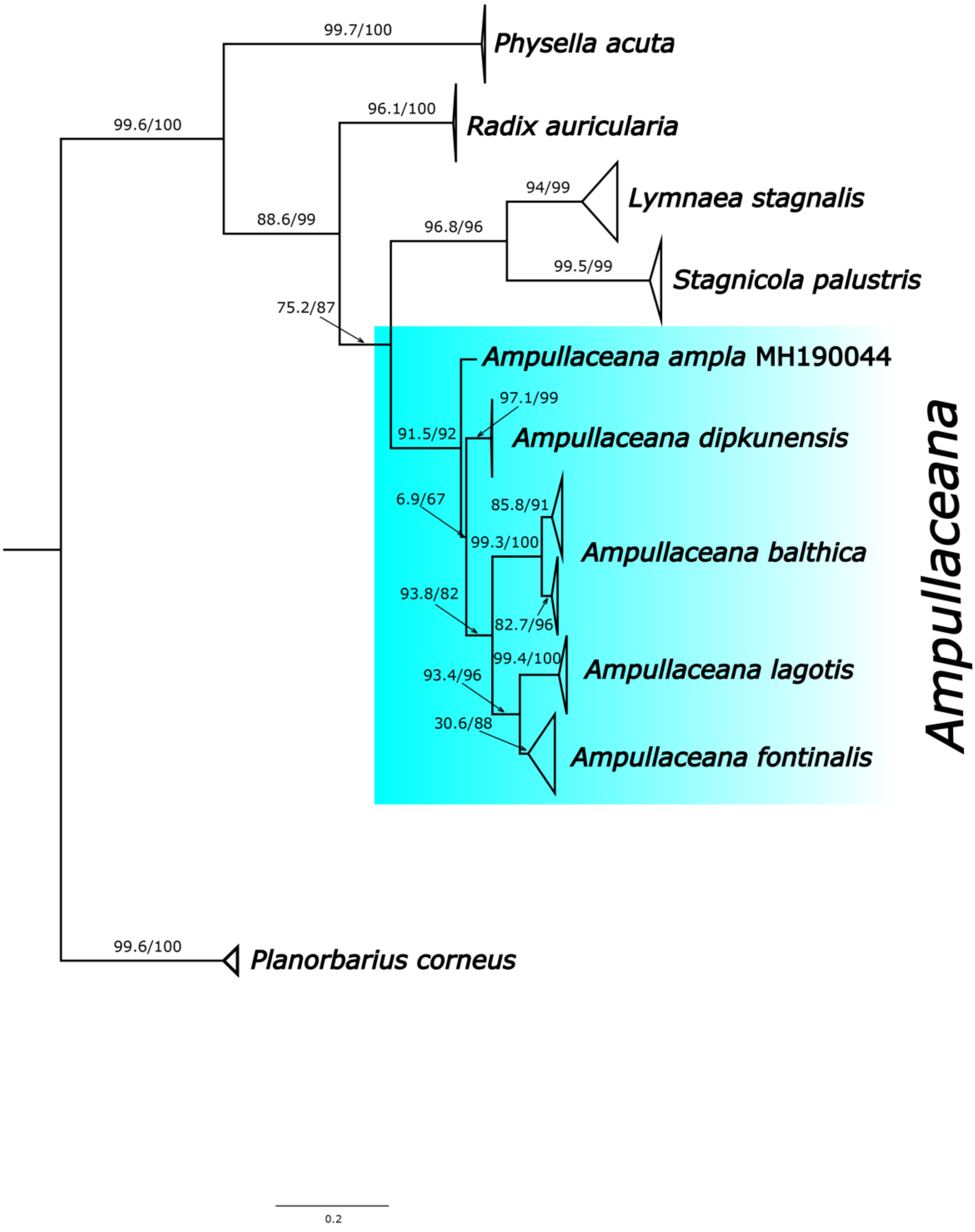

Figure 3. Phylogenetic relationships within the genus *Ampullaceana* obtained using the maximum likelihood optimality criterion based on COI sequences (Log-likelihood of the tree = -4245.7847). Values of both the SH-like approximate likelihood-ratio test (SH-aLRT) and ultrafast bootstrapping are shown for branches.

The COI sequences were aligned with MAFFT ver. 7.490 (Katoh et al. 2019) with FFT-NS-i strategy set to Auto. Stop codons were checked and examined at the amino acid level using MEGA X (Kumar et al., 2018).

A total of 156 sequences were analysed. The length of COI aligned sequences was 658 base pairs (see Table 1).

The phylogenetic tree was obtained using the maximum likelihood method in the IQTREE v1.6.12 program (Nguyen et al. 2015). Statistical branch support was estimated using 1000 iterations of both the SH-like approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010) and the ultrafast boot-strapping algorithm (Minh et al. 2013).

According to the Bayesian Information Criterion (BIC), the most appropriate substitution models were determined: TIM2+F+G for the first, TPM2+F for the second, and TPM2+F+G for the third codon position of the COI gene.

Within and between group mean uncorrected distances, as well as their standard errors, which were obtained by a bootstrap procedure (1000 replicates), were calculated using MEGA X (Kumar et al. 2018).

Results

Morphology

Externally, the shell of *A. fontinalis* bears a significant resemblance to that of *A. lagotis*. However, the height of the curl in *A. fontinalis* is lower, and the width of the last shell whorl is smaller than in the compared species. The shells of our samples are small, fragile, translucent, light brown, the shape of the shells is oval-conical. The penultimate whorls in the whorl are truncated-conical, with slight swelling of the lateral walls, not symmetrical, the sutures between the whorls are shallow. The upper margin of the last whorl is oval, slightly shifted to the right from the central axis of the shell. The part of the last whorl, rising above the aperture, does not seem strongly swollen, descends gently towards the mouth. The outer periphery of the last whorl is regularly rounded, without visible corners and shoulders. The parietal and upper edges of the palatal margin of the aperture form an acute angle, which is why the aperture has a drop-shaped (or pointed-oval) shape. The line connecting the parietal and columellar margins of the aperture has a slight S-shaped curve. The columellar fold covers the umbilicus with a lapel, but does not close it completely, so that a narrow duct remains. The columellar margin of the aperture smoothly passes into the basal and further into the palatal, forming a regular, widely rounded lower margin of the aperture. The lower part of the columellar, basal, and lower and middle parts of the palatal margins of the aperture are slightly turned outwards, their margins are sharp. On the outer surface of the shells, there are low radial folds formed by the outer conchiolin layer. The inner surface of the free edge of the aperture is smooth and shiny; folds of the outer surface of the shell are visible in the light. Iridization of the inner layer of the shell (hypostracum) is not noticeable. The dimensions of our samples (PS42 and PS43 respectively): SH – 12.2 and 21.5+ mm, SW – 8.3 and 14.7+ mm, AH – 9.5 and 16.0 mm, AW – 6.0 and 10.7+ mm, CH – 2.7 and 5.5+ mm, LHA – 1.5 and 3.6 mm, LWA – 5.7 and 11.1 mm.

The prepuce (praeputium) and the sheath of the penis are light colored, their length is almost the same. Mean copulatory apparatus index (ICA) values are slightly above 1.0 (Kruglov 2005; Vinarski et al. 2020, fig. 8D).

DNA barcoding and phylogeny

The obtained phylogenetic tree shows that all three sequences joined the clade that corresponds to *Ampullaceana fontinalis* and contains samples originating from Europe (Germany, Russia and the Crimean Peninsula in southern Ukraine) and Asia (Turkey, Tajikistan and Iran) (Fig. 4).

This clade has a low statistical support (30.6/88) and the short root branch. Its number of base differences per site from averaging sequence pairs within the clade is 0.0286 ± 0.0041. The genetic distances between the newly generated sequences and the other members of the *Ampullaceana fontinalis* clade do not exceed the average intragroup genetic distance in this clade (Table 2). Samples PS42 and PS43 coming from the same locality in Luhansk region are almost identical. The PS3 sample from Donetsk region differs from the previous two and joined another subclade along with sequences from the Crimean Peninsula (southern Ukraine) and Germany.

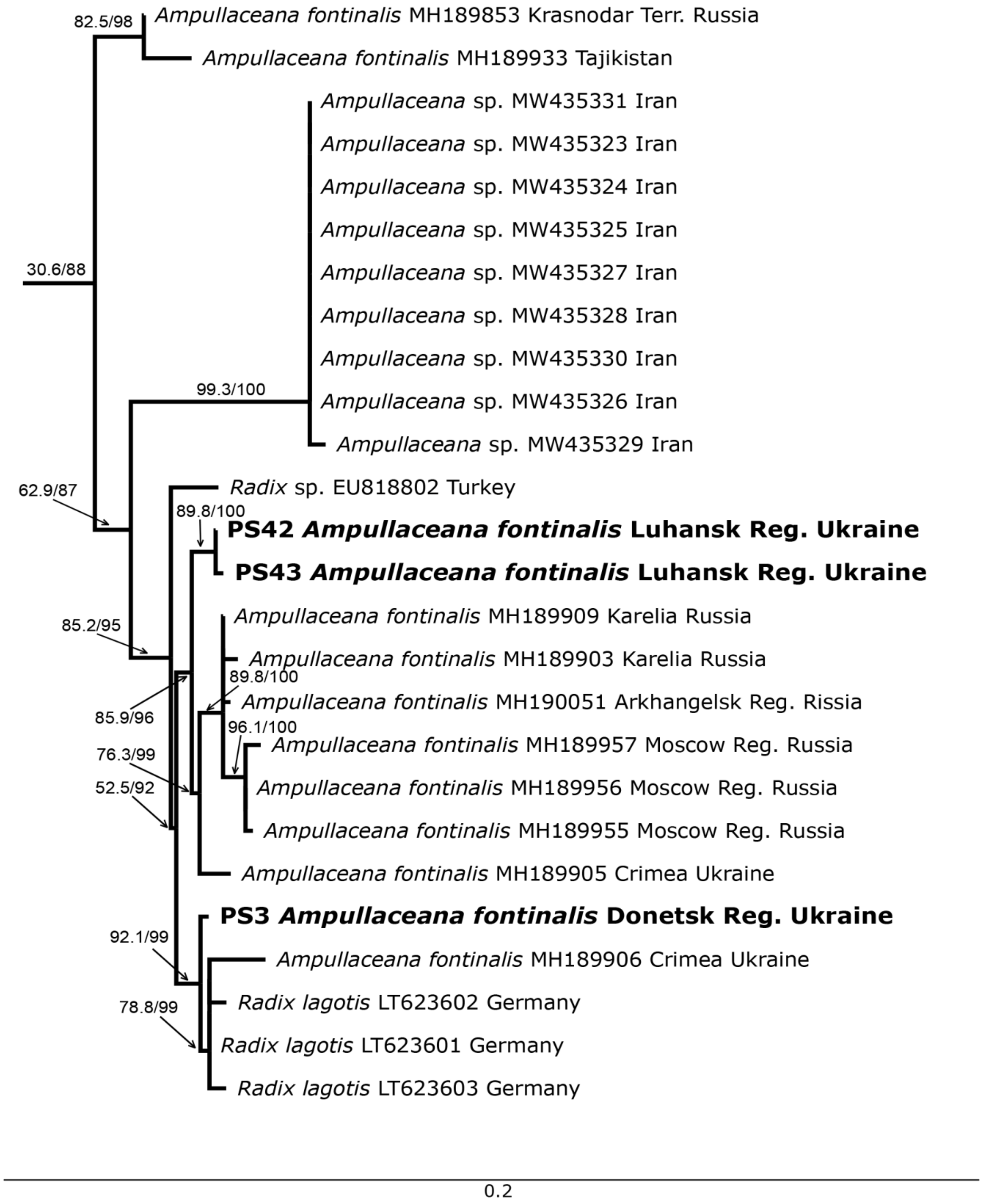

Figure 4. Phylogenetic relationships within the clade of *Ampullaceana fontinalis* (see Fig 3).

Table 2. Estimates of evolutionary divergence over sequence pairs between newly generated sequences of *Ampullaceana fontinalis* and the other members of the species' clade (see Table 1 for sequence codes).

	PS3	PS42	PS43
PS3			
PS42	0.0137 ± 0.0045		
PS43	0.0152 ± 0.0048	0.0015 ± 0.0016	
Rest of *Ampullaceana fontinalis* clade	0.0259 ± 0.0043	0.0266 ± 0.0043	0.0274 ± 0.0045

Discussion

The molecular phylogenetic analysis made it possible to attribute the pond snails examined to *A. fontinalis*. Since the beginning of malacological studies in the Siverskyi Donets River Basin, this taxon has not been distinguished as a separate species. Previous researchers recorded *Lymnaea ovata* sensu lato for the study area (Krynicki 1837; Siemaschko 1847; Radkevich 1878; Lindholm 1901). For the first time, this taxon was mentioned by Beletsky (1918) as a form of *L. ovata* from the environs of Kharkiv. Between the 1920s and 1980s, *A. fontinalis* did not appear in records on molluscs of the study region, although Zhadin (1933) recorded "*L. ovata* var. *fontinalis*" for the European part of the former USSR, including the territory of Ukraine. Moreover, *A. fontinalis* might be implicitly assigned to *L. ovata* sensu lato in a number of records (Zhadin 1929; Fadeev 1929; Booth 1940; Solodovnikov 1940; Popova 1950). Since the early 1980s, *L. fontinalis* was constantly present in the lists of the malacofauna of the area studied (Zatravkin 1980), however, neither anatomical, nor molecular studies on it were carried out at that time. Therefore, the current research resolves those ambiguities and uncertainties of the previous nomenclature.

Our molecular phylogenetic analysis confirms that the Siverskyi Donets River basin in eastern Ukraine, as a habitat for *A. fontinalis*, is an integral part of its species range. Specimens recorded under the name *A. fontinalis* and sequenced from many European countries (Poland, Germany, Switzerland, Hungary, and Bulgaria), from North Asia, Southeast Europe, Turkey and Iran have significant genetic similarity and form their own clade at the level of a separate species (Kruglov & Starobogatov 1993; Aksenova et al., 2018; our data). Conchologically, these specimens are identical to the lectotype of *Limnaea fontinalis* Studer, 1820 (Studer 1820) from Switzerland (Forcart 1957; Vinarski et al. 2020).

At the same time, there is an alternative opinion suggesting that the name *A. fontinalis* is problematic (Hubendick 1951; Glöer 2002; Welter-Schultes 2012). This implies that the populations of the pond snails are not conspecific. In accordance with this opinion, Switzerland and adjacent European countries should be excluded from the range of the species, and the name *A. fontinalis* of Eastern European and Asian samples should be accompanied with the caution «*sensu* Kruglov & Starobogatov (1993) ». Obviously, further studies using more specimens from the type locality are needed to resolve this issue.

The presented description of the shell morphology and images of the specimens examined may serve as a guide for identifying more individuals of *A. fontinalis*, since our identification is confirmed at the molecular level. Minor differences from the earlier descriptions of this species (Kruglov & Starobogatov 1993; Stadnichenko 2004; Kruglov 2005) may be explained by the wide variability of the conchological parameters of this species.

The genetic variability of the samples examined turned out to be quite unexpected. Despite the fact that all the studied samples entered the species-level clade of *A. fontinalis*, the samples collected from the left tributaries flowing from north to south (Borova River, Luhansk region) joined the subclade formed mainly by samples originating from areas situated north of the study area. The PS3 sample taken from the right tributary flowing from south to north (Kazennyi Torets, Donetsk region) joined the subclade consisting of samples originating from Western Europe and Crimea. The presence of several distinct haplotypes in such a small area is of particular interest and probably indicates separate evolutionary histories of the populations of *A. fontinalis*.

Our phylogenetic analysis revealed the genus *Ampullaceana* consisting of *A. ampla*, *A. dipkunensis*, *A. balthica*, *A. lagotis* and *A. fontinalis*. The latter two species are sister groups according to the phylogenetic tree obtained. These taxonomy and phylogeny are in agreement with recent studies on the pond snails (Aksenova et al. 2018).

The low statistical support of the clade of *A. fontinalis* suggests that further analyses that use more mitochondrial and nuclear markers are needed to identify the phylogenetic relationships of this species and its systematic status.

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