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The taxonomic status and phylogenetic relationships of the genus *Aenigmomphiscola* Kruglov and Starobogatov, 1981 (Gastropoda: Pulmonata: Lymnaeidae)

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The taxonomic status and phylogenetic relationships of the lymnaeid genus *Aenigmomphiscola* Kruglov and Starobogatov, 1981 are studied for the first time using molecular taxonomic methods. Snails of this genus have strong conchological similarity to representatives of the genus *Omphiscola* Rafinesque, 1815, but differ from other lymnaeids by an advanced inner structure of the praeputium. In spite of clear morphological differences between *Aenigmomphiscola* and *Omphiscola*, some authors have proposed their probable synonymy. The use of four molecular markers (two nuclear and two mitochondrial) and two phylogenetic tree-building algorithms allowed us to conclude that *Aenigmomphiscola* is an independent genus within Lymnaeidae that is closely allied to *Omphiscola*. These genera are vicarious, as the first of them inhabits the eastern, and the second the western part of the Palaearctic. Conchological and radular characters are not sufficient to distinguish between *Aenigmomphiscola* and *Omphiscola*.

Keywords: *Aenigmomphiscola*; Lymnaeidae; morphology; molecular systematics

Introduction

There has long been a controversy between molluscan taxonomists on how many genera should be delineated within the family Lymnaeidae Rafinesque, 1815. The first approach is to place most species of this family into a large single genus *Lymnaea* Lamarck, 1799, while separating some morphologically advanced forms into a separate genus. This approach has been adopted widely and some authors, such as Hubendick (1951), Kruglov and Starobogatov (1981) and Jackiewicz (1998), developed their own versions of this two-genus system. No agreement was reached on which species should not be classified within *Lymnaea*. For example, Hubendick (1951) regarded *Lanx* Clessin, 1882 as a separate lymnaeid genus, with all other lymnaeids belonging to the genus *Lymnaea*. The conchological and anatomical traits of *Lanx* are so distinct from other lymnaeids (Baker 1925) that some authors accepted a special family Lancidae Hannibal, 1912 for this genus (Baker 1925; Taylor and Sohl 1962; Harry 1964; Starobogatov 1967, 1970). Jackiewicz (1998) divided the European

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lymnaeids into two genera: *Lymnaea* and *Omphiscola* Rafinesque, 1815. In contrast, Kruglov and Starobogatov (1981), who regarded *Omphiscola* as a subgenus of *Lymnaea*, established a new genus *Aenigmomphiscola* Kruglov et Starobogatov, 1981 to include all species whose copulative apparatus has an unusual structure (see below). In spite of these differences, all the above authors predominantly used data on reproductive anatomy, and most lymnaeid species were lumped in a single genus because of striking uniformity in their genital structures. Following Hubendick's (1951) opinion, most malacologists have treated the conchological differences between species as not having any importance in genus delimitation (Kruglov 2005). Radular structure has also proved to be almost useless for this purpose (Hubendick 1951).

The opposite approach is to split the family into a series of genera, members of which share a similar shell appearance and are ecologically more or less similar. The first attempt to create a multi-genus system of Lymnaeidae on the basis of conchological and anatomical characteristics was that of Baker (1911). Recently, the multi-genus approach has prevailed in European and North American taxonomy. Most modern taxonomic surveys and checklists dealing with Holarctic lymnaeid snails include six to seven distinct genera in this family (Burch 1989; Falkner et al. 2001; Glöer 2002; see also Ponder and Waterhouse 1997).

In spite of the differences, both taxonomic approaches have used macromorphological data only. Recent developments in molecular taxonomy allow us to use DNA sequences to determine phylogenetic relationships within the family Lymnaeidae. To date, several papers on molecular systematics of European lymnaeid snails have been published (Bargues and Mas-Coma 1997, 2005; Remigio and Blair 1997; Rybska et al. 2000, 2008; Remigio 2002; Bargues et al. 2001, 2003, 2006). Jackiewicz's (1998) hypothesis concerning the distinct phylogenetic position of *Omphiscola* has been rejected, since members of this genus do not form a separate sister clade to "other" *Lymnaea* (Bargues et al. 2003; Bargues and Mas-Coma 2005). However, the taxonomic status of the genus *Aenigmomphiscola* has not been checked using molecular tools. This is the main aim of the present study.

The genus *Aenigmomphiscola* has no conchological diagnosis, since the shells of its members are almost indistinguishable from those of the (sub-)genus *Omphiscola* (Kruglov 2005, 2008). The only reliable distinction lies in a quite unusual structure of the copulative organ that sharply delineates *Aenigmomphiscola* from all other European species of Lymnaeidae (Kruglov and Starobogatov 1981). *Aenigmomphiscola* species possess a so-called "praeputial organ" within the praeputium that is regarded to be an asymmetrically enlarged velum. All other Palaearctic lymnaeid species, including *Omphiscola glabra*, have a praeputium of simpler structure, without the praeputial organ (see Figure 1F). Moreover, the penis sheath of *Aenigmomphiscola* is divided into a proximal thin-walled part and a distal part (see Figure 1E) with walls consisting of glandular tissue (Kruglov and Starobogatov 1981). The genus is an endemic taxon of the former USSR territory. Kruglov and Starobogatov (1981) separated as many as three distinct species distributed in European Russia, south-western Siberia and northern Kazakhstan.

In the phylogenetic scheme presented by Kruglov (2005), *Aenigmomphiscola* is not a sister genus of the genus *Lymnaea*. Kruglov and Starobogatov (1987) proposed that the striking conchological similarity between *Aenigmomphiscola* and *Omphiscola* is not a trait inherited from their common ancestor. Instead, they postulated that this is the result of convergent evolution in two separate phylogenetic clades of lymnaeids

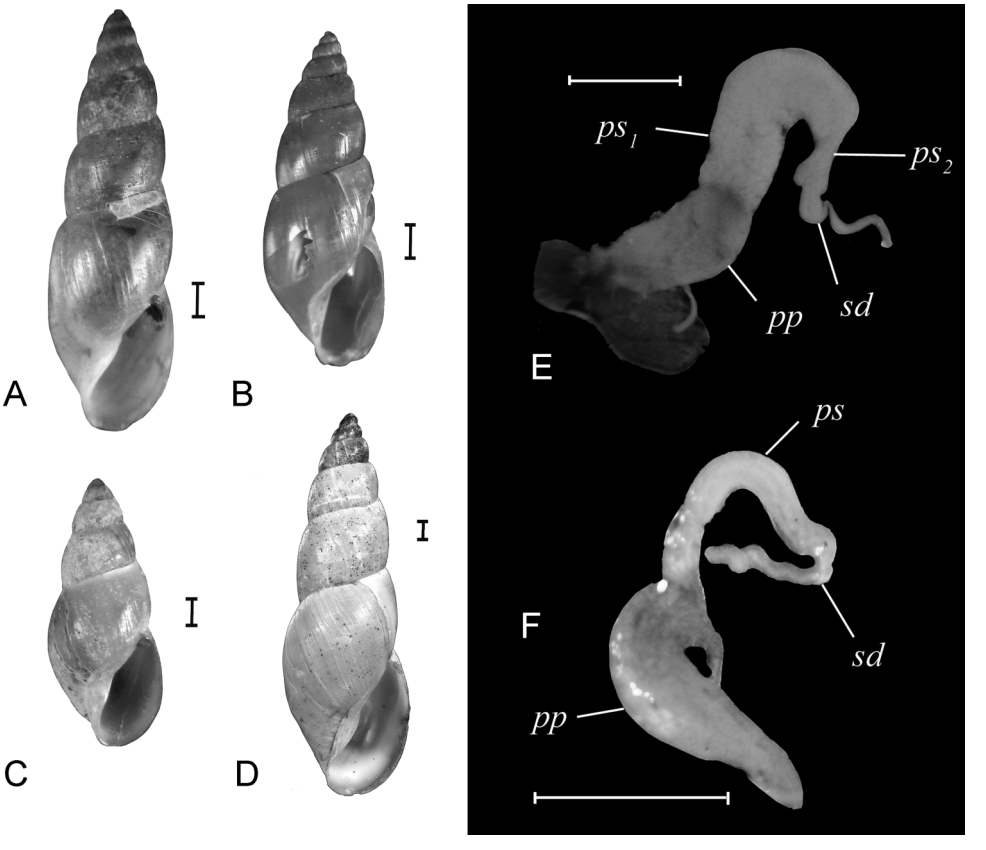

Figure 1. Shells and copulative apparatuses of molluscs of the genera *Aenigmomphiscola* (A–C, E) and *Omphiscola* (D, F). (A–B, E) *Ae. europaea* (Russia, Moscow region); (C) *Ae. kazakhstanica* (Russia, Mountain Altay); (D, F) *O. glabra* (Germany, Saxony). Scale bars 1 mm. Labels: *pp*, praeputium; *ps*, penis sheath; ps_1, ps_2, two parts of the penis sheath in *Ae. europaea*; *sd*, spermiduct.

that developed a similar shell habitus as an adaptation to their occurrence in temporary water bodies. These authors suggested that *Aenigmomphiscola* originated from ancestors of the subgenus *Lymnaea* (*Stagnicola*), and that there is no close phylogenetic affinity between *Aenigmomphiscola* and *Lymnaea* (*Omphiscola*) (Kruglov and Starobogatov 1981).

In spite of the strong morphological differences between *Aenigmomphiscola* and other Palearctic lymnaeids, no malacologists working outside the former USSR accepted this phylogenetic hypothesis. The genus is absent in the most recent European taxonomic surveys and checklists (Falkner et al. 2001; Bank 2011; the species are interpreted as part of the genus *Omphiscola* (?)) and it is accepted by Russian malacologists only. The discovery of two new habitats of *Aenigmomphiscola* in Russia in 2008–2009 allowed us to acquire fresh tissue material for DNA sequencing and morphological studies, in order to evaluate the taxonomic independence of this genus and clarify its position in the system.

Materials and methods

Lymnaeid samples and morphological approach

Snails of the genus *Aenigmomphiscola* from two habitats situated in European and Asiatic parts of Russia were analysed (Table 1, Figure 1). According to their morphological traits, these snails belong to two species; one of them, *Ae. europaea* Kruglov and Starobogatov, 1981, has a European distribution, and the other, *Ae. kazakhstanica* Kruglov and Starobogatov, 1981, has a south-western Siberian range (Kruglov 2005). A series of European lymnaeid species, including *Omphiscola glabra*, were included in the molecular analyses (see Table 1, Figure 1) for comparison. We followed the current European checklists for the taxonomy (Falkner et al. 2001; Bank 2011), in which *Galba* Schrank, 1803, *Lymnaea*, *Omphiscola*, *Radix* Montfort, 1810 and *Stagnicola* Jeffreys, 1830 are distinct genera within the family Lymnaeidae. Representatives of *Planorbarius corneus* (L., 1758) from the family Planorbidae Rafinesque, 1815 and *Aplexa hypnorum* (L., 1758) from the family Physidae Fitzinger, 1833 were used as outgroups. These molluscs belong to families of basommatophoran snails (Gastropoda: Pulmonata) allied to the family Lymnaeidae.

The voucher specimens are placed in collections of the Senckenberg Naturhistorische Sammlungen Dresden (SNSD, Germany) and (*Aenigmomphiscola* only) the Museum of Siberian Aquatic Molluscs (Russia, Omsk State Pedagogical University).

Scanning electron micrographs of the radulas of *Ae. europaea*, *Ae. kazakhstanica* and *O. glabra* were taken using a Zeiss EVO 50 Scanning electronic microscope. Analyses were performed using the variable pressure mode.

Molecular techniques

Tissue samples were taken under a microscope from the feet of the snails and fixed in 100% ethanol. The samples were registered in the tissue collection of the SNSD with a new collection number and the collection number of the specimen in the molluscan collection of SNSD, and stored at –80°C.

DNA was extracted using DTAB (dodecyl trimethyl ammonium bromide) buffer (Gustincich et al. 1991). The tissue samples were washed with 100 µl Tris EDTA buffer and subsequently incubated with 500 µl preheated DTAB for 30 min at 65°C. The incubation was continued after adding 10 µl proteinase K (50 mg/ml) for 20–24 hours, followed by a short incubation with 10 µl RNase (10 mg/ml) for 30 min at 37°C. Remaining tissue fragments disintegrate after vortexing. For cleaning, 550 µl chloroform/isoamyl alcohol (24/1) was used. The samples were vortexed for 20 sec and the phases subsequently separated again at 12,000 g for 3 min. The procedure was repeated with the upper aqueous phase. Then 100 µl 169,56 g/l LiCl (lithium chloride) and 400 µl isopropanol were added to the aqueous phase for precipitation. The samples were cooled at –20°C for 30 min and subsequently the DNA was pelleted by centrifugation at 11,200 g for 20 min at 4°C. The liquid was disposed of and the pellets were dried by inverting the tubes on a paper towel. The pellets were cleaned twice with 200 µl ice-cold 70% ethanol. The DNA pellets were then dried for 10 min at 50°C and subsequently redissolved in 50 µl of TE buffer.

Table 1. Material used in the molecular genetic studies.

Code	Collection No. SNSD	Locality	ENA No			
			cyt-b	COI	ITS-2	18S
Planorbarius corneus (L., 1758)						
P. corn-1	Moll 52556	Germany, Saxony, Linz, pond Goldgrubenteich, 13°43′09″E 51°19′45″N	FR797880	FR797857	FR797830	FR797811
P. corn-2	Moll 52557	Germany, Saxony, Linz, pond Goldgru-benteich, 13°43′09″E 51°19′45″N	FR797881	FR797858	FR797831	FR797812
Aplexa hypnorum (L., 1758)						
A. hypn-1	Moll S348	Germany, Mecklenburg-Vorpommern, lake Nebel, 12°42′02″E 53°15′32″N	FR797882	FR797859	FR797832	FR 797813
A. hypn-2	Moll S350	Germany, Mecklenburg-Vorpommern, lake Nebel, 12°42′02″E 53°15′32″N	FR797883	FR797860	FR797833	FR797814
Lymnaea stagnalis (L., 1758)						
L. stag-1	Moll 53108	Germany, Baden-Württemberg, Konstanz- Egg, ditch Hockgraben, 9°11′34.2″E 47°40′57.3″N	FR797894	FR797865	FR797834	FR797829
L. stag-2	Moll 53109	Germany, Baden-Württemberg, Konstanz- Egg, ditch Hockgraben, 9°11′34.2″E 47°40′57.3″ N	FR797895	FR797866	FR797835	FR797823
L. stag-3	Moll 53093	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00′04″E 47°43′52″N	FR797896	FR797867	FR797836	FR797824
L. stag-4	Moll 53094	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00′04″E 47°43′52″N	FR797897	FR797868	FR797837	FR797825
Stagnicola palustris (O.F. Müller, 1774)						
St. pal-1	Moll S1345	Germany, Mecklenburg-Vorpommern, lake Grosser Plaetschsee, south bank, 12°19′18″E 53°26′25″N	——	—	FR797838	FR797826

(*Continued*)

Table 1. (Continued).

Code	Collection No. SNSD	Locality	ENA No			
			cyt-b	COI	ITS-2	18S
St. pal-2	Moll S1346	Germany, Mecklenburg-Vorpommern, lake Grosser Plaetschsee, south bank, 12°19′18″E 53°26′25″N	——	——	FR797839	——
St. pal-3	Moll 48715	Germany, Saxony, wetland west of Burghausen, 12°14′44″E 51°21′33″N	FR797898	FR797869	FR797840	FR797827
St. pal-4	Moll 48716	Germany, Saxony, wetland west of Burghausen, 12°14′44″E 51°21′33″N	FR797899	FR797870	FR797841	——
St. pal-5	Moll S381	Sweden, Södermanlands Län, Askö Island, near Biostation in 0,2 m depth, N58.827° O17.631°	FR797900	——	——	FR797828
St. pal-6	Moll S382	Sweden, Södermanlands Län, Askö Island, near Biostation in 0,2 m depth, N58.827° O17.631°	FR797901	——	——	——
St. pal-7	Moll 53095	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00′04″E 47°43′52″N	——	FR797871	——	——
St. pal-8	Moll 53096	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00′04″E 47°43′52″N	——	FR797872	——	——
Radix auricularia (L., 1758)						
R. aur-1	Moll 53070	Germany, Bavaria, Weichering, pond in riverside forest, 11°19′23.6″E 48°43′34.1″N	FR797902	FR797879	FR797842	——
R. aur-2	Moll 53071	Germany, Bavaria, Weichering, pond in riverside forest, 11°19′23.6″E 48°43′34.1″N	FR797903	——	FR797843	FR797817
R. aur-3	Moll 53072	Germany, Bavaria, Weichering, pond in riverside forest, 11°19′23.6″E 48°43′34.1″N	FR797904	——	FR797844	FR797818
R. aur-4	Moll S1332	Germany, Mecklenburg-Vorpommern, Plauer See near Zislow, 12°18′33″E 53°25′53″N	——	FR797876	——	——

R. aur-5	Moll S1333	Germany, Mecklenburg-Vorpommern, lake Plauer See near Zislow, 12°18′33″E 53°25′53″N	——	FR797877	——	——
R. aur-6	Moll S1313	Germany, Mecklenburg-Vorpommern, lake Plauer See, southeast bank of peninsula Plauer Werder, 12°20′08.46″E 53°28′46.34″N	——	FR797878	——	——
Galba truncatula (O.F. Müller, 1774)						
G. tru-1	Moll S1130	Bulgaria, Osogovo Mountains, Smolichane Village, karst spring, 22°48′25.2″E 42°07′ 58.1″N	FR797890	FR797873	FR797845	—
G. tru-2	Moll S1131	Bulgaria, Osogovo Mountains, Smolichane Village, karst spring, 22°48′25.2″E 42°07′ 58.1″N	FR797891	—	FR797846	——
G. tru-3	Moll 52543	Germany, Saxony, Oelsnitz/Erzgebirge former pond, 12°42′04″E 50°43′02″N	—	FR797874	—	FR797815
G. tru-4	Moll 52544	Germany, Saxony, Oelsnitz/Erzgebirge former pond, 12°42′04″E 50°43′02″N	—	FR797875	—	FR797816
G. tru-5	Moll 52545	Germany, Saxony, Oelsnitz/Erzgebirge former pond, 12°42′04″E 50°43′02″N	FR797892	—	FR797847	——
G. tru-6	Moll 52546	Germany, Saxony, Oelsnitz/Erzgebirge former pond, 12°42′04″E 50°43′02″N				
Aenigmomphiscola europaea Kruglov and Starobogatov, 1981						
Ae. eu-1	Moll S1150	Russia, Moscow region, near Konobeevo village, flood plain of river Moscow 38°34.2′ E 55°22.8′ N	FR797884	—	FR797849	—-
Ae. eu-2	Moll S1151	Russia, Moscow region, near Konobeevo village, flood plain of river Moscow 38°34.2′ E 55°22.8′ N	FR797885	FR797861	FR797850	—
Ae. eu-3	Moll S1153	Russia, Moscow region, near Konobeevo village, flood plain of river Moscow 38°34.2′ E 55°22.8′ N	—	—	—	FR797821
Aenigmomphiscola kazakhstanica Kruglov and Starobogatov, 1981						
Ae. kaz-1	Moll S233	Russia, Altai Republic, river Bija, 51°47.16′ N 87°13.86′ E	—	—	FR797851	——
Ae. kaz-2	Moll S234	Russia, Altai Republic, river Bija, 51°47.16′ N 87°13.86′ E	FR797886	—	FR797852	FR797822

(*Continued*)

Table 1. (Continued).

Code	Collection No. SNSD	Locality	ENA No			
			cyt-b	COI	ITS-2	18S
Omphiscola glabra (O.F. Müller, 1774)						
O. gla-1	Moll S303	Germany, Hamburg, Kollau, Mühlenau, 09°55′33″E 53°36′34″N	FR797887	—	FR797853	—
O. gla-2	Moll S304	Germany, Hamburg, Kollau, Mühlenau, 09°55′33″E 53°36′34″N	FR797888	FR797862	FR797854	—
O. gla-3	Moll S305	Germany, Hamburg, Kollau, Mühlenau, 09°55′33″E 53°36′34″N	FR797889	FR797863	FR797855	—
O. gla-4	Moll S306	Germany, Hamburg, Kollau, Mühlenau, 09°55′33″E 53°36′34″N	—	FR797864	FR797856	FR797819
O. gla-5	Moll S307	Germany, Hamburg, Kollau, Mühlenau, 09°55′33″E 53°36′34″N	—	—	—	FR797820

Polymerase chain reaction (PCR) and purification of PCR products

The PCRs were carried out in a final volume of 20 μl with quantities of DNA from 0.5–5.0 μl depending on the concentration estimated by gel electrophoresis, 2 μl 10 × PCR buffer (Bioron Germany, Ludwigshafen, incomplete), 1 μl $MgCl_2$ (magnesium chloride) (Bioron, 0.055 μS/cm), 1 μl of each primer (10 pmol/μl), 0.5 μl dNTP (10 mM), 0.2 μl Taq DNA polymerase (DFS-Taq, Bioron) and the corresponding volume of sterile H_2O.

The primers used for COI (fragment of about 638 base pairs) were LCO1490 and HCO2198 (Folmer et al. 1994), and the temperature profile used for COI was 92°C 2 min (92°C 40 s, 50°C 1 min, 72°C 90 s) × 30, 72°C 8 min, 8°C hold.

The 18S rRNA gene was amplified in three fragments using the following primer pairs: Lym1 (for) and Lym658 (rev), Lym511 and Lym1307, Lym1112 and Lym1822 (Bargues and Mas-Coma 1997). The temperature profile used was 94°C 5 min (94°C 1 min, 54°C 1 min, 72°C 2 min) × 37, 72°C 10 min, 8°C hold.

From the cyt-b gene a region of circa 370 bp (base pairs) was amplified with the primers UCyt*b*151F and UCyt*b*270R (Merritt et al. 1998) and a temperature profile of 94°C 4 min (94°C 40 s, 48°C 40 s, 72°C 1.15 min) × 40, 72°C 6 min, 8°C hold.

The primers used for the nuclear gene ITS-2 were LT1 (Bargues et al. 2001) and ITS2-Rixo (Almeyda-Artigas et al. 2000). The temperature profile used was 94°C 4 min (94°C 30 s, 50°C 30 s, 72°C 1 min) × 40, 72°C 7 min, 8°C hold.

PCR products were purified with 0.1 μl Exo Sap-It Exonuclease I and Shrimp Alkaline Phosphatase, USB Corporation, Cleveland, Ohio, USA plus 4 μl bidestilled H_2O and incubation for 30 min at 37°C, then deactivation for 15 min at 80°C.

The primers used for the cycle sequencing were UCyt*b*151F for cyt-b, LCO1490 for COI, LT1 for ITS-2, and for 18S rRNA Lym658, Lym511 and Lym1112. The quantity of PCR product used for cycle-sequencing ranged from 0.5–5.0 μl depending on the concentration estimated by gel electrophoresis. Then 0.5 μl BigDye T-Mix (ABI, Applied Biosystems, Foster City, California, USA), 2.25 μl BigDye buffer (5 ×), 0.5 μl primer (10 pmol/μl) and sterile H_2O were added to a total volume of 10 μl. The following temperature profile was used: (96°C 10 s, 50°C 5 s, 60°C 4 min) × 25, 8°C hold. The products were purified by adding 1 μl 246,09 g/l NaAc (sodium acetate) (pH 4.6) and 25 μl 100% ethanol, centrifuging at 13,000 g for 15 min, inverting the tubes on a paper towel and washing with 200 μl 70% ethanol. After removing the ethanol, the pellets were dried for 10 min at 50°C. Samples were sequenced on an ABI 3130 xl (Applied Biosystems).

Because the specimens of *Aenigmomphiscola* are very small (shell height is less than 12 mm) there was not enough DNA to get sequences of all genes from all specimens.

Alignment was performed by eye using BioEdit Sequence Alignment Editor (Hall 1999). This was demanding for ITS-2 sequences, so it was repeated 10 times independently. Since the results were the same in all ten trials, the alignment was accepted for analyses.

Phylogenetic analyses of sequences

For maximum-likelihood (ML) analyses, including bootstrap support, we used raxmlGUI 0.9 beta 2 (RAxML) Randomized Axelerated Maximum Likelihood (Silvestro

and Michalak 2010; Stamatakis et al. 2005). The settings were "ML+thorough bootstrap" with 100 (replicate) runs and 1000 (bootstrap) repetitions.

Maximum-parsimony (MP) trees were reconstructed using PAUP (phylogenetic analysis using parsimony) (version 4.0b10; Swofford 2002; settings: gapmode=NewState, addseq=closest, maxtree=100). For presentation of the MP results, one of the best trees was chosen to be able to illustrate branch lengths (one showing the same overall topology as the majority-rule consensus tree was chosen). In maximum-parsimony analysis gaps were treated as a fifth state.

Genetic distances of cyt-b and COI were calculated using MEGA (molecular evolutionary genetics analysis) version 4 (Tamura et al. 2007) using p-distance (nucleotide substitutions). Missing information sites were treated using option "Pairwise-Deletion".

All DNA sequences have been placed in the European Nucleotide Archive (ENA, see http://www.ebi.ac.uk/ena/) under accession numbers FR 797811–FR 797794.

Results

Molecular genetics

Genetic distances from pair-wise comparisons of cyt-b sequences (fragment of about 370 bp) are shown in Table 2. Differences between species of the different families

Table 2. Evolutionary distances of the cyt-b gene fragment (about 370 bp) calculated using MEGA version 4 (Tamura et al. 2007).

	P. corneus	*A. hypnorum*	*Ae. europaea*	*Ae. kazakhstanica*	*O. glabra*	*G. truncatula*	*L. stagnalis*	*St. palustris*	*R. auricularia*
Planorbarius corneus	–	–	–	–	–	–	–	–	–
Aplexa hypnorum	0.312	–	–	–	–	–	–	–	–
Ae. europaea	0.296	0.290	–	–	–	–	–	–	–
Ae. kazakhstanica	0.260	0.269	0.090	–	–	–	–	–	–
Omphiscola glabra	0.276	0.264	0.151	0.131	–	–	–	–	–
Galba truncatula	0.294	0.263	0.221	0.199	0.203	–	–	–	–
Lymnaea stagnalis	0.284	0.286	0.244	0.222	0.223	0.224	–	–	–
Stagnicola palustris	0.322	0.331	0.283	0.258	0.257	0.267	0.226	–	–
Radix auricularia	0.307	0.271	0.244	0.223	0.226	0.166	0.259	0.280	–

Table 3. Evolutionary distances of the COI gene fragment (about 638 bp) calculated using MEGA version 4 (Tamura et al. 2007).

	P. corneus	*A. hypnorum*	*Ae. europaea*	*O. glabra*	*L. stagnalis*	*St. palustris*	*G. truncatula*	*R. auricularia*
Planorbarius corneus	–	–	–	–	–	–	–	–
Aplexa hypnorum	0.171	–	–	–	–	–	–	–
Ae. europaea	0.166	0.166	–	–	–	–	–	–
Omphiscola glabra	0.177	0.159	0.121	–	–	–	–	–
Lymnaea stagnalis	0.184	0.169	0.167	0.167	–	–	–	–
Stagnicola palustris	0.184	0.182	0.167	0.148	0.143	–	–	–
Galba truncatula	0.173	0.158	0.151	0.152	0.147	0.158	–	–
Radix auricularia	0.190	0.179	0.172	0.199	0.174	0.188	0.180	–

Planorbidae, Physidae and Lymnaeidae (outgroup comparison) ranged between 33.1% and 26.0%.

Among the six genera of Lymnaeidae the lowest values (15.1% and 13.1%) are between *Omphiscola* and the two *Aenigmomphiscola* species. Between other genera the values ranged from 16.6% to 28.3%. The lowest value of 9.0% is between the species *Ae. europaea* and *Ae. kazakhstanica*.

Genetic distances from pair-wise comparisons of the second mitochondrial gene analysed, COI (fragment of about 638 bp), are shown in Table 3. In this gene, the differences between species of the different families Planorbidae, Physidae and Lymnaeidae (outgroup comparison) ranged between 19.0% and 15.8%. As in cyt-b, the lowest value (12.1%) is within the genera of Lymnaeidae, between *Omphiscola* and *Aenigmomphiscola*. Between the other genera, the values ranged from 19.9% to 14.3%.

The maximum-parsimony (MP) tree of the nuclear marker ITS-2 (tree length = 1827, consistency index = 0.6869, retention index = 0.9162) is illustrated in Figure 2B. Although two basal branches have less than 60% bootstrap support, all other basal branches and the clades of the species themselves have full bootstrap support. A sister group consisting of *Lymnaea* and *Stagnicola* is sister to the other genera of Lymnaeidae analysed. Among the latter, *Radix* is the sister group to *Galba*, *Aenigmomphiscola* and *Omphiscola*. The two *Aenigmomphiscola* species analysed group sister to *O. glabra*. Within *Aenigmomphiscola*, the two species *europaea* and *kazakhstanica* are separated with nearly full bootstrap support.

The MP tree of the second nuclear marker 18S rRNA (tree length = 158, consistency index = 0.9494, retention index = 0.9760) (not shown) as well as the RAxML

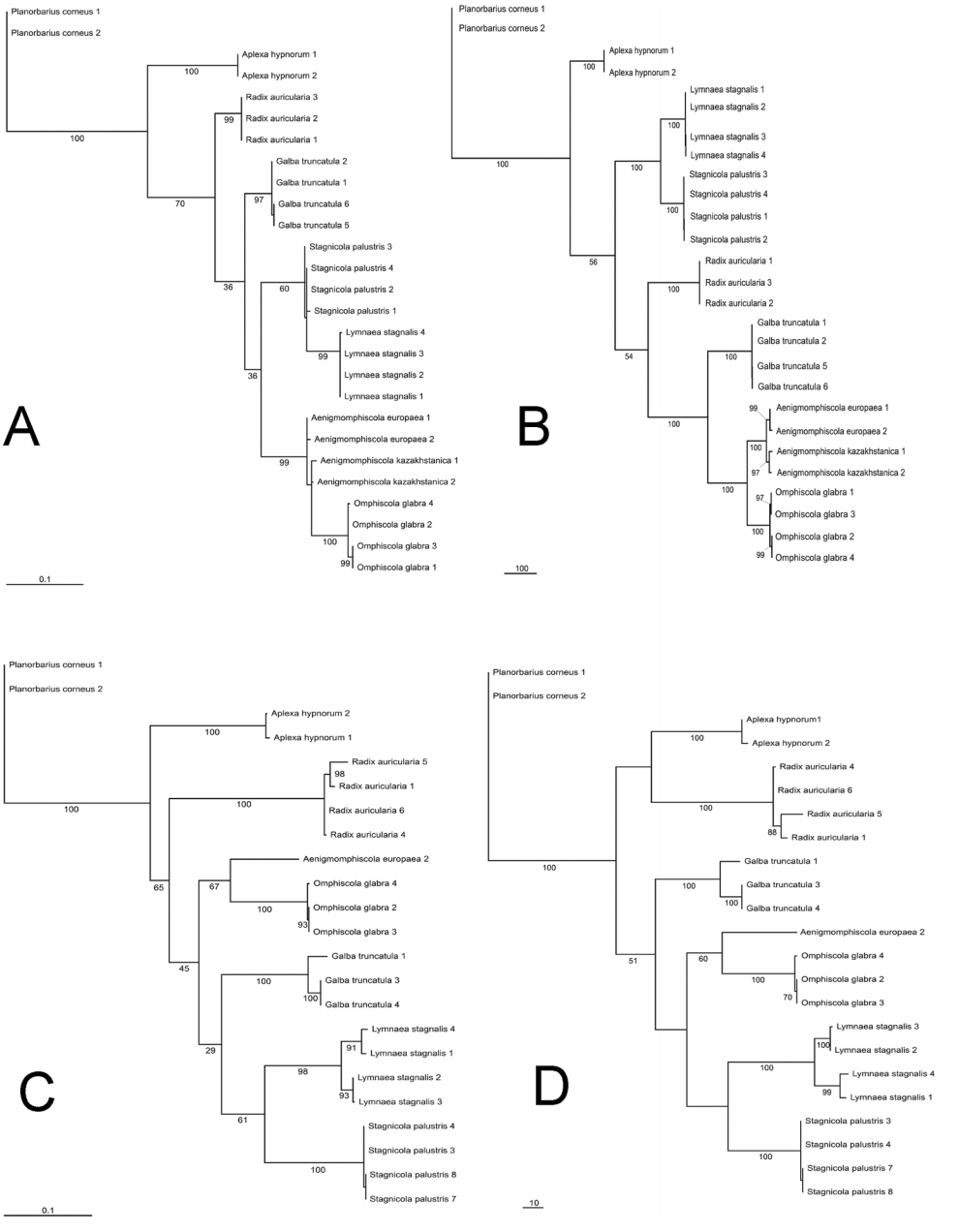

Figure 2. Phylogenetic trees of the lymnaeid species studied, obtained using the different molecular markers and different algorithms of tree building. (A) ITS-2 tree based on ML algorithm; (B) ITS-2 tree based on MP algorithm; (C) COI tree based on ML algorithm; (D) COI tree based on MP algorithm. Numbers below branches are bootstrap scores.

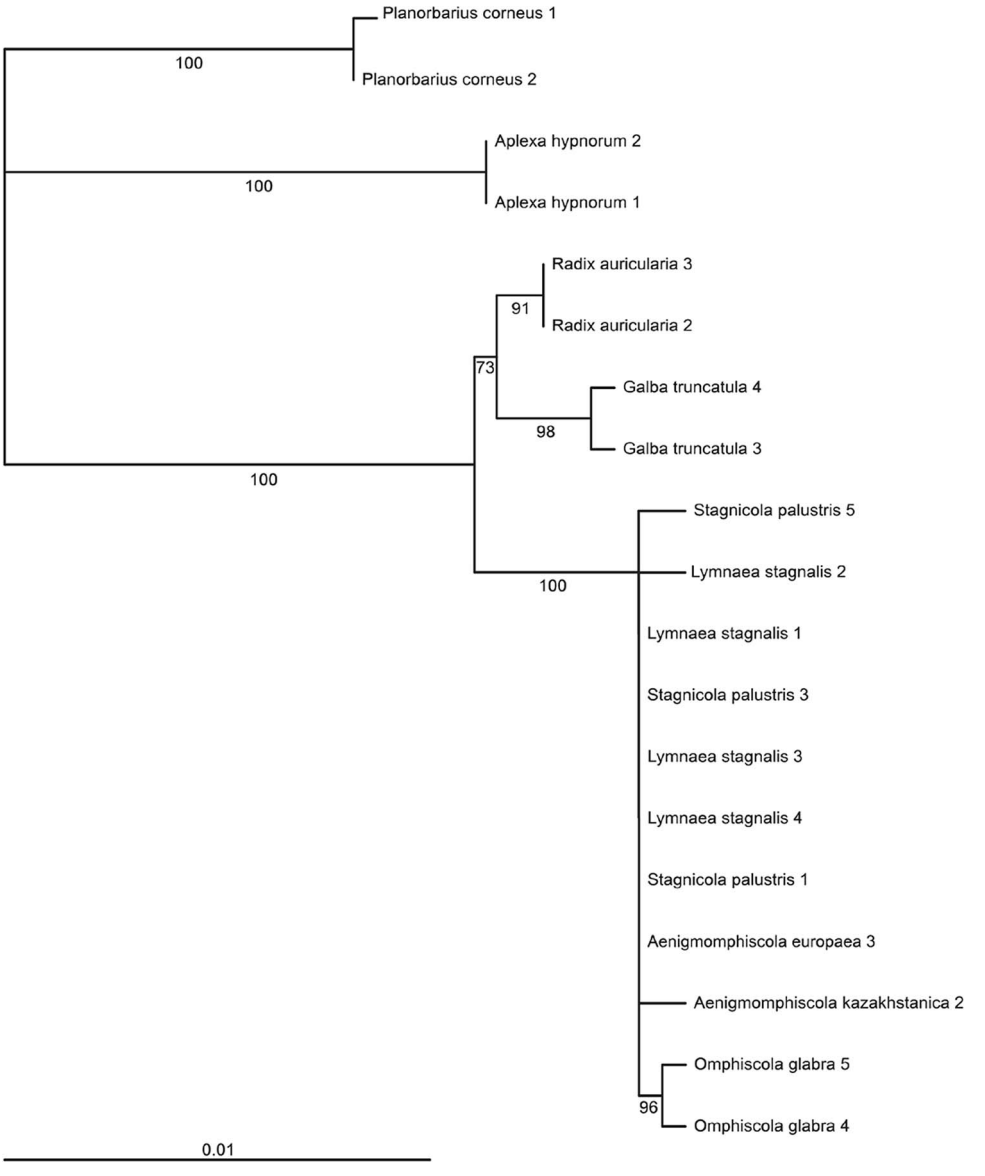

Figure 3. 18S rRNA phylogenetic tree obtained using ML algorithm. Numbers below branches are bootstrap scores.

tree (Figure 3) show a fully supported polytomy of *Ae. europaea*, *Ae. kazakhstanica*, *O. glabra*, *L. stagnalis* and *St. palustris*. The only difference in the two reconstructions is that in the MP tree, *G. truncatula* groups sister to *R. auricularia* and the clade of the other Lymnaeidae species, whereas in the RAxML tree, *G. truncatula* is the sister group to *R. auricularia*. In both trees, these basal branches are not well supported. All other basal branches have full bootstrap support. The clades of the species are highly supported.

The MP tree of the cyt-b sequences (tree length = 1827, consistency index = 0.6869, retention index = 0.9162) (not shown), also has well supported clades of the species themselves, as observed in the MP tree of ITS-2. However, two basal branches are also not well supported. In the MP tree, as well as in the RAxML tree of cyt-b (not shown), the *Aenigmomphiscola* species group sister to *O. glabra*. *Lymnaea* groups as sister genus to *Stagnicola*, and *Radix* to *Galba*. In the RAxML tree, two of the basal branches only have around 40% bootstrap support: *Aplexa hypnorum*, as a representative of the family Physidae, is not in the outgroup together with *Planorbarius corneus* as in the MP of the cyt-b tree, but forms a sister-group to *Radix auricularia* and *Galba truncatula* within the Lymnaeidae. The clades of the species are highly supported or have full bootstrap support.

The MP tree of the second mitochondrial marker COI (tree length = 530, consistency index = 0.6358, retention index = 0.8449) (Figure 2D) as well as the RAxML tree (Figure 2C), have less than 70% bootstrap support at the basal branches. This poor support is underlined by a polytomy of four branches, with *G. truncatula*, *L. stagnalis*, *St. palustris* as well as *O. glabra* with *Ae. europaea* as sister groups in the strict consensus MP tree (not shown). Also, *Aplexa*, as representative of the family Physidae, groups sister to *R. auricularia*. The clades of the species themselves are highly supported or have full bootstrap support. In the RAxML tree, *Ae. europaea* also groups sister to *O. glabra*. In this reconstruction, *Radix* is the sister group to all other genera of Lymnaeidae analysed. *Lymnaea* is the sister group to *Stagnicola* and both together group sister to *G. truncatula*.

Morphology

Aenigmomphiscola differs from all other lymnaeids in having a praeputial organ. Moreover, the penis sheath in this genus is separated externally into two distinct parts; the proximal part has thin walls and the distal part has walls of glandular tissue (Kruglov and Starobogatov 1981).

According to our data, the central radular tooth in *Aenigmomphiscola* is bicuspid with clear disparity in sizes of the cusps (Figure 4). The lateral teeth are tricuspid, with weakly developed endoconus. The same traits are found in *Omphiscola* (see Figure 4). It should be noted, however, that the radular structure of *Ae. uvalievae* was not studied here, therefore conclusions on the whole genus are based only on two of the three species.

Discussion

The results of our molecular genetic analyses of the nuclear marker ITS-2 and the two mitochondrial markers, the cyt-b fragment (about 370 bp) and COI, allow the conclusion that *Aenigmomphiscola* appears to constitute a separate clade among lymnaeids that is sister to the genus *Omphiscola* (Figure 2) and differs from the other examined species of Lymnaeidae at the level of a genus. This result is consistent in all trees generated: it is supported by three genes – ITS-2, cyt-b and COI – and two different algorithms of phylogeny reconstruction (maximum parsimony, maximum likelihood).

The genetic distance of 15.1% between *Aenigmomphiscola europaea* and *Omphiscola* in the cyt-b fragment (about 370 bp) is approximately comparable to that between *Radix auricularia* and *Galba truncatula* (16.6%), as well as other lymnaeid taxa of generic rank.

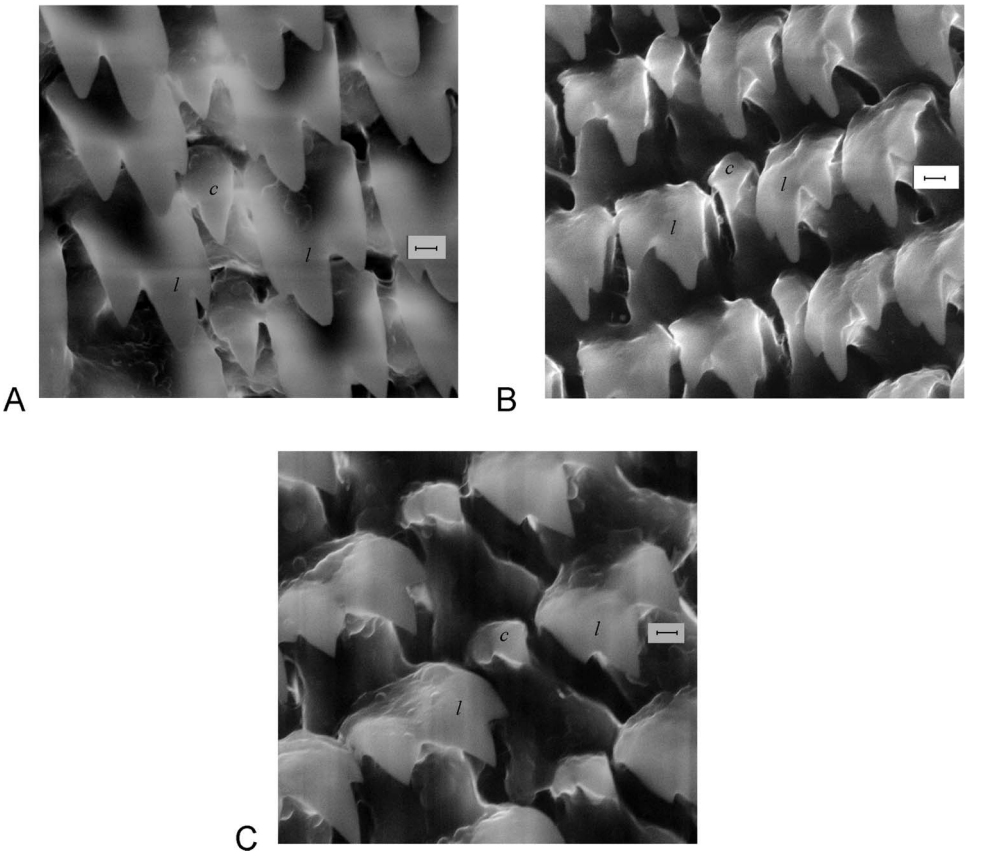

Figure 4. The radular morphology of *Aenigmomphiscola* and *Omphiscola* species. (A) *Ae. kazakhstanica*; (B) *Ae. europaea*; (C) *Omphiscola glabra*. Labels: *c*, central tooth; *l*, lateral teeth. Scale bars 2 μm.

The molecular distance of 9% between the species *Ae. europaea* and *Ae. kazakhstanica* in the cyt-b fragment is comparable to those between closely related species of the genus *Radix*, for example *R. balthica* (L., 1758) and *R. lagotis* (Schrank, 1803) (Schniebs et al., unpublished data).

According to the Hennigian principles of phylogenetic systematics, sister taxa should bear equal taxonomic rank (Hennig 1966). Hence, *Aenigmomphiscola* must be considered as a separate lymnaeid genus, at least within the taxonomic framework that is commonly accepted in European taxonomy (Falkner et al. 2001; Glöer 2002; Bank 2011).

Falkner et al. (2001) have questioned the generic independence of *Aenigmomphiscola*, regarding it as a probable synonym of the genus *Omphiscola*. However, there are several reasons for not synonymizing these taxa. Firstly, the molecular distances between them (reported above) indicate that they have a degree of genetic divergence comparable to those between other "good" genera of Lymnaeidae. Secondly, the number of internal prostate folds is not the same in *Aenigmomphiscola* and *Omphiscola*, and some malacologists (for example, Kruglov 2005, 2008) regard this trait as specific for delineation of higher taxa in Lymnaeidae. Thirdly, the presence

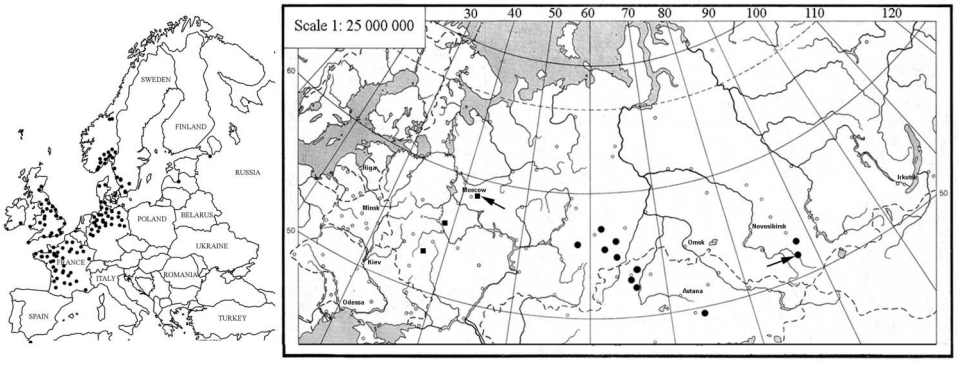

Figure 5. The ranges of *Omphiscola* (left, after distributional data presented by Hubendick 1951; Kruglov, Starobogatov 1981 and Økland 1990) and *Aenigmomphiscola* (right). On the right map, black squares (■) indicate known localities of *Ae. europaea* and black circles (●) indicate known localities of *Ae. kazakhstanica* and *Ae. uvalievae.* The two latter species are almost indistinguishable by their conchological and anatomical traits and probably are synonyms (Khokhutkin et al. 2009). The arrows on the right map indicate habitats of *Aenigmomphiscola* species used in the molecular analyses.

of the praeputial organ (see above) in *Aenigmomphiscola* differentiates it sharply from all another lymnaeids of the Palaearctic. Additionally, the structure of the penis sheath is characteristic for this genus only.

Finally, the ranges of the two genera do not overlap: *Omphiscola* is distributed predominantly in western Europe and is relatively rare in central European countries (Figure 5), for example Germany (Glöer 2002), Poland (Piechocki 1979; Jackiewicz 1998) and Ukraine (Stadnichenko 2004). In Russia, the only known record of this genus was made more than a century ago in 1895, in the vicinity of St Petersburg (Kruglov 2005). This may be the easternmost habitat of this genus in Europe. *Aenigmomphiscola* has an eastern-European–southern-Siberian distribution (see Figure 5) and its westernmost record is from the vicinity of Moscow. Hence, the two genera are separated geographically.

We thus consider the genus *Aenigmomphiscola* to be a "good" genus within the family Lymnaeidae, which is closely allied to the genus *Omphiscola* but can be clearly distinguished from the latter by both anatomical and molecular traits. No conchological or radular characters are useful to delimit these taxa. We cannot report any reliable distinction in the radular morphology between A*enigmomphiscola* and *Omphiscola*. Only small differences in relative sizes of separate cusps of teeth can be found in the *Omphiscola* radula as compared to that of *Aenigmomphiscola*.

The representatives of *Aenigmomphiscola* are recorded from eastern Europe and southern Siberia within the boundaries of the Russian Federation, therefore this taxon should be included in all European surveys and checklists dealing with the diversity of freshwater molluscs.

The phylogenetic hypothesis proposed by Kruglov and Starobogatov (1981), which considers the (sub-)genus *Stagnicola* as the most probable ancestor of *Aenigmomphiscola*, can evidently be rejected by our results. The phylogenetic trees almost concordantly show that the genera *Aenigmomphiscola, Omphiscola, Stagnicola*, and even *Lymnaea* and possibly *Galba* are all the descendants of one common ancestor

(see Figures 2, 3), but that the *Aenigmomphiscola*-*Omphiscola* clade developed independently. Judging from their geographic ranges, they could be viewed as a vicarious pair of genera that inhabit correspondingly the western and the eastern parts of the Palaearctic region. The closest extant relatives of the two genera are still unknown, since support for deeper nodes is so low in our reconstructions.

It should be stressed, however, that the ranks of taxa above species level cannot be established objectively. According to some authors (Mayr 1969; Shaposhnikov 1974), the higher taxa are real but their taxonomic Linnaean rank may be determined in different ways. There are no definite criteria for how to delimit genera in molluscan systematics (Meier-Brook 1993), and there is no generally recognized genus-concept in zoology (Dubois 1988).

In the case of *Omphiscola*, there is a dilemma in deciding whether to use "genus" or "subgenus", which depends on which taxonomic methodology is accepted by a particular systematicist. The two-genus system of the family Lymnaeidae proposed by Kruglov and Starobogatov (1981) and Kruglov (2005, 2008) is not based on cladistic methodology. The basis of this system is a rather different approach known as "evolutionary systematics", which opposes the Hennigian one in many important points (Skarlato and Starobogatov 1974; Mayr 1998; Grant 2003). Evolutionary systematics does not accept the principle of rank equality for sister taxa, and uses the "principle of the same degree of difference" instead: taxa of the same rank should be separated by an equal level of distinctiveness (Mayr 1969; Skarlato and Starobogatov 1974; Golikov and Starobogatov 1988).

These approaches are logically equivalent, since they are based on alternative and mutually supplemental grounds (Pavlinov 2003). Thus, a malacologist who wishes to follow evolutionary systematics could place all European lymnaeids except for *Aenigmomphiscola* into the large genus *Lymnaea sensu lato* with a plethora of subgenera (Kruglov 2008). Since conchological characters are traditionally regarded as having less taxonomic value than anatomical ones (Baker 1911; Hubendick 1951), the sharp differences in the structure of the copulative organ of *Aenigmomphiscola* allow us to separate it as an independent genus, in spite of its striking conchological similarity to snails of *Omphiscola*. On the other hand, *Omphiscola* should be considered a subgenus of *Lymnaea* since the structure of its copulative apparatus is typical for most lymnaeids (Kruglov and Starobogatov 1981).

The molecular data obtained in this study do not give us grounds to synonymize the species *Ae. europaea* and *Ae. kazakhstanica* in spite of the relatively low genetic distance separating them. The reasons for this are as follows. The morphological differences between these species described by Kruglov and Starobogatov (1981) and revealed by one of the authors (Vinarski and Grebennikov, unpublished data) are stable and include both conchological and anatomical traits. It appears that morphological differences are of high importance in combined molecular and morphological studies of molluscan taxonomy; lymnaeid species that are obviously distinct by their morphological traits, for example *Stagnicola palustris* (O.F. Müller, 1774) and *S. turricula* (Held, 1836), but were shown to be almost indistinguishable with the genetic markers analysed, were still regarded as subspecies (Bargues et al. 2003, 2006). Furthermore, there is no standard level of genetic divergence corresponding to the species or subspecies, or even generic rank (Lee 2004; Abramson 2009; but see Baker and Bradley 2006; Lefébure et al. 2006). Thus, low genetic divergence in itself is hardly enough to corroborate the conspecificity hypothesis in this case. However, it

should be noted here that only one sample of each *Aenigmomphiscola* species was used in this work. Thus, possible intraspecific variability of genetic traits has been not detected and there is no firm molecular evidence for the specific distinctness of the two *Aenigmomphiscola* species studied. The third species of the genus, *Aenigmomphiscola uvalievae* Kruglov et Starobogatov, 1981, is very similar to *Ae. kazakhstanica* in its morphology. It has been suggested that the latter two species are in fact synonyms (Khokhutkin et al. 2009) but we have no soft tissues of *Ae. uvalievae* in order to test this suggestion.

The question of how many species there are in the genus *Aenigmomphiscola* should be answered by thorough examination of numerous diverse morphological traits on the basis of large samples. The study of intraspecific genetic varibility will be suitable for this as well.

On the basis of our results it can be shown, however, that 18S rRNA is not suitable as a marker for differentiation between species of the genera *Lymnaea*, *Stagnicola*, *Aenigmomphiscola* and *Omphiscola* (see Figure 3).

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