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Review

European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences☆

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Abstract

Freshwater snails of the family Lymnaeidae are of a great parasitological importance because of the very numerous helminth species they transmit, mainly trematodiases of large medical and veterinary impact. The present knowledge on the genetics of lymnaeids and on their parasite–host inter-relationships is far from being sufficient. The family is immersed in a systematic–taxonomic confusion. The necessity for a tool which enables species distinction and population characterization is evident. This paper aims to review the European Lymnaeidae basing on the second internal transcribed spacer ITS-2 of the nuclear ribosomal DNA. The ITS-2 sequences of 66 populations of 13 European and 1 North American lymnaeid species, including the five generic (or subgeneric) taxa *Lymnaea sensu stricto*, *Stagnicola*, *Omphiscola*, *Radix* and *Galba*, have been obtained. The ITS-2 proves to be a useful marker for resolving supraspecific, specific and population relationships in Lymnaeidae. Three different groupings according to their ITS-2 length could be distinguished: *Radix* and *Galba* may be considered the oldest taxa (370–406 bp lengths), and *Lymnaea s. str*., European *Stagnicola* and *Omphiscola* (468–491 bp lengths) the most recent, American *Stagnicola* and *Hinkleyia* being intermediate (434–450 bp lengths). This hypothesis agrees with the phylogeny of lymnaeids based on palaeontological data, chromosome numbers and radular dentition. ITS-2 sequences present a conserved central region flanked by two variable lateral regions corresponding to the 5′ and 3′ ends. The number of repeats of two microsatellites found in this conserved central region allows to differentiate *Radix* from all other lymnaeids. Phylogenetic trees showed four clades: (A) *Lymnaea s. str*., European *Stagnicola* and *Omphiscola*; (B) *Radix* species; (C) *Galba truncatula*; and (D) North American stagnicolines. ITS-2 results suggest that retaining *Stagnicola* as a subgenus of *Lymnaea* may be the most appropriate and that genus status for *Omphiscola* is justified. *Radix* shows a complexity suggesting different evolutionary lines, whereas *G. truncatula* appears to be very homogeneous. North American and European stagnicolines do not belong to the same supraspecific taxon; the genus *Hinkleyia* may be used for the American stagnicolines. Genetic distances and sequence differences allowed us to distinguish the upper limit to be expected within a single species and to how different sister species may be. *S. palustris*, *S. fuscus* and *S. corvus* proved to be valid species, but *S. turricula* may not be considered a species independent from *S. palustris*. Marked nucleotide divergences and genetic distances detected between different *S. fuscus* populations may be interpreted as a process of geographic differentiation developping in the present. Among *Radix*, six valid species could be distinguished: *R. auricularia*, *R. ampla*, *R. peregra* (= *R. ovata*; = *R. balthica*), *R. labiata*, *R. lagotis* and *Radix* sp. The information which the ITS-2 marker furnishes is of applied interest concerning the molluscan host specificity of the different trematode species. The phylogenetic trees inferred from the ITS-2 sequences are able to differentiate between lymnaeids transmitting and those non-transmitting fasciolids, as well as between those transmitting *F. hepatica* and those transmitting *F. gigantica*. The *Fasciola* specificity is linked to the two oldest genera which moreover cluster together in the phylogenetic trees, suggesting an origin of the *Fasciola* ancestors related to the origin of this branch. European *Trichobilharzia* species causing human dermatitis are transmitted only by lymnaeids

☆ A total of 31 new nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers listed in the first table.

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of the *Radix* and *Lymnaea s. str.–Stagnicola* groups. Results suggest the convenience of reinvestigating compatibility differences after accurate lymnaeid species classification by ITS-2 sequencing. Similarly, ITS-2 sequencing would allow a step forward in the appropriate rearrangement of the actual systematic confusion among echinostomatids.

Keywords: Mollusca; *Lymnaea*; rDNA ITS-2 sequences; Genus, species and population relationships; Phylogeny; Human trematodiases; Europe

1. Introduction

1.1. Lymnaeids in parasite transmission

Basommatophoran pulmonate gastropod molluscs are not only of a malacological interest, but also of a great parasitological importance because of the very numerous helminth species they transmit. Freshwater snails of the family Lymnaeidae (Rafinesque, 1815) are, besides planorbids, those known to be involved in a greater number of helminth life cycles. They are used as intermediate hosts by numerous digenean trematode species (mainly as first intermediate hosts, but also as second intermediate hosts as for instance in echinostomatids) and, although less frequently, by nematodes, mainly Protostrongylidae and hymenolepidid cestodes.

Lymnaeids participate in the life cycles of at least 71 trematode species belonging to 13 different families whose members use birds and both domestic and sylvatic mammals as definitive hosts (Brown, 1978), and this without counting digeneans of lower vertebrates such as amphibians. A compilation by Erasmus (1972) of the numbers of cercariae species recorded from freshwater molluscs in Europe showed the importance of several species of the genus *Lymnaea* Lamarck, 1799 *sensu lato*: 29 different species in *Lymnaea stagnalis*, 17 in *L. palustris*, three in *L. truncatula*, seven in *L. ovata*, six in *L. auricularia*, and 20 in *L. peregra*. Sometimes, the same lymnaeid species is even used by more than one digenean species simultaneously (Moukrim et al., 1993). Trematode species transmitted by a given lymnaeid species differ depending from the geographic region considered (i.e. Adam and Lewis, 1993; Toledo et al., 1998a) and local ecological characteristics (Esch and Fernandez, 1993), but mainly depend on parasite–host specificity. Trematode-snail specificity concerns concepts as infectivity, susceptibility, resistance, immunity, compatibility, host finding, host recognition, host attraction, phylogeny, and genetic variability (Yoshino and Vasta, 1996; Adema and Loker, 1997; Haas and Haberl, 1997; Sapp and Loker, 2000a,b).

Lymnaeids are also of applied interest because of transmitting several trematode species of large medical and veterinary impact. The fasciolids *Fasciola hepatica* and *Fasciola gigantica* are the most important (Mas-Coma and Bargues, 1997). In recent years, this interest has markedly increased because of the detection of true human endemics, ranging from low to very high prevalences and intensities, indicating that human fascioliasis must no longer be considered merely as a secondary zoonotic disease, but be included in the list of important human parasitic diseases (Mas-Coma et al., 1999a,b). Moreover, several lymnaeid species are infected by schistosomatids of birds and mammals whose cercariae they shed cause dermatitis or swimmer's itch in humans (Degentile et al., 1996; Kolárová et al., 1997; Horák and Kolárová, 2001). Echinostomatidae are another group of trematodes including species developing in lymnaeids, of applied interest because of their capacity to infect humans (Sen-Hai and Mott, 1994; Graczyk and Fried, 1998). *Fascioloides magna* and *Paramphistomum daubneyi* are examples of other trematodes of great economic importance (in veterinary medicine) which also use lymnaeids in their life cycles (Erhardova, 1961; Horák, 1971; Moukrim et al., 1993).

1.2. The systematic–taxonomic confusion in Lymnaeidae

Despite the applied interest of lymnaeid snails in epidemiological and control studies concerning the important trematode parasitic diseases they transmit, the present knowledge on the genetics of this gastropod group as well as on their parasite–host inter-relationships is far from being sufficient.

A good example of this situation is the systematic–taxonomic confusion in which this molluscan family is immersed. About 1800 species and 34 genera of lymnaeids have been named in the past (Hubendick, 1951; Te, 1976), with classifications recognizing a single genus (Walter, 1968), two genera (Hubendick, 1951; Jackiewicz, 1998), or more than two genera (Zilch, 1959–1960; Burch, 1965, 1980, 1982a,b; Malek, 1985; Jackiewicz, 1993; Glöer and Meier-Brook, 1998). The multigeneric scheme of Burch (1965, 1980, 1982a) serves as a convenient means for species-group identification (Burch and Lindsay, 1973a; Burch, 1982a). Several approaches have been used to evaluate the taxonomy and relationships within the family: morphology (Hubendick, 1951; Walter, 1968; Burch, 1982a,b, 1988), palaeontology (Zilch, 1959–1960; Inaba, 1969), karyology (Burch, 1965; Inaba, 1969), experimental cross-breeding (Burch and Ayers, 1973), enzyme electrophoresis (Rudolph and Burch, 1989), and immunology (Burch, 1968b; Burch and Lindsay, 1968). However, a consensus has not yet been reached because of the little systematic resolution of the information, as with chromosome numbers (Patterson and Burch, 1978), or the disagreement of the results furnished by morphological studies on shell, radula, and prostate gland with those from karyological and biochemical methods (see reviews by Davis, 1978 and Patterson and Burch, 1978), suggesting that morphological homoplasy is common among lymnaeids. At any rate, reproductive tract characteristics sometimes proved to be

useful for lower taxonomic unit distinction between very closely related lymnaeid species (i.e. Jackiewicz, 1988, 1989b; Glöer and Meier-Brook, 1998).

At lymnaeid species level, the problems are found mainly because of the interspecific morphological and anatomic uniformity numerous species show, usually giving serious difficulties in specimen classification, sometimes even impeding it (e.g. Oviedo et al., 1995). Moreover, intraspecific variation of shell shape is particularly well marked within lymnaeids according to environmental conditions (Burch, 1968a; Burch and Lindsay, 1973b), although a genetic component in shell shape has been shown at least in some lymnaeid populations (Samadi et al., 2000). In Europe, there are many specimen classification problems, mainly concerned with species of the "stagnicola" and "radix" type groups (Glöer and Meier-Brook, 1998).

1.3. The present situation in the systematics of European Lymnaeidae

From the view-point of phylogenetic systematics (Hennig, 1966), there are but few supraspecific taxa within Lymnaeidae that are actually based on undoubted autapomorphies. One example is the genus *Radix* Montfort, 1810 (type species: *R. auricularia*). In a vast literature such groups are treated as subgenera of the almost only genus *Lymnaea sensu lato*; there is no acceptible criterion to decide on the level of subgenera, genera and any other supraspecific taxa, and consequently each one treats this question as he deems it practicable. At any rate, in families with great species numbers it appears useful in communication between scientists to group species in subcategories. Genus names have been used for lymnaeids since decades and in the one or other case also subgenera may help to unite species with closer relationship.

An apomorphy of *Radix* species is the chromosome number of $n = 17$. As it does not occur in any lymnaeid species apart from this taxon, it is an autapomorphy of this genus. In no other case autapomorphies of species groups are clear enough to secure taxa of their own.

Three widely distributed species are usually accepted in Europe: *R. auricularia* (Linnaeus, 1758), *R. peregra* (Müller, 1774) and *R. ovata* (Draparnaud, 1805). *Radix auricularia* is regarded by Hubendick (1951) to form a superspecies of widest distribution in the Palaearctic. This species, together with the majority of lymnaeids, bears a long bursa duct, which appears to be the plesiomorphic state. Both *R. peregra* and *R. ovata*, in contrast, almost lack a bursa duct. Hubendick (1945) believed that only one species with this character state exists. As he investigated this question in Sweden only, where, up to that time, two species had occasionally been distinguished, he correctly stated that he did not find any consistent species differences. In the German literature (e.g. Ehrmann, 1933; Glöer and Meier-Brook, 1998), however, a commonly distributed and euryecious species was called *R. ovata*, and a stenoecious and rarer species has been treated under the name of *R. peregra*, a name coined by Müller in 1774. Since most authors, who distinguished these two, claimed that both are ranging as far northward as Iceland and North Scandinavia, in the second half of the past century everybody followed Hubendick (1945, 1951) considering them as conspecific. In the 1970s, Meier-Brook (unpublished data) found evidence for the "two species concept", viz. in constant differences in shell colour and thickness, in pigmentation, in particular on the mantle roof (cf. figures in Glöer and Meier-Brook, 1994, 1998; descriptions also in earlier editions starting in 1985), but in front of all in electrophoretic behaviour of several enzymes, most conspicuously in PGI (phosphoglucose–isomerase). After re-actualisation of this view, it became evident that the central European species *ovata* is identical with all what is called *peregra* in north Europe. And new research by Falkner (unpublished data) has revealed that Müller, who probably had never known the southern European species called "*peregra*" by German malacologists, must have had the north European species in view when describing his *Buccinum peregrum*. This means that British and Scandinavian systematists correctly used the name *peregra* for their species (whose nomenclatorally valid name is *R. balthica* (Linnaeus, 1758) according to Falkner, personal communication, who will soon treat the nomenclatorial questions of lymnaeids), that *ovata* is a junior synonym and that we have to use another name for the species living south of the north German lowland. Thus, the present situation in the pair of short duct species is as follows: whole Europe is inhabitated by *R. peregra* (Müller, 1774), and the central European highlands and the area southward including the circummediterranean countries are inhabitated by another *Radix* species.

Both *R. peregra* (in the sense of its synonym *R. ovata*) and *R. auricularia* under certain environmental conditions, as in a habitat of fine mud in large lakes, tend to be selected towards enlargement of the aperture enabling better hold on their soft substrate. They are known under a variety name *ampla* (Hartmann, 1821). The question if a species to carry this name as an independant taxonomic unit exists in fact, as suggested by Falkner (1990a, p. 101), deserves further investigation.

The nominate genus *Lymnaea* Lamarck, 1799 *sensu stricto* contains not more than very few species, which have a characteristic concave outline of the spire in common, giving them an acute apex. Within Europe hitherto only one species has been recognized: the type species *L. stagnalis* (Linnaeus, 1758).

Stagnicola Jeffreys, 1830 (= *Stagnicola* Leach, 1830; = *Limnophysa* Fitzinger, 1833) (type species: *S. palustris*) is a genus of snails of medium size, with gradually increasing whorls, and anthracite black pigmentation. Up to 1959 only one species was accepted in Europe, when Jackiewicz (1959) discovered a heterogeneity of the *palustris* complex. Now five species are recognized within this group: *S. palustris* (Müller, 1774), *S. corvus* (Gmelin, 1791), *S. fuscus* (Pfeiffer, 1821) (= *S. vulnerata* Küster, 1862), *S. turricula* (Held, 1836), and *S. occulta* (Jackiewicz, 1959).

These are distinguished by proportions in male copulatory organs, forms of the bursa duct, and numbers of folds in the prostate gland which vary between one, two or many. In North America there are numerous species that have been also included in *Stagnicola*.

A slender species, *Omphiscola glabra* (Müller, 1774), relatively rare and stenoecious, usually is separated in a genus of its own, *Omphiscola* Rafinesque, 1819 (= *Omphiscola* Beck, 1837; = *Leptolimnaea* Swainson, 1840) (type species: *O. glabra*), on the fact that the prostate gland lacks any fold (Jackiewicz, 1989b, 1998). A close relationship with *Stagnicola* species, however, was repeatedly supposed.

There remains a genus named *Galba* Schrank, 1803 including one species, *G. truncatula* (Müller, 1774) in the old world, with its closest relatives known from the Nearctic under the generic name *Fossaria* Westerlund, 1885. The difference in genus ascription is a problem of nomenclature rather than one of systematics. All these species play a major role in serving the liver fluke, *F. hepatica*, as first intermediate host. Close relationship with species of the genus *Stagnicola* has often been supposed, as is suggested by former use of the name *Galba* for the *Stagnicola* species (Jackiewicz, 1959).

Finally, the unusual species *Myxas glutinosa* (Müller, 1774), only member of the genus *Myxas* Sowerby, 1882, completes the list of European lymnaeids. Its mantle is enlarged to an extent that the shell is entirely covered by it. The shell is extremely fragile. This species is highly endangered. In some respect it resembles the Philippine *Bullastra cumingiana* (Pfeiffer, 1845), but similarities are most probably due to convergent evolution.

1.4. Genetic and molecular techniques applied to lymnaeids

The necessity for a tool which enables species distinction and population characterization within lymnaeids is evident. Some genetic and molecular techniques have proven to be useful tools for studies of lymnaeids. Isoenzyme electrophoresis and DNA microsatellites are useful at population level and have shown that a very large range of situations can be found in lymnaeids, from heterogeneous, polymorphic populations (Rudolph and Burch, 1989; Jarne and Delay, 1990a; Coutellec-Vreto et al., 1994) to completely homogeneous, monomorphic populations (Jabbour-Zahab et al., 1997; Trouve et al., 2000; Meunier et al., 2001), a phenomenon related to both selfing and crossing capacities of these freshwater snails (Jarne and Delay, 1990b; Jarne et al., 1993). RAPD analysis did not show, however, to be sufficiently concluent when applied to lymnaeids (Rybska et al., 2000) despite having been used on other gastropod groups; Backeljau et al. (1995) already emphasized that results of RAPD studies should be used with great caution in taxonomic analyses.

DNA sequencing of well known markers has proved to be the best tool up to the moment. The 18S rRNA gene proved to be useful mainly for supraspecific relationships of lymnaeids, with the E10-1 helix of the V2 variable region of the secondary structure of this gene enabling the distinction between lymnaeid species which transmit and which do not transmit fasciolid parasites, as well as to distinguish between those species which transmit *F. hepatica* from those which transmit *F. gigantica* (Bargues and Mas-Coma, 1997; Bargues et al., 1997). At any rate, this gene is markedly conserved and is usually not very useful for the differentiation of lymnaeid species which are very similar (Bargues et al., unpublished); moreover, its validity for lymnaeid taxonomy and phylogeny has recently been questioned owing to single nucleotide polymorphisms detected in *Lymnaea natalensis* and intraspecific versus interspecific divergence levels it shows in certain groups (Stothard et al., 2000), although further studies on the 18S rRNA gene of lymnaeids are evidently needed. Sequence analyses of the large subunit (16S) mitochondrial ribosomal DNA enabled to distinguish between several lymnaeid species and to analyze their phylogenetic relationships (Remigio and Blair, 1997a). The internal transcribed spacers of the nuclear ribosomal DNA have shown to be the markers most useful at species level, enabling distinction between four systematically problematic, very closely related North American stagnicoline species (Remigio and Blair, 1997b).

The present paper aims to review the European Lymnaeidae basing on the usefulness of the second internal transcribed spacer ITS-2 of the nuclear ribosomal DNA. This spacer was selected because of its well-known usefulness in species identification among several eukaryothic organism groups, including parasites and vectors (Mas-Coma, 1999; Almeyda-Artigas et al., 2000a; Marcilla et al., 2001; Bargues et al., 2001) such as trematodes (Luton et al., 1992; Morgan and Blair, 1995; Bowles et al., 1995; Blair et al., 1997; Mas-Coma et al., 2001) and molluscs (Stothard et al., 1996; Remigio and Blair, 1997b; Mas-Coma et al., 2001). We analyse the sequences of several European lymnaeid species, namely those most common and known to participate in trematode life cycles, to verify the information this spacer can furnish about their phylogeny, as well as to analyse relationships between genera, closely related species and different populations within a given species. This study includes all lymnaeid species whose taxonomic validity is presently accepted in Europe, excepting *S. occulta* and *M. glutinosa* (unfortunately the only materials available of these two species did not allow DNA extraction).

2. Materials and methods

2.1. Lymnaeid materials

A total of 66 populations of 13 European and 1 North American lymnaeid species, including the five generic (or subgeneric) taxa *Lymnaea*, *Stagnicola*, *Omphiscola*, *Radix* and *Galba* were studied (Table 1). The DNA was extracted from more than one specimen of a given population and from

Table 1
European lymnaeid species and populations studied, including geographic origins, nucleotide length of the ITS-2 sequences obtained and corresponding GenBank accession numbers

Species-genotype code	Population number	Preliminary classification	Locality	Country	ITS-2 length (bp)	GenBank accession number
Lymnaea (*Lymnaea*) *stagnalis*: four different sequences/seven populations studied						
Ls-GT1	1	*L. stagnalis*	Lab. cult., orig. Bad Münstereifel, Erschweiler	Germany	489	AJ319614
Ls-GT1	2	*L. stagnalis*	Lab. cult., orig. Aisch, Höchstadt, Erlangen, Nürnberg	Germany	489	AJ319614
Ls-GT2	3	*L. stagnalis*	Jouy-le-Potier, Val de Loire	France	490	AJ319615
Ls-GT3	4	*L. stagnalis*	Lac d'Annecy, rive Ouest, Haute Savoie	France	491	AJ319616
Ls-GT4	5	*L.* "*stagnalis*"	Lab. cult., orig. Lago di Trasimeno	Italy	490	AJ319617
Ls-GT4	6	*L. stagnalis*	Brenne, Indre	France	490	AJ319617
Ls-GT4	7	*L. stagnalis*	München, Bavaria	Germany	490	AJ319617
Lymnaea (*Stagnicola*) *turricula*: two different sequences/two populations studied						
St-GT1	8	*S. turricula*	Wallersee, near Salzburg	Austria	473	AJ319618
St-GT2	9	*S. fuscus*	Wallersee, near Salzburg	Austria	473	AJ319619
Lymnaea (*Stagnicola*) *palustris*: one sequence/three populations studied						
Sp	10	*L.* sp. indet.	Tatihou Island, Cotentin, St. Vaast-la-Hougue, Normandie	France	473	AJ319620
Sp	11	*S. turricula*	Danube Valley, Bavaria	Germany	473	AJ319620
Sp	12	*L. palustris*	Beetsterzwaag, Prov. Friesland	The Netherlands	473	AJ319620
Lymnaea (*Stagnicola*) *fuscus*: four different sequences/seven populations studied						
Sf-GT1	13	*L. palustris*	Bou, Val de Loire	France	472	AJ319621
Sf-GT2	14	*S. fuscus*	Herten, Nordrhein, Westfallen	Germany	472	AJ319622
Sf-GT2	15	*S. fuscus*	Spitzberg, Tübingen	Germany	472	AJ319622
Sf-GT3	16	*R. peregra*	Puch, near Salzburg	Austria	468	AJ319623
Sf-GT3	17	*L. palustris*	Bocca di Razzali, Corsica Island	France	468	AJ319623
Sf-GT4	18	*S. corvus*	Albufera population 1, Valencia	Spain	468	AJ319624
Sf-GT4	19	*S. fuscus*	Albufera population 2, Valencia	Spain	468	AJ319624
Lymnaea (*Stagnicola*) *corvus*: one sequence/one population studied						
Sc	20	*S. corvus*	Wallersee, near Salzburg	Austria	484	AJ319625
Omphiscola glabra: two different sequences/three populations studied						
Og-GT1	21	*O. glabra*	Klüsser Mühle, Ludwigslust, Mecklenburg	Germany	481	AJ319626
Og-GT1	22	*O. glabra*	Schlosspark, Ludwigslust, Mecklenburg	Germany	481	AJ319626
Og-GT2	23	*L. glabra*	Indre	France	481	AJ319627
Radix auricularia: five different sequences/ten populations studied						
Ra-GT1	24	*R. auricularia*	Podkadovsky Pond, Kadov	Czech Republic	401	AJ319628
Ra-GT1	25	*R. auricularia*	Jistebnice	Czech Republic	401	AJ319628
Ra-GT1	26	*R. auricularia*	Schönau, southeast of Vienna	Austria	401	AJ319628
Ra-GT1	27	*R. auricularia*	Wallersee, near Salzburg	Austria	401	AJ319628
Ra-GT1	28	*R. auricularia*	Doddington Pool, Keele, Midlands	UK	401	AJ319628
Ra-GT2	29	*R. auricularia*	Horni Cerekev	Czech Republic	403	AJ319629
Ra-GT2	30	*R. auricularia*	Schwanzenberg Pond, Veselí n. L.	Czech Republic	403	AJ319629
Ra-GT3	31	*L. auricularia*	Reserve de Guazza, Bastia, Corsica Island	France	406	AJ319630
Ra-GT4	32	*L. auricularia*	Lac d'Annecy, rive Ouest, Haute Savoie	France	404	AJ319631
Ra-GT5	33	*L. auricularia*	Combourg, Ile-et-Vilaine	France	402	AJ319632

Table 1 (*Continued*)

Species-genotype code	Population number	Preliminary classification	Locality	Country	ITS-2 length (bp)	GenBank accession number
Radix peregra (=*R. ovata*; =*R. balthica*): three different sequences/fourteen populations studied						
Rp-GT1	34	*R. ovata*	Lac d'Annecy, rive Ouest, Haute Savoie	France	395	AJ319633
Rp-GT1	35	*R. ovata*	Courbouzon, near Beaugency, Val de Loire	France	395	AJ319633
Rp-GT1	36	*R. ovata*	Courbouzon, near Beaugency, Val de Loire	France	395	AJ319633
Rp-GT1	37	*R. ovata*	Confluence Loire-Loiret, near Orléans, Val de Loire	France	395	AJ319633
Rp-GT1	38	*R. ovata*	Baie du Mont Saint Michel, Manche	France	395	AJ319633
Rp-GT1	39	*R. peregra*	Perpignan, Pyrénées Orientales	France	395	AJ319633
Rp-GT1	40	*R. limosa*	Les Bouillouses (2000 m), Pyrénées Orientales	France	395	AJ319633
Rp-GT1	41	*R. peregra*	Lac d'Annecy, Haute Savoie (infected population)	France	395	AJ319633
Rp-GT1	42	*R. peregra*	Annecy, Haute Savoie (yellow foot population)	France	395	AJ319633
Rp-GT1	43	*R. ovata*	Beetsterzwaag, Prov. Friesland	The Netherlands	395	AJ319633
Rp-GT1	44	*R. ovata*	Albufera, Valencia	Spain	395	AJ319633
Rp-GT1	45	*R. ?peregra*	Family Park, Laugardalur, Reykjavík	Iceland	395	AJ319633
Rp-GT2	46	*R. peregra*	Lac Léman, Haute Savoie (grey–black foot population)	France	395	AJ319634
Rp-GT3	47	*R. peregra*	Rivière Lot, Nérac, Agen, Lot-et-Garonne	France	395	AJ319635
Radix labiata: two different sequences/three populations studied						
Rl-GT1	48	*R. peregra*	Lab. strain, Charles Univ. Prague (orig. from Podkadovsky Pond, Kadov)	Czech Republic	370	AJ319636
Rl-GT1	49	*R. peregra* s.E.	Lab. cult., orig. Bozdag, Söke, Aydin Sira Daglari	Turkey	370	AJ319636
Rl-GT2	50	*R. peregra* s.E.	Bad Münstereifel	Germany	370	AJ319637
Radix lagotis: two different sequences/five populations studied						
Rla-GT1	51	*R. peregra*	Lab. strain, Charles Univ. Prague (resistant to *T. regenti*) (orig. from Podkadovsky Pond, Kadov)	Czech Republic	378	AJ319638
Rla-GT1	52	*R. ovata*	Faculty Garden Pond, Charles Univ. Prague (orig. from Podkadovsky Pond, Kadov)	Czech Republic	378	AJ319638
Rla-GT1	53	*R. peregra*	Vusi Pond, Kadov	Czech Republic	378	AJ319638
Rla-GT1	54	*R. ovata*	Podkadovsky Pond, Kadov	Czech Republic	378	AJ319638
Rla-GT2	55	*R. ovata*	Schönau, southeast of Vienna	Austria	378	AJ319639
Radix ampla: one sequence/one population studied						
Ram	56	*R. ampla*	Wallersee, near Salzburg	Austria	387	AJ319640
Radix sp.: one sequence/one population studied						
Rsp	57	*R. peregra*	Söke, Aydin Sira Daglari	Turkey	373	AJ319641
Galba truncatula: two different sequences/eight populations studied						
Gt-GT1	58	*L. truncatula*	Sueca, Albufera, Valencia	Spain	401	AJ243017
Gt-GT1	59	*L. truncatula*	Benicasim, Castellón	Spain	401	AJ243017
Gt-GT1	60	*L. truncatula*	Minho	Portugal	401	AJ243017
Gt-GT1	61	*L. truncatula*	Le Taulard, Lausanne	Switzerland	401	AJ243017
Gt-GT2	62	*L. truncatula*	Sierra de Javalambre (1500 m), Castellón	Spain	401	AJ296271
Gt-GT2	63	*L. truncatula*	Beira	Portugal	401	AJ296271
Gt-GT2	64	*L. truncatula*	Monacia, Corsica Island	France	401	AJ296271
Gt-GT2	65	*L. truncatula*	Beetsterzwaag, Prov. Friesland	The Netherlands	401	AJ296271
Hinkleyia catascopium: one sequence/one population studied						
RspW	66	*Radix* sp.	Winnebago Lake, Oshkosh, Wisconsin	USA	449	AJ319642

more than one population of a given species when necessary for sequence conservation verification studies, mainly in cases of microsatellite detection or in cases of unexpected results.

2.2. Molecular techniques

2.2.1. DNA extraction

Snail feet fixed in 70% ethanol and maintained at 4 °C for several weeks were used for DNA extraction according to the phenol-chloroform method (Sambrouk et al., 1989). After dissection under a microscope, half of the foot was suspended in 400 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS)) containing 500 μg/ml proteinase K (Promega, Madison, WI, USA) and digested for 2 h at 55 °C with alternate shaking each 15 min. The following steps were performed according to methods outlined previously (Bargues and Mas-Coma, 1997). The lysed preparation was gently mixed and then incubated for 4 h at 55 °C with alternate shaking each 15 min. For the extraction of total DNA, three steps were followed. In the first there was an equal volume of phenol; in the second 200 μl of phenol and 200 μl of chloroform/isoamyl alcohol (24/1) were used; in the third, 400 μl of chloroform/isoamyl alcohol (24/1) were employed. After each extraction step, phases were separated at 12,000 × *g* for 3 min. The aqueous phase was precipitated with 1/10 vol. of 3 M sodium acetate and 2.5 vol. of 100% ethanol and refrigerated at −20 °C. The spooled DNA or pellet obtained was washed in 70% ethanol, centrifuged at 12,000–13,000 × *g* for 5–10 min at 4 °C, and briefly air dried. The precipitated DNA was redissolved in a small volume (20–50 μl) of sterile TE buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA) and stored at −20 °C until use. When working on snails, protocols including a centrifugation step after precipitation risk incorporating a white flocculate substance (probably polysaccharides) and melanic pigments to the DNA pellet, which usually inhibits the polymerase chain reaction (PCR) or causes amplification of non-specific products (Gasser et al., 1993; Bargues et al., 1997). To avoid this problem, only the snail foot was used.

2.2.2. rDNA sequence amplification

The fragment corresponding to the ITS-2 of each lymnaeid was amplified by the PCR using 4–6 μl of lymnaeid genomic DNA for each 50 μl PCR reaction, according to methods outlined previously (Almeyda-Artigas et al., 2000a). The PCR amplification was performed using primers designed in conserved positions of 5.8S and 28S rRNA genes of several eukaryote Metazoa species. The primers used were as described by Almeyda-Artigas et al. (2000a). Only one additional primer, LT1 (forward) 5′-TCGTCTGTGTGAGGGTCG was designed for amplification and sequencing purposes. Amplifications were generated in a GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT, USA), by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, preceded by 30 s at 94 °C and followed by 7 min at 72 °C. Ten microliters of the reaction mixture were examined by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

2.2.3. Purification and quantification of PCR products

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA purification system (Promega) according to the manufacturer's protocol and resuspended in 50 μl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

2.2.4. DNA sequencing

The sequencing of the ITS-2 of the rDNA was performed on both strands by the dideoxy chain-termination method (Sanger et al., 1977), and was carried out with the Taq dye-terminator chemistry kit for ABI 373A (Perkin-Elmer, Foster City, CA, USA), using PCR primers.

2.3. Software programs used

2.3.1. For sequence alignment

Sequences were aligned using CLUSTAL-W version 1.8 (Thompson et al., 1994) and introducing sequences in different orders at random to reduce biases (Lake, 1991). The alignments were made including the lymnaeid species studied together with other known lymnaeid sequences. The following rDNA ITS-2 present in GenBank–EMBL were used: *Stagnicola* (*Stagnicola*) *catascopium* (Say, 1817) (accession No. AF013143), *S.* (*S.*) *elodes* (Say, 1821) (accession No. AF013138), *S.* (*S.*) *emarginata* (Say, 1821) (accession No. AF013142), and *S.* (*Hinkleyia*) *caperata* (Say, 1829) (accession No. AF013139) (Remigio and Blair, 1997b).

2.3.2. For phylogenetic analysis

Maximum parsimony (MP), distance and maximum-likelihood (ML) methods were used in phylogeny reconstruction. All these analyses were performed using algorithms provided in PAUP version 4.0b6 (Swofford, 2001).

Maximum parsimony analysis was performed using the heuristic algorithm. To assess the relative support for internal nodes, a bootstrap-resampling approach (with 1000 replicates) was used. Alignment gaps were treated as missing character states for the analyses. Only minimal length trees were kept. Polytomies were permitted. Accelerated transformation was used for character-state optimization.

For distance analysis, neighbor-joining (NJ) trees (Saitou and Nei, 1987) were generated from a Kimura-2 (K-2) parameter (Kimura, 1980) distance matrix. NJ analysis using gamma-corrected Kimura distances was performed and statistical support of each NJ tree was assessed with bootstrap-resampling technique (Felsenstein, 1985) over 1000 replications.

Maximum-likelihood trees were constructed utilising the HKY85 model of DNA substitution assuming that all sites evolve at the same rate and the transition/transversion rate

is equal to 2 ($\kappa = 4.049$). To provide an assessment of the precision of the trees, a quartet puzzling analysis was employed (with 1000 puzzling steps).

3. Results

3.1. Sequence analyses according to populations and species

A total of 31 different sequences were found among the 66 lymnaeid populations studied. ITS-2 nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers noted in Table 1. The lengths of the ITS-2 sequences varied between 370 and 491 bp (see Table 1). Three different groupings according to their ITS-2 length can be distinguished: (A) *Radix* and *Galba* groups including 370–406 bp lengths; (B) the lymnaeid from Wisconsin enters in the group of the North American *Stagnicola* and *Hinkleyia* (Remigio and Blair, 1997b), in which the sequences are 434–450 bp long; (C) *Lymnaea s. str*., European *Stagnicola*, and *Omphiscola* with 468–491 bp lengths. The nucleotide compositions of the sequences appeared uniformly G + C biased: 55.0–61.5%

Table 2
ITS-2 sequence differences detected in pairwise comparisons between proximal populations among a species[a]

Species and compared populations	Alignment length Number of bp	Nucleotide differences		Substitutions				Insertions + deletions	
		Number	Percentage	Transitions		Transversions		Number	Percentage
				Number	Percentage	Number	Percentage		
L. (*Lymnaea*) *stagnalis*									
Ls-GT1 vs. Ls-GT2	491	5	1.02	1	0.20	0	0.00	4	0.81
Ls-GT1 vs. Ls-GT3	491	4	0.81	1	0.20	0	0.00	3	0.61
Ls-GT1 vs. Ls-GT4	491	7	1.42	3	0.61	0	0.00	4	0.81
Ls-GT2 vs. Ls-GT3	491	3	0.61	2	0.40	0	0.00	1	0.20
Ls-GT2 vs. Ls-GT4	490	4	0.81	4	0.81	0	0.00	0	0.00
Ls-GT3 vs. Ls-GT4	491	3	0.61	2	0.40	0	0.00	1	0.20
L. (*Stagnicola*) *turricula*									
St-GT1 vs. St-GT2	473	3	0.63	2	0.42	1	0.21	0	0.00
L. (*Stagnicola*) *fuscus*									
Sf-GT1 vs. Sf-GT2	472	1	0.21	0	0.00	1	0.21	0	0.00
Sf-GT1 vs. Sf-GT3	473	9	1.90	2	0.42	1	0.21	6	1.27
Sf-GT1 vs. Sf-GT4	473	12	2.53	3	0.63	3	0.63	6	1.27
Sf-GT2 vs. Sf-GT3	473	10	2.11	3	0.63	1	0.21	6	1.27
Sf-GT2 vs. Sf-GT4	473	11	2.32	3	0.63	2	0.42	6	1.27
Sf-GT3 vs. Sf-GT4	468	2	0.42	0	0.00	2	0.42	0	0.00
Omphiscola glabra									
Og-GT1 vs. Og-GT2	481	7	1.45	1	0.21	3	0.62	3	0.62
Radix auricularia									
Ra-GT1 vs. Ra-GT2	403	2	0.49	0	0.00	0	0.00	2	0.49
Ra-GT1 vs. Ra-GT3	406	9	2.21	3	0.74	1	0.25	5	1.23
Ra-GT1 vs. Ra-GT4	404	4	0.99	0	0.00	1	0.25	3	0.74
Ra-GT1 vs. Ra-GT5	402	1	0.25	0	0.00	0	0.00	1	0.25
Ra-GT2 vs. Ra-GT3	406	7	1.72	3	0.74	1	0.25	3	0.74
Ra-GT2 vs. Ra-GT4	404	2	0.49	0	0.00	1	0.25	1	0.25
Ra-GT2 vs. Ra-GT5	406	3	0.74	0	0.00	0	0.00	3	0.74
Ra-GT3 vs. Ra-GT4	406	7	1.72	3	0.74	2	0.49	2	0.49
Ra-GT3 vs. Ra-GT5	406	8	1.97	3	0.74	1	0.25	4	0.98
Ra-GT4 vs. Ra-GT5	404	3	0.74	0	0.00	1	0.25	2	0.49
Radix peregra (= *R. ovata*; = *R. balthica*)									
Rp-GT1 vs. Rp-GT2	395	3	0.76	2	0.50	1	0.25	0	0.00
Rp-GT1 vs. Rp-GT3	395	4	1.01	0	0.00	4	1.01	0	0.00
Rp-GT2 vs. Rp-GT3	395	5	1.26	2	0.50	3	0.76	0	0.00
Radix labiata									
Rl-GT1 vs. Rl-GT2	370	1	0.27	1	0.27	0	0.00	0	0.00
Radix lagotis									
Rla-GT1 vs. Rla-GT2	378	1	0.26	0	0.00	1	0.26	0	0.00
Galba truncatula									
Gt-GT1 vs. Gt-GT2	401	1	0.25	1	0.25	0	0.00	0	0.00

[a] GT: genotype.

Table 3
ITS-2 sequence differences detected in pairwise comparisons between proximal species

Compared species	Alignment length Number of bp	Nucleotide differences		Substitutions				Insertions + deletions	
		Number	Percentage	Transitions		Transversions		Number	Percentage
				Number	Percentage	Number	Percentage		
Species of the subgenus *Stagnicola*									
S. palustris vs. *S. turricula*	473	2–3	0.42–0.63	1	0.21	1–2	0.21–0.42	0	0.00
S. palustris vs. *S. fuscus*	479	19–24	3.96–5.01	4–5	0.83–1.04	7–9	1.46–1.88	7–11	1.46–2.29
S. palustris vs. *S. corvus*	485	36	7.42	9	1.85	14	2.88	13	2.68
S. turricula vs. *S. fuscus*	479	21–27	4.38–5.63	6	1.25	8–10	1.67–2.09	7–11	1.46–2.29
S. turricula vs. *S. corvus*	485	38–39	7.83–8.04	8–10	1.65–2.06	15–18	3.09–3.71	13	2.68
S. fuscus vs. *S. corvus*	489	36–42	7.36–8.59	11	2.24	9–11	1.84–2.25	16–22	3.27–4.50
Species of the genus *Radix*									
R. peregra vs. *R. labiata*	401	60–67	14.96–16.71	12–17	2.99–4.24	11–14	2.74–3.49	37	9.22
R. peregra vs. *R. lagotis*	396	38–42	9.59–10.60	9–11	2.27–2.77	10–14	2.52–3.53	19	4.80
R. peregra vs. *R. ampla*	395	27–30	6.83–7.59	11–13	2.78–3.29	8–10	2.02–2.53	8	2.02
R. peregra vs. *Radix* sp.	402	68–69	16.91–17.16	15–16	3.73–3.98	18–19	4.48–4.72	34	8.46
R. labiata vs. *R. lagotis*	384	45–50	11.72–13.02	16–19	4.16–4.95	9–13	2.34–3.38	20	5.21
R. labiata vs. *R. ampla*	393	58–59	14.76–15.01	13–14	3.31–3.56	16	4.07	29	7.38
R. labiata vs. *Radix* sp.	378	55–56	14.55–14.81	22–23	5.82–6.08	20	5.29	13	3.44
R. lagotis vs. *R. ampla*	388	27	6.96	9–10	2.32–2.57	6–7	1.54–1.80	11	2.83
R. lagotis vs. *Radix* sp.	390	54	13.84	12–13	3.07–3.33	21–22	5.38–5.64	20	5.13
R. ampla vs. *Radix* sp.	393	56	14.25	14	3.56	16	4.07	26	6.61

(mean 58.5%). The mean nucleotide compositions of the 31 different sequences were: A, 17.2%; C, 30.5%; G, 28.0%; T, 24.3%.

Several populations originally classified as belonging to different species showed identical ITS-2 sequences, and other populations originally classified as pertaining to the same species presented different ITS-2 sequences (compare preliminary classifications in Table 1 with results in Tables 2 and 3). Sometimes the sequence differences were very few, suggesting intraspecific variability. But sometimes differences detected among populations classified as pertaining to the same species were numerous, sufficient as to consider different species involved. Moreover, the number of sequence differences between species appeared sometimes lower than that between populations of the same species.

Consequently, additional specimens from the European populations were again anatomically studied and appropriately compared, and finally definitive species classifications were obtained enabling population groupings according to sequence similarities (Tables 1–3).

The lymnaeid from Wisconsin is very close to American stagnicoline species. It only differs from *S. emarginata* by two transitions, from *S. elodes* by two transitions and one transversion, and from *S. catascopium* by three transitions, one transversion and four insertions/deletions.

When comparing the different sequences in alignments, several interesting microsatellites were found. Polymorphic microsatellites presenting a different number of repeats, related to different sequence lengths between populations among a given species or proximal species group, were only found in *R. auricularia* and in the American stagnicolines. In *R. auricularia*, the genotypes GT2, GT3 and GT4 present $(GT)_6$, whereas GT1 and GT5 have only $(GT)_5$ between positions 108 and 119 of the alignment of all *R. auricularia* populations. In the alignment of the American stagnicoline species, including the lymnaeid from Wisconsin and excluding *H. caperata*, the interrupted microsatellite (AT) showed a different number of repeats between positions 431 and 438: *S. catascopium* has $(AT)_2$, whereas *S. emarginata*, *S. elodes* and the lymnaeid from Wisconsin have $(AT)_4$; the final part of the ITS-2 sequence, where these microsatellite repeats are found, is absent in *H. caperata*.

In the 581-position-long alignment obtained when comparing the 31 ITS-2 sequences representing all lymnaeid species and populations examined plus four ITS-2 sequences of North American stagnicolines, 247 sites were constant and 311 were parsimony-informative. Gaps, indicating insertions and deletions, were present throughout the sequences, although there was a block of 126 positions at the middle of the sequence alignment (between positions 249 and 375) in which sequences had no, or only a very few, gaps. This block corresponds to a conserved central region present in all lymnaeids analysed, North American stagnicoline species included. Variable sites were mainly located in both lateral regions corresponding to the 5′ and 3′ ends of the ITS-2 sequence. Similarities detected were 9.64% in the 5′ region (249 bp), 73.01% in the central region (126 bp), and 4.85% in the 3′ region (206 bp).

Two microsatellites found in this conserved central region are worth mentioning. All European *Radix* populations presented $(GT)_2$ in positions 284–288 and $(GGC)_3$ in positions 324–332, whereas all other lymnaeids presented only $(GT)_1$ and $(GGC)_2$ in the same respective positions (the sequence

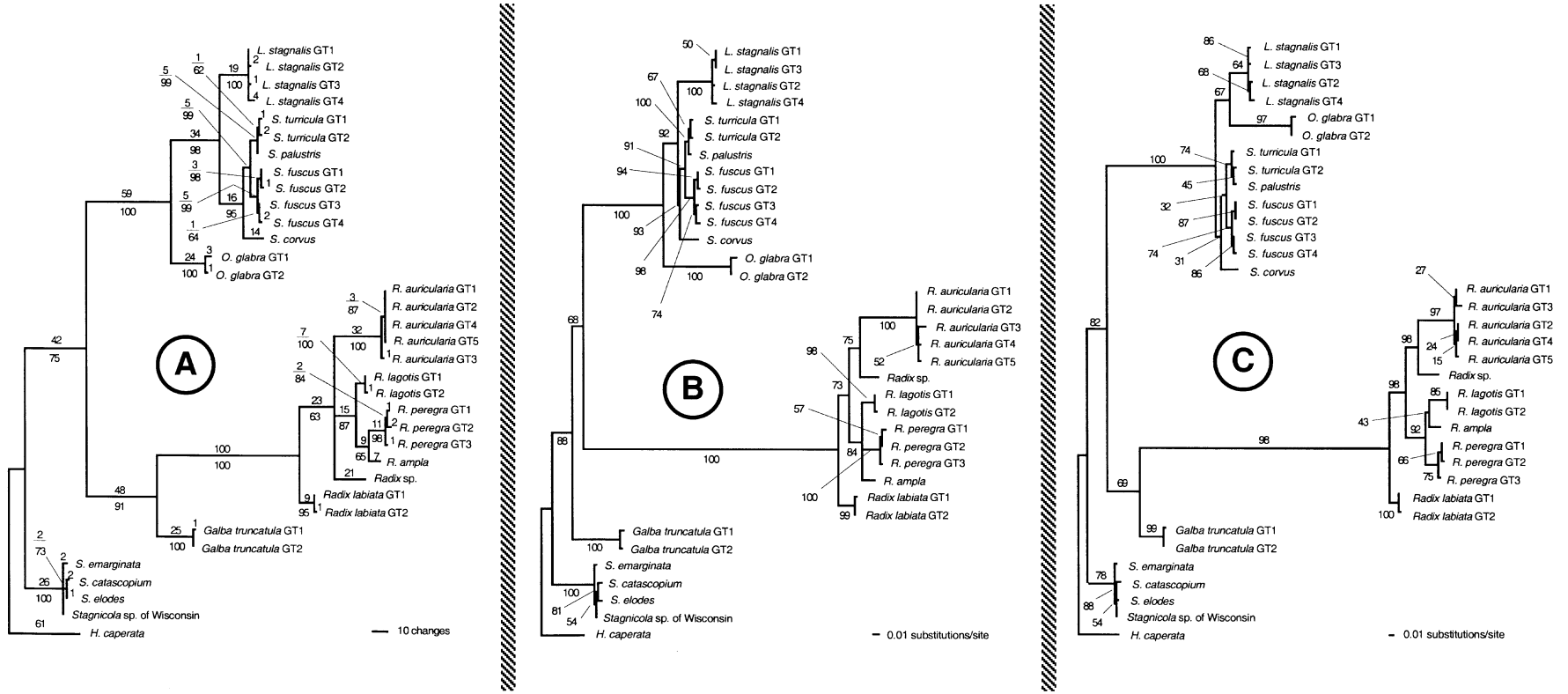

Fig. 1. Phylogenetic trees of the lymnacid species studied, obtained using *Hinkleyia caperata* as outgroup: (A) based on MP analysis using the heuristic option; numbers above the line indicate branch lengths (steps); numbers below the line represent the percentage of 1000 bootstrap replicates; (B) inferred from the distance data (K-2 parameter) using the NJ method; scale bar indicates the number of substitutions per sequence position; numbers indicate the frequency of a particular branch cluster in 1000 bootstrap replicates; (C) derived from the ML model; scale bar indicates the number of substitutions per sequence position; numbers represent the percentage of 1000 puzzling replicates.

Table 4
Pairwise comparisons of nucleotide divergences according to K-2 parameter model for the whole set of 35 ITS-2 sequences from the lymnaeid populations analyzed[a]

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Ls-GT1	–	0.00409	0.00204	0.00818	0.07889	0.08102	0.07676	0.08955	0.09168	0.08172	0.08387	0.08805	0.16241	0.15385	0.49280	0.49280	0.49857	0.49425
2	Ls-GT2	2	–	0.00611	0.00815	0.08298	0.08511	0.08085	0.09149	0.09362	0.08369	0.08584	0.08577	0.16628	0.15777	0.48851	0.48851	0.49429	0.48997
3	Ls-GT3	1	3	–	0.00611	0.07856	0.08068	0.07643	0.09130	0.09130	0.08137	0.08351	0.08977	0.16359	0.15509	0.49284	0.49284	0.49858	0.49429
4	Ls-GT4	4	4	3	–	0.08085	0.08298	0.07872	0.09149	0.09149	0.08155	0.08369	0.08577	0.16859	0.16009	0.48851	0.48851	0.49429	0.48997
5	St-GT1	37	39	37	38	–	0.00633	0.00422	0.03212	0.03212	0.02778	0.02778	0.05074	0.17340	0.16507	0.46705	0.46705	0.47293	0.46857
6	St-GT2	38	40	38	39	3	–	0.00633	0.03426	0.03426	0.02991	0.02991	0.05285	0.17577	0.16746	0.46705	0.46705	0.47293	0.46857
7	Sp	36	38	36	37	2	3	–	0.02784	0.02784	0.02350	0.02778	0.04651	0.17102	0.16268	0.46705	0.46705	0.47293	0.46857
8	Sf-GT1	42	43	43	43	15	16	13	–	0.00211	0.00855	0.01282	0.04915	0.15663	0.15217	0.47674	0.47674	0.48266	0.47826
9	Sf-GT2	43	44	43	43	15	16	13	1	–	0.00855	0.01282	0.04915	0.15663	0.15217	0.47674	0.47674	0.48266	0.47826
10	Sf-GT3	38	39	38	38	13	14	11	4	4	–	0.00426	0.04904	0.15904	0.15459	0.47384	0.47384	0.47977	0.47536
11	Sf-GT4	39	40	39	39	13	14	13	6	6	2	–	0.05330	0.15663	0.15217	0.47965	0.47965	0.48555	0.48116
12	Sc	42	41	43	41	24	25	22	23	23	23	25	–	0.17290	0.16471	0.46705	0.46705	0.47009	0.46571
13	Og-GT1	70	72	71	73	73	74	72	65	65	66	65	74	–	0.00835	0.50700	0.50700	0.50833	0.50838
14	Og-GT2	66	68	67	69	69	70	68	63	63	64	63	70	4	–	0.50000	0.50000	0.50140	0.50141
15	Ra-GT1	171	170	172	170	163	163	163	164	164	163	165	163	181	177	–	0.00000	0.00995	0.00000
16	Ra-GT2	171	170	172	170	163	163	163	164	164	163	165	163	181	177	0	–	0.00990	0.00000
17	Ra-GT3	174	173	175	173	166	166	166	167	167	166	168	165	183	179	4	4	–	0.00988
18	Ra-GT4	172	171	173	171	164	164	164	165	165	164	166	163	182	178	0	0	4	–
19	Ra-GT5	172	171	173	171	164	164	164	165	165	164	166	163	182	178	0	0	4	0
20	Rl-GT1	149	149	149	148	139	139	139	143	143	142	144	140	158	157	48	49	51	50
21	Rl-GT2	148	148	148	147	138	138	138	142	142	141	143	139	157	156	47	48	50	49
22	Rla-GT1	152	152	153	152	145	145	145	146	146	145	147	146	164	160	50	50	51	50
23	Rla-GT2	153	153	154	153	146	146	146	147	147	146	148	147	164	160	50	50	51	50
24	Rp-GT1	160	159	160	159	152	152	152	153	153	152	153	158	173	168	58	58	60	59
25	Rp-GT2	159	158	159	158	151	151	151	152	152	151	152	157	172	167	58	58	60	59
26	Rp-GT3	159	158	159	158	151	151	151	152	152	151	152	157	172	167	56	56	58	57
27	Ram	156	155	156	155	149	149	149	150	150	149	149	151	162	160	53	53	54	53
28	Rsp	148	147	148	147	144	144	144	146	146	145	147	143	159	157	41	41	42	42
29	Gt-GT1	99	99	101	101	100	102	101	97	97	97	98	104	94	93	135	135	137	136
30	Gt-GT2	98	98	100	100	99	101	100	96	96	96	97	103	93	92	134	134	136	135
31	Semar	122	122	123	124	112	113	113	105	105	106	107	120	105	106	140	140	142	141
32	Scata	122	122	123	124	112	113	113	105	105	106	107	119	107	108	141	141	143	142
33	Selod	123	123	124	125	113	114	114	106	106	107	108	121	107	108	140	140	142	141
34	RspW	121	121	122	123	111	112	112	104	104	105	106	119	106	107	140	140	142	141
35	Hcape	109	109	110	111	95	97	96	95	95	95	96	106	117	117	154	154	159	155

Table 4 (*Continued*)

		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	Ls-GT1	0.49425	0.44478	0.44179	0.45104	0.45401	0.45845	0.45559	0.45559	0.45217	0.44985	0.25515	0.25258	0.28438	0.28571	0.28671	0.28205	0.27387
2	Ls-GT2	0.48997	0.44478	0.44179	0.44970	0.45266	0.45429	0.45143	0.45143	0.44798	0.44681	0.25385	0.25128	0.28306	0.28438	0.28538	0.28074	0.27250
3	Ls-GT3	0.49429	0.44478	0.44179	0.45266	0.45562	0.45584	0.45299	0.45299	0.44957	0.44985	0.25831	0.25575	0.28472	0.28605	0.28704	0.28241	0.27500
4	Ls-GT4	0.48997	0.44179	0.43881	0.44970	0.45266	0.45429	0.45143	0.45143	0.44798	0.44681	0.25897	0.25641	0.28770	0.28904	0.29002	0.28538	0.27750
5	St-GT1	0.46857	0.41617	0.41317	0.42899	0.43195	0.43182	0.42898	0.42898	0.42939	0.43769	0.25641	0.25385	0.26730	0.26923	0.26969	0.26492	0.24548
6	St-GT2	0.46857	0.41617	0.41317	0.42899	0.43195	0.43182	0.42898	0.42898	0.42939	0.43769	0.26154	0.25897	0.26969	0.27163	0.27208	0.26730	0.25065
7	Sp	0.46857	0.41617	0.41317	0.42899	0.43195	0.43182	0.42898	0.42898	0.42939	0.43769	0.25897	0.25641	0.26969	0.27163	0.27208	0.26730	0.24806
8	Sf-GT1	0.47826	0.42943	0.42643	0.43713	0.44012	0.44092	0.43804	0.43804	0.43860	0.44923	0.25260	0.25000	0.25424	0.25610	0.25666	0.25182	0.24934
9	Sf-GT2	0.47826	0.42943	0.42643	0.43713	0.44012	0.44092	0.43804	0.43804	0.43860	0.44923	0.25260	0.25000	0.25424	0.25610	0.25666	0.25182	0.24934
10	Sf-GT3	0.47536	0.42643	0.42342	0.43413	0.43713	0.43804	0.43516	0.43516	0.43567	0.44615	0.25260	0.25000	0.25604	0.25791	0.25845	0.25362	0.24934
11	Sf-GT4	0.48116	0.43243	0.42943	0.44012	0.44311	0.44092	0.43804	0.43804	0.43567	0.45231	0.25521	0.25260	0.25845	0.26034	0.26087	0.25604	0.25197
12	Sc	0.46571	0.41916	0.41617	0.43195	0.43491	0.44507	0.44225	0.44225	0.43516	0.43465	0.26463	0.26209	0.27907	0.27934	0.28140	0.27674	0.26904
13	Og-GT1	0.50838	0.47164	0.46866	0.48094	0.48094	0.49288	0.49003	0.49003	0.46552	0.47181	0.23980	0.23724	0.24941	0.25416	0.25416	0.25178	0.27725
14	Og-GT2	0.50141	0.46866	0.46567	0.47337	0.47337	0.48276	0.47989	0.47989	0.46377	0.47006	0.23907	0.23650	0.25298	0.25776	0.25776	0.25537	0.27924
15	Ra-GT1	0.00000	0.13115	0.12842	0.13298	0.13298	0.15223	0.15223	0.14698	0.13984	0.11051	0.43408	0.43087	0.43478	0.43789	0.43478	0.43478	0.45161
16	Ra-GT2	0.00000	0.13351	0.13079	0.13228	0.13228	0.15144	0.15144	0.14621	0.13911	0.11051	0.43408	0.43087	0.43478	0.43789	0.43478	0.43478	0.45161
17	Ra-GT3	0.00993	0.13859	0.13587	0.13456	0.13456	0.15625	0.15625	0.15104	0.14136	0.11290	0.43910	0.43590	0.43963	0.44272	0.43963	0.43963	0.46221
18	Ra-GT4	0.00000	0.13587	0.13315	0.13193	0.13193	0.15365	0.15365	0.14844	0.13874	0.11290	0.43590	0.43269	0.43653	0.43963	0.43653	0.43653	0.45322
19	Ra-GT5	–	0.13351	0.13079	0.13263	0.13263	0.15445	0.15445	0.14921	0.13947	0.11290	0.43590	0.43269	0.43653	0.43963	0.43653	0.43653	0.45322
20	Rl-GT1	49	–	0.00270	0.10221	0.10221	0.10137	0.10137	0.09315	0.09642	0.10335	0.38095	0.37755	0.39542	0.39869	0.40196	0.39542	0.42991
21	Rl-GT2	48	1	–	0.10497	0.10497	0.10411	0.10411	0.09589	0.09917	0.10615	0.37755	0.37415	0.39216	0.39542	0.39869	0.39216	0.42679
22	Rla-GT1	50	37	38	–	0.00264	0.05556	0.05556	0.05291	0.05291	0.10440	0.41528	0.41196	0.42765	0.43087	0.42765	0.42765	0.43598
23	Rla-GT2	50	37	38	1	–	0.05820	0.05820	0.05556	0.05556	0.10440	0.41860	0.41528	0.43087	0.43408	0.43087	0.43087	0.43902
24	Rp-GT1	59	37	38	21	22	–	0.00758	0.01010	0.05412	0.10714	0.41325	0.41325	0.44817	0.45399	0.45122	0.44817	0.44713
25	Rp-GT2	59	37	38	21	22	3	–	0.01263	0.05670	0.10714	0.40694	0.40379	0.44207	0.44785	0.44512	0.44207	0.44411
26	Rp-GT3	57	34	35	20	21	4	5	–	0.04897	0.10440	0.41325	0.41009	0.44207	0.44785	0.44512	0.44207	0.44411
27	Ram	53	35	36	20	21	21	22	19	–	0.10989	0.40645	0.40323	0.43125	0.43437	0.43125	0.43125	0.43769
28	Rsp	42	37	38	38	38	39	39	38	40	–	0.40136	0.39796	0.41447	0.41776	0.42105	0.41447	0.43210
29	Gt-GT1	136	112	111	125	126	131	129	131	126	118	–	0.00249	0.17188	0.16754	0.17448	0.16927	0.17857
30	Gt-GT2	135	111	110	124	125	131	128	130	125	117	1	–	0.16927	0.16492	0.17188	0.16667	0.17582
31	Semar	141	121	120	133	134	147	145	145	138	126	66	65	–	0.01126	0.01116	0.00446	0.14677
32	Scata	142	122	121	134	135	148	146	146	139	127	64	63	5	–	0.00676	0.00676	0.14925
33	Selod	141	123	122	133	134	148	146	146	138	128	67	66	5	3	–	0.00670	0.14925
34	RspW	141	121	120	133	134	147	145	145	138	126	65	64	2	3	3	–	0.14677
35	Hcape	155	138	137	143	144	148	147	147	144	140	65	64	59	60	60	59	–

[a] Below diagonal: total character differences; above diagonal: mean character differences. For codes of European species and RspW see Table 1; Semar: *Stagnicola emarginata*; Scata: *S. catascopium*; Selod: *S. elodes*; Hcape: *Hinkleyia caperata*.

GGCGGTGGT of *H. caperata* in the positions 324–332 is the only exception and should be revised).

3.2. Phylogenetic analyses

Different outgroups were assayed. The most consistent phylogenetic topologies were obtained by using *S.* (*Hinkleyia*) *caperata* or *L.* (*G.*) *truncatula* as outgroups. The convenience of using the first as outgroup lies in that North American lymnaeids appear clustered and in a clade different from those of European and Asian species in phylogenetic trees inferred from mitochondrial rDNA 16S sequences (Remigio and Blair, 1997a) and in the generic validity of *Hinkleyia* proved by ITS-1 and ITS-2 sequences (Remigio and Blair, 1997b). The appropriateness of using *L.* (*G.*) *truncatula* is related to the fact that *Galba* is the earliest lymnaeid represented in the fossil record (Zilch, 1959–1960). At any rate, phylogenetic trees obtained in both cases showed similar branch distributions and bootstrap supports.

When using *S.* (*H.*) *caperata* as outgroup, parsimony analysis, using the heuristic option, of the aligned sequences yielded a single most-parsimonious tree (Fig. 1A). The tree obtained was 659 steps long. The consistence index (CI) and the homoplasy index (HI) were 0.778 and 0.221, respectively. CI and HI excluding uninformative characters were 0.768 and 0.231, respectively. The retention index (RI) and the rescaled consistency index (RC) were 0.946 and 0.736, respectively. The North American stagnicolines formed a monophyletic unit different from that of the European lymnaeids. The European species clustered into two groupings. In one branch supported by a 91% bootstrap *G. truncatula* was located basal to the *Radix* grouping; all species included in *Radix* appeared clustered together and supported by a 100% bootstrap value; in this clade, *Radix labiata* appeared basal to the other species included in a paraphyly, with *R. auricularia* in a branch, *R. lagotis*, *R. peregra* and *R. ampla* in a second branch and *Radix* sp. in a third one. In another branch, species of *Lymnaea s. str*., *Stagnicola* (including *S. corvus* basal to *S. turricula*, *S. palustris* and *S. fuscus*) and *Omphiscola* clustered together with a 100% bootstrap, *O. glabra* appearing basal.

The topology of the phylogenetic tree derived from the distance data (Table 4) and bootstrap values using the NJ method (Fig. 1B) were similar to those of the MP tree (Fig. 1A). Slight differences were only found in *G. truncatula*, which appeared basal to the dicotomy of *Radix* and *Lymnaea s. str*.*–Stagnicola–Omphiscola*, and in the *Radix* branch, in which *Radix* sp. clustered with *R. auricularia*.

Maximum-likelihood analyses generated a tree (likelihood is equal to −3681.70185) with topology (Fig. 1C) similar to those from parsimony and distance analyses. The number of quartets examined was 52360 using least-squares method with ML distances. Frequency distribution of lengths of 1000 evaluated random trees provided $g1 = -0.5474$ and $g2 = -0.1802$ values indicating that the data set contained a significant degree of cladistic structure (Hillis and Bull, 1993). *G. truncatula* appeared in the same branch of *Radix* (as in MP), *Radix* sp. clustered with *R. auricularia* (as in NJ), and *O. glabra* appeared in a branch together with *Lymnaea stagnalis*, stagnicolines appearing thus basal to the latter *Lymnaea s. str*.*–Omphiscola* branch.

When using *G. truncatula* as outgroup, the characteristics of the MP, NJ and ML analyses were similar to those obtained by using *H. caperata* as outgroup. Supports were also very high and topologies concerning European lymnaeids were identical to those obtained in the corresponding methods when using *H. caperata* as outgroup. In the trees obtained by using *G. truncatula* as outgroup, the American stagnicolines appeared always monophyletic, although their location differed according to the method: in the same branch of and basal to the European *Lymnaea s. str*.*–Stagnicola–Omphiscola* grouping in MP and ML, and basal to all remaining European lymnaeids in NJ.

4. Discussion

4.1. The rDNA ITS-2 marker in lymnaeids

The results of sequence comparisons and MP, distance and ML analyses, indicate that the ITS-2 spacer is a useful marker for resolving supraspecific, specific and population relationships in Lymnaeidae, even in spite of the G + C bias. A similar G + C bias was already found in both the ITS-1 (57.0–57.9%) and ITS-2 (59.8–60.8%) of the four North American stagnicoline lymnaeid species by Remigio and Blair (1997b). This G + C bias contrasts with the absence of G + C bias (51.5–51.8%) found in the 18S nuclear rDNA sequences and with the A + T bias (66–73%) found in the 16S mitochondrial rDNA sequences of lymnaeids (Bargues and Mas-Coma, 1997; Bargues et al., 1997; Remigio and Blair, 1997a). In general, balanced DNA regions seem better for taxonomic and phylogenetic studies since substitutions become more easily detectable when bases occur with approximately similar frequency (25%) (Luton et al., 1992). Although the G + C composition of internal transcribed spacers tends to be around 50% in many organisms (e.g. Ellis et al., 1986; Luton et al., 1992; Morgan and Blair, 1995), nucleotide compositions biased towards G + C or A + T have often been found in the ITSs. Examples of ITS-2 biased towards G + C include 88% in *Xenopus laevis* (Torres et al., 1990), 83% in humans (Gonzalez et al., 1990), and between 60 and 80% in angiosperms (Hershkovitz and Zimmer, 1996).

The lengths of the lymnaeid ITS-2 spacers sequenced varied between a very large range (Table 1), although it falls within the range reported for various animals (Luton et al., 1992; Remigio and Blair, 1997b; Mas-Coma, 1999). The existence of three lymnaeid groups according to ITS-2 lengths, with *Galba* included in that presenting the shortest sequences, is worth noting. The oldest lymnaeid fossil

known is *Galba* from the Jurassic (Zilch, 1959–1960), which suggests that a shorter ITS-2 would be the plesiomorphic condition and that an increase in ITS-2 length occurred during lymnaeid evolution. This agrees with the general pattern known in ITSs of eucaryothes, in which the spacers become shorter when going deep in the evolutionary branches, so that they can be of only a few tenths of bp in protozoans and up to even the ITS-2 being absent (Mas-Coma, 1999). In this way, *Radix* and *Galba* may be considered the oldest taxa (370–406 bp lengths), and *Lymnaea s. str*., European *Stagnicola* and *Omphiscola* (468–491 bp lengths) the most recent, American *Stagnicola* and *Hinkleyia*, including the lymnaeid from Wisconsin, being intermediate (434–450 bp lengths). Interestingly, this hypothesis fully agrees with the only previously published phylogeny of lymnaeids proposed by Inaba (1969) basing on palaeontological data, chromosome numbers and radular dentition.

Usually different species included in the same subgenus or genus present ITS-2 sequences of the same or very similar size and, when length variation appear, it is usually related to different microsatellite repeat numbers (Mas-Coma, 1999; Almeyda-Artigas et al., 2000b; Marcilla et al., 2001). These are the cases of the species studied in *Lymnaea s. str*., European *Stagnicola*, and *Omphiscola*, and also American stagnicolines, although the length (484 bp) in *S. corvus* is a little far away from the lengths of the *S. turricula–S. palustris–S. fuscus* group (468–473 bp). However, this is not the case of the *Radix* populations studied here, in which the pronounced ITS-2 length variation (370–406 bp) is surprising. The high number of insertions/deletions in *Radix* may also be related to the old origin of this taxon.

4.2. Differentiation of populations and species

The analysis of genetic distances and sequence differences found between the distinct populations and taxa studied allows us to distinguish the upper limit to be expected within a single species and to how different sister species can be expected to be at rDNA ITS-2 sequence level.

4.2.1. Conspecific entities

As example of conspecific entities we can use the numerous widely geographically distributed populations studied within a species (Table 1). Absolute ITS-2 nucleotide differences detected in pairwise comparisons between proximal populations among a species ranged between 1 and 12 (0.21–2.53% in two-sequence alignments of 370–491 bp lengths), including 0–4 transitions (0.00–0.81%), 0–4 transversions (0.00–1.01%) and 0–6 insertions + deletions (0.00–1.27%) (Table 2). Genetic distances in pairwise comparisons between these populations ranged from 0.00000 to 0.01282, corresponding to 0–6 total character differences (0.00–1.26%) according to PAUP (Table 4).

These data confirm the conclusions of Remigio and Blair (1997b), who considered *S. catascopium*, *S. elodes* and *S. emarginata* to be conspecific according to the very few differences in their ITS-1 and ITS-2 sequences: only 2.23% absolute differences in their 448 bp-long ITS-2 alignment, including 1.34% substitutions and 0.89% insertions + deletions. This agrees with results previously obtained using morphological, ecological and allozyme data (Hubendick, 1951; Clarke, 1973; Rudolph and Burch, 1989). Moreover, the very few differences between these American stagnicolines and the lymnaeid from Wisconsin indicate that the latter must be classified as *S. catascopium* (synonym: *S. elodes* and *S. emarginata*).

Similarly, the very few absolute differences in the ITS-2 sequence (0.42–0.63%) and the small genetic distances (0.00422–0.00633) between *S. palustris* and *S. turricula* (see Tables 3 and 4) indicate that the latter must be considered a synonym of the first, although it could be retained as subspecies of *S. palustris* according to its anatomy (morphological characteristics of the male copulatory apparatus) and delimited geographical distribution (Danube valley in Germany, Austria and Hungary; Bieszczady Mountains, SE Poland) (Falkner, 1984, 1985; Jackiewicz, 1989a, 1996; Kilias, 1992; Glöer and Meier-Brook, 1998; Rybska et al., 2000).

Among European *L. stagnalis*, Remigio and Blair (1997a) suggested that the Italian population from Lago di Trasimeno could belong to a taxon different than that of the German population from Bad Münstereifel, owing to their sequence divergence and genetic distance detected in the 16S mitochondrial rRNA gene. These authors further speculated about the Alpine orogeny having influenciated the geographic separation of both entities. The study of ITS-2 sequences of different European *L. stagnalis* populations demonstrate that nucleotide differences and genetic distances are not sufficient as to differentiate them in different taxa, and, moreover, prove that the same Italian genotype (GT4) is also present in France (Indre) and southern Germany (Munich).

Where ITS sequences are almost identical, the entities in question may be so closely related that gene flow can occur between them, as in trematodes (Despres et al., 1992; Morgan and Blair, 1995). The North American stagnicolines are apparently reproductively isolated according to cross-breeding experiments (Burch and Ayers, 1973), although selfing characteristics, well known in lymnaeids as well as in other hermaphrodite freshwater gastropods such as the planorbid *Bulinus* (Jarne and Delay, 1990b; Jarne et al., 1993), may not be forgotten. Genetic distances found in ITSs of *Bulinus* species belonging to the same *Bulinus* species group (Stothard et al., 1996) were greater (0.02) than that (0.005–0.011) seen among these three stagnicoline species (Remigio and Blair, 1997b).

4.2.2. Species and sister species

ITS-2 sequences confirm that *L. stagnalis*, *O. glabra* and *G. truncatula* are well-established lymnaeid species. Interestingly, European *G. truncatula* populations seem to be very homogeneous, contrarily to the cases of *L. stagnalis* and

O. glabra in which geographically separated populations show marked divergences (compare Tables 1 and 2).

As examples of sister species we can use very closely related taxa within the *Stagnicola* and the *Radix* groups (see Falkner, 1995; Jackiewicz, 1998; Glöer and Meier-Brook, 1998). Our ITS-2 sequencing results allow us to understand the actual systematic confusion in the family Lymnaeidae. Sequence results agree with Glöer and Meier-Brook (1998) in that, in Europe, the problems in specific classification of specimens and populations are mainly found in the *Stagnicola* and *Radix* species groups.

In *Stagnicola*, absolute ITS-2 nucleotide differences detected in pairwise comparisons between proximal species (excluding the comparison *S. palustris* versus *S. turricula*) ranged between 19 and 42 (3.96–8.59% in two-sequence alignments of 479–489 bp lengths), including 4–11 transitions (0.83–2.24%), 7–18 transversions (1.46–3.71%) and 7–22 insertions + deletions (1.46–4.50%) (Table 3). Genetic distances in pairwise comparisons between these populations ranged from 0.02350 to 0.05330, corresponding to 11–25 total character differences (2.29–5.14%) according to PAUP (Table 4). Nucleotide differences and genetic distances between *S. palustris* (including *S. turricula*), *S. fuscus* and *S. corvus* are sufficient to consider the three taxa as valid species. Interestingly, nucleotide divergences (Table 2) and genetic distances (Table 4) detected between the four different *S. fuscus* genotypes found are the highest among different genotypes belonging to a given species and may be interpreted as a process of geographic differentiation developping in the present (GT1 in France, GT2 in Germany, GT3 in Austria and Corsica, and GT4 in Spain).

In *Radix*, absolute ITS-2 nucleotide differences detected in pairwise comparisons between proximal species ranged between 27 and 69 (6.83–17.16% in two-sequence alignments of 378–402 bp lengths), including 9–23 transitions (2.27–6.08%), 6–22 transversions (1.54–5.64%) and 8–37 insertions + deletions (2.02–9.22%) (Table 3). Genetic distances in pairwise comparisons between these populations ranged from 0.04897 to 0.10989, corresponding to 19–40 total character differences (4.80–10.15%) according to PAUP (Table 4). These differences between *Radix* taxa are somewhat greater than those between *Stagnicola* taxa and agree with the hypothesis of their respective old and recent origins.

R. auricularia appears to be a species well separated from the other *Radix* taxa (Table 4). Marked divergences among different European populations of this species (genotypes GT1–GT5) are found (see Table 2) and remembers that already Hubendick (1951) considered *R. auricularia* to be a superspecies.

Nucleotide differences and genetic distances between *Radix* entities indicates that *R. ampla* may be considered a valid species (Tables 3 and 4), as already suggested by Falkner (1990a,b), despite the difficulties in its differentiation because of morphological similarities with other European *Radix* species (see Glöer and Meier-Brook, 1998).

Moreover, ITS-2 sequences demonstrate that, besides *R. auricularia* and *R. ampla*, there are at least other three *Radix* species in Europe and another fourth species in Turkey (see Tables 3 and 4):

1. One species includes the west-European populations studied from Iceland, The Netherlands, France and Spain; the Iceland population presented a sequence identical to that of snails from the latter three countries (GT1), suggesting a very recent introduction into Iceland, most probably from western Europe and related to human activities; this species is able to reach high altitudes of about 2000 m, as in the French Pyrenees; we ascribe this species to *R. peregra* (= *R. ovata*; = *R. balthica*).
2. Another species includes the populations systematically grouped under the taxon *R. peregra sensu* Ehrmann, 1933 and includes the population studied from Bad Münstereifel (Germany), a laboratory strain maintained in the Parasitology Department of the Charles University in Prague (Czech Republic), and the population from Bozdag, Söke (Turkey) whose 16S mitochondrial rDNA was also studied by Remigio and Blair (1997a); after reviewing all (more than 160) old names proposed for European species synonymized with *peregra* by Hubendick (1951), we decided to ascribe this species to the binomen *Radix labiata* (Rossmaessler, 1835) according to Falkner (personal communication).
3. A third species includes the central-European populations along the river Danube basin, in the Czech Republic and Schönau, near Vienna (Austria); according to Falkner (personal communication), we call this species *Radix lagotis* (Schrank, 1803).
4. A fourth species includes only the population from Söke (Turkey), which appears somewhat related to *R. auricularia* (see trees inferred from the NJ and ML methods); we prefer to leave the systematic classification of this species open (*Radix* sp.), pending the study of Asian lymnaeids.

4.3. Supraspecific level phylogeny and taxonomic classification according to ITS-2 sequences

All phylogenetic trees obtained show four clear clades: (A) species belonging to the *Lymnaea s. str*., European *Stagnicola* and *Omphiscola* groups; (B) species belonging to the *Radix* group; (C) *G. truncatula*; (D) the North American stagnicoline species.

4.3.1. The Lymnaea s. str., *European* Stagnicola *and* Omphiscola *clade*

Nucleotide differences and genetic distances between the species of *Lymnaea s. str*., European *Stagnicola* and *Omphiscola* are similar to those between the different *Radix* entities (Table 4). This fact is reflected in the trees, where all these species always cluster very closely together (Figs. 1 and 2). This generates doubts as to whether *Stagnicola* may be considered a valid genus or not. ITS-2 results

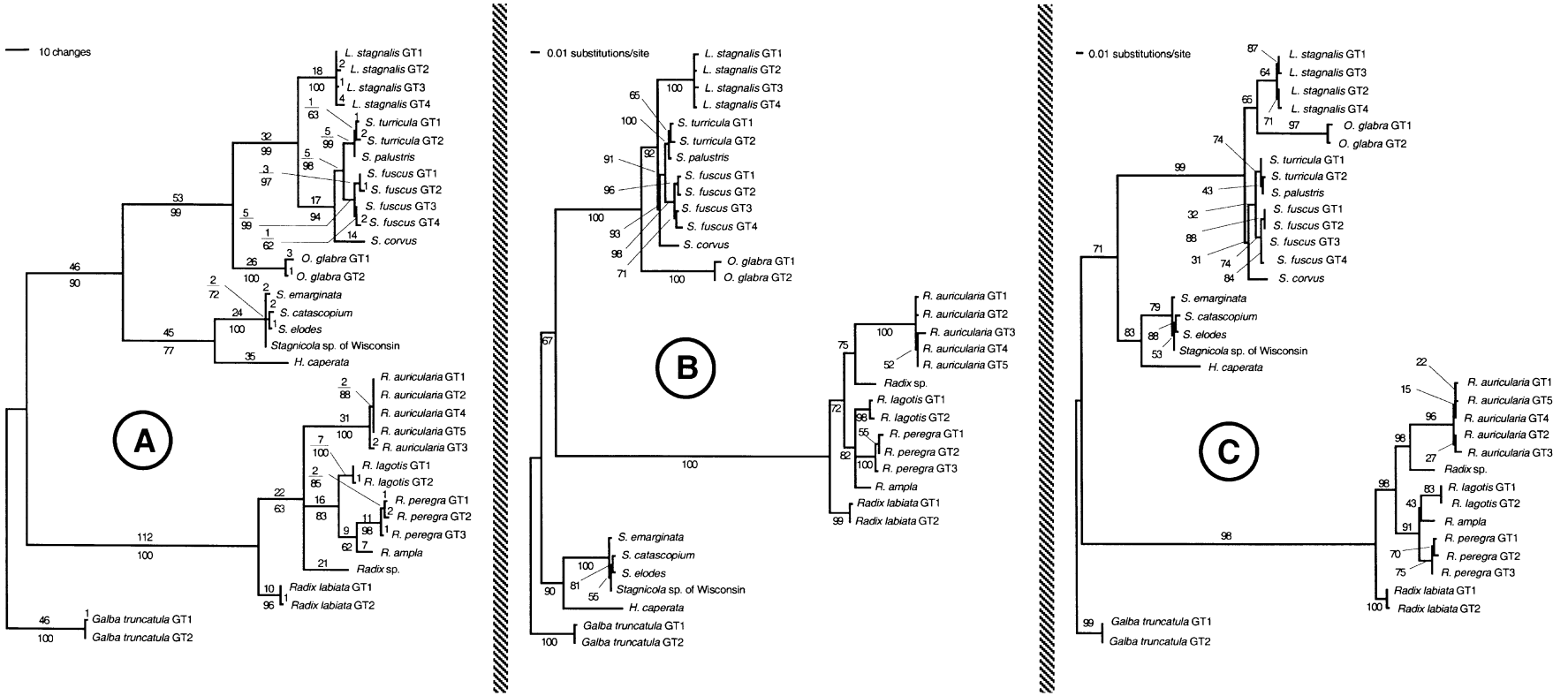

Fig. 2. Phylogenetic trees of the lymnaeid species studied, obtained using *Galba truncatula* as outgroup: (A) based on MP analysis using the heuristic option; numbers above the line indicate branch lengths (steps); numbers below the line represent the percentage of 1000 bootstrap replicates; (B) inferred from the distance data (K-2 parameter) using the NJ method; scale bar indicates the number of substitutions per sequence position; numbers indicate the frequency of a particular branch cluster in 1000 bootstrap replicates; (C) derived from the ML model; scale bar indicates the number of substitutions per sequence position; numbers represent the percentage of 1000 puzzling replicates.

suggest that retaining it as a subgenus of *Lymnaea* may be the most appropriate for the time being. Results inferred from the 18S rRNA gene suggested that *Stagnicola* does not merit genus status (Bargues and Mas-Coma, 1997) and a similar conclusion is obtained when 16S mitochondrial rDNA results are taken into account (Remigio and Blair, 1997a).

Similar arguments can be noted concerning *Omphiscola*. The different situation of *O. glabra* in the ML trees where it clusters with *L. stagnalis* (Figs. 1C and 2C), contrarily to its situation basal to *Lymnaea s. str*. and European *Stagnicola* in MP and NJ trees (Figs. 1A and B and 2A and B), also indicates that retaining it as a subgenus of *Lymnaea s. str*. could be appropriate. At any rate, nucleotide differences and genetic distances between *Omphiscola* and *Lymnaea s. str*. and between *Omphiscola* and *Stagnicola* are markedly higher than those between *Lymnaea s. str*. and *Stagnicola* (see Table 4) and genus status for *Omphiscola* may thus be justified, as already proposed by Jackiewicz (1989b, 1998).

4.3.2. *The* Radix *clade*

The monophyletic clustering of all European *Radix* species reflect the great nucleotide differences and genetic distances between them and all other lymnaeids (Table 4). This supports the taxonomic validity of the genus *Radix*, in which we will most probably be able to distinguish different evolutionary lines (subgenera?) once the sequences of other Asian and African species of this group become known. The ITS-2 results of a Wisconsin lymnaeid preliminarily classified as *Radix* species prove that in fact it was a stagnicoline species.

Nucleotide divergences and genetic distances between several *Radix* entities are even greater than those between *Lymnaea s. str*. and *Stagnicola* (Table 4). This *Radix* complexity was already suggested by the topology of the species *R. peregra* from Söke, Turkey (= *Radix* sp. in the present paper), *R. quadrasi* (von Moellendorff, 1898) from Philippines and *R. rubiginosa* (Michelin, 1831) from Malaysia (the latter two considered geographical races or subspecies of the widespread Eurasian *L. auricularia* by Hubendick, 1951) obtained in the phylogenetic tree inferred from partial sequences of the 16S mitochondrial rDNA (Remigio and Blair, 1997a).

4.3.3. *The* truncatula *clade*

G. truncatula shows a great number of sequence differences and high genetic distances when compared to the other lymnaeids (Table 4) and appears always alone in a clade. These results are similar to those obtained with complete 18S rDNA sequences (Bargues and Mas-Coma, 1997; Bargues et al., 1997) and support the necessity of genus status. There is a great confusion around the generic names proposed for *truncatula* and proximal species in the Americas (the Central American *cubensis*, the North American *humilis* and *bulimoides*, and the South American *viatrix* and *diaphana*, only to mention the most important), all of which share the capacity of transmitting *F. hepatica*.

The species *truncatula* has traditionally been included in *Galba* by European specialists (see Bargues and Mas-Coma, 1997; Jackiewicz, 1998; Glöer and Meier-Brook, 1998; only to mention the most recent review articles) following the review paper of Hubendick (1951) in which *truncatula* appears as its type species. However, a recent review of the original description (Schrank, 1803) allowed Bargues et al. (1997) to verify that *G. pusilla* Schrank, 1803 (and not *truncatula*) was initially proposed as type species for *Galba*. Regardless, *truncatula* was also proposed (see Hubendick, 1951) and has been extensively used (see Burch, 1982b) as type species for another lymnaeid genus (or subgenus): *Fossaria* Westerlund, 1885. Consequently, the taxon *Fossaria* should be retained with *truncatula* as type species.

Although usually included in the genus *Fossaria* by several American authors (Burch, 1982b; Malek, 1985; Burch and Cruz-Reyes, 1987; Cruz-Reyes and Malek, 1987), in fact *cubensis* was originally proposed as the type species of the genus *Nasonia* Baker, 1928 (see Hubendick, 1951), later also of the subgenus *Fossaria Bakerilymnaea* Weyrauch, 1964 (see Burch, 1982a,b; Burch and Cruz-Reyes, 1987), and even of the recently described subgenus *Lymnaea* (*Afrogalba*) Kruglov et Starobogatov, 1985 (Kruglov and Starobogatov, 1985). Significant nucleotide substitutions in helix E10-1 of the variable region V2 of the secondary structure of the 18S rRNA gene already suggested the need for separating *cubensis* from *truncatula* at least in a different subgenus (Bargues et al., 1997). ITS-2 sequencing studies now under way will clarify whether American species similar to *truncatula* merit a generic status independent from that of this European species or not. If supraspecific differentiation is needed, the taxons *Pectinidens* Pilsbry, 1911 (type species: *diaphana*), *Simpsonia* Baker, 1911 (type species: *humilis*) and *Pseudogalba* Baker, 1913 (new name for *Simpsonia*) (see Hubendick, 1951) should also be taken into account.

In the present paper *truncatula* is ascribed to *Galba* following the European tradition and pending the definitive systematic decision to be taken after the appropriate together analysis of the European and American species.

4.3.4. *The North American stagnicoline clade*

The North American stagnicoline species cluster together in all phylogenetic trees obtained. When using *H. caperata* as outgroup, they appear always external and basal to all European lymnaeids (Fig. 1A–C). When using *G. truncatula* as outgroup, they are again situated far away from the European stagnicolines in the NJ tree (Fig. 2B) and only basal to them in the MP and ML trees. These phylogenetic results, together with the great nucleotide differences and genetic distances (Table 4), suggest that North American and European stagnicolines do not belong to the same supraspecific taxon, despite shell morphology and visceral anatomy similarities which may be homoplasic. Similar

conclusions were reached in trees inferred from 16S mitochondrial rDNA sequences (Remigio and Blair, 1997a). Interestingly, nucleotide differences and genetic distances between American stagnicolines and *G. truncatula* are pronouncedly lower than those between American and European stagnicolines and similar to those between *Omphiscola* and *Lymnaea s. str*. and those between *Omphiscola* and European *Stagnicola* (Table 4).

The genus *Hinkleyia* Baker, 1928 (type species: *caperata*) may be used for the American stagnicolines. The conclusion reached by Remigio and Blair (1997b) after the study of the ITS-1, 5.8S and ITS-2 nuclear rDNA sequences about the convenience of ascribing *catascopium–elodes–emarginata* and *caperata* to two different genera does not seem appropriate now according to the nucleotide differences and genetic distances detected between the European supraspecific taxa (Table 4). Only a subgeneric differentiation could be justified at present.

4.4. Interest in disease epidemiology

At first intermediate host level, trematodes show a marked snail host specificity, from usually oioxenous (one digenean species/one snail species) or stenoxenous (one digenean species/a few, closely related snail species) to less frequently oligoxenous (one digenean species/numerous, family-, subfamily- or tribe-related snail species) (Wright, 1973; Kalbe et al., 1997; Adema and Loker, 1997). Moreover, variability studies on the susceptibility of a given snail species to infection by a given digenean species have recently shown that it may differ between snail populations as well as between individuals among a given snail population (Rollinson and Southgate, 1985; Adema and Loker, 1997). Compatibility differences between a given trematode species and different geographic populations of the same first intermediate snail host species are already known in several digenean species following an aquatic life cycle, such as for instance in species of *Schistosoma* (Preston and Southgate, 1994), *Fasciola* (Kendall and Parfitt, 1959; Boray, 1969, 1978; Perez-Reyes et al., 1985) and *Echinostoma* (Fried et al., 1987). Among lymnaeids, there are pronounced differences in susceptibility between snail populations that occur in close proximity to one another (Perez-Reyes et al., 1985), and some snail populations even show a total lack of susceptibility (Kendall and Parfitt, 1959).

The information which the ITS-2 marker furnishes is of applied interest concerning the molluscan host specificity of the different trematode species, owing to the ITS-2 capacity of distinguishing between proximal species and populations of lymnaeids. However, the ITS-2 marker does not seem to be useful for the differentiation of susceptible and resistant individuals among a lymnaeid population or susceptible and resistant populations among a given lymnaeid species, according to the results here obtained on different laboratory strains of *R. lagotis* reared in the Parasitology Department of the Charles University in Prague, among which individuals susceptible and others resistant to the infection by *Trichobilharzia regenti* are present. The results on *R. peregra* (= *R. ovata*; = *R. balthica*) GT1 snails from Lac d'Annecy (France) naturally infected and non-infected by ocellate cercariae unfortunately cannot be concluent.

4.4.1. Fasciolids

F. hepatica is present in the five continents. It has a preferred snail transmitting species in Europe: *G. truncatula*. However, there are other different European lymnaeid species which have also been found transmitting it under special natural conditions: *O. glabra* and *L.* (*S.*) *palustris*. In the laboratory *O. glabra*, *L.* (*S.*) *palustris* and *L.* (*S.*) *fuscus*, and even *L.* (*L.*) *stagnalis*, *R. peregra* and *M. glutinosa* can be extremely infected if miracidium infection takes place during the first few days of the snail's life, although a high mortality level is obtained (see reviews by Oviedo et al., 1996 and Dreyfuss et al., 2000). Natural infections with *F. hepatica* have also been reported in Polish populations of *L.* (*S.*) *occulta* and *L.* (*S.*) *p. turricula* (Czapski, 1962, 1977), although the status of these lymnaeid species as intermediate hosts of *F. hepatica* needs to be revised.

Other main or obligatory intermediate snail hosts mentioned for *F. hepatica* in other continents are: *G. truncatula* and *P. columella* in Africa; *F. humilis*, *F. bulimoides* and *F. cubensis* in North America; *F. cubensis* and *Pseudosuccinea columella* in Central America; *F. viatrix* (= *F. viator*), *L. diaphana*, *F. cubensis* and *G. truncatula* in South America; *G. truncatula* and *Austropeplea ollula* (*A. viridis*) in Asia; *L. tomentosa* in Australia; *L. tomentosa*, *P. columella* and *G. truncatula* in New Zealand; *A. ollula* in Hawaii, Papua New Guinea, Philippines and Japan. Alternate or facultative host species cited in other continents are: *P. columella* in North and South America; *P. columella* and *A. ollula* (= *A. viridis*) in Australia; and *L. gedrosiana* in Iran (Boray, 1982; Malek, 1985; Mas-Coma and Bargues, 1997).

F. gigantica is mainly distributed in Africa and Asia. Less important endemic areas of *F. gigantica* are the southern parts of Europe, Turkey, the near east, and some southern states of the old USSR, particularly Armenia, and has only been sporadically mentioned in North America. Principal or obligatory intermediate snail hosts mentioned for *F. gigantica* are: *R. natalensis* in Africa, *R. auricularia* sspp. in near east, middle east, far east and southern states of old USSR, *F. cubensis* in North America gulf coast, *R. rufescens* in Asia and Indian subcontinent, *R. rubiginosa* in the far east and Malaysia, *R. swinhoei* in south east Asia and Philippines, and *A. ollula* (= *A. viridis*) in Hawaii and Japan. Alternate or facultative host species are: *G. truncatula* in Africa, *R. peregra* in near east, middle east, and southern states of old USSR, *P. columella* in North America gulf coast, and *A. ollula* (= *A. viridis*) in the far east (Boray, 1982; Mas-Coma and Bargues, 1997). *R. caillaudi* (junior synonym of *R. natalensis*) in Egypt, *R. gedrosiana* in Iran, *R. euphratica* in Iraq, *R. luteola* in Nepal, and *R. bactriana*,

R. tenera and *R. subdisjuncta* in Turkmenia were more recently added (Mas-Coma and Bargues, 1997).

It has been suggested that in the early stages of digenean evolution, specificity with respect to the molluscan host was less marked than what is presently observed (Gibson and Bray, 1994). This marked *Fasciola*/lymnaeid species specificity suggests an old parasite–host relationship of *F. hepatica* with *Galba* (*Fossaria*) and *F. gigantica* with *Radix*. The monophyletisms shown by both *Galba* and *Radix* in our phylogenetic trees are in agreement with our knowledge on *Fasciola*/lymnaeid species relationships and suggest a parallel evolution of each liver fluke species with both different lymnaeid branches towards an increasing specificity. The phylogenetic trees obtained from the ITS-2 sequences are consequently able to differentiate between lymnaeids transmitting and those non-transmitting fasciolids, as well as between those transmitting *F. hepatica* and those transmitting *F. gigantica*, similarly as trees inferred from 18S rDNA sequences did (Bargues and Mas-Coma, 1997; Bargues et al., 1997).

Interestingly, the specificity is linked to the two genera which appear to be the oldest among lymnaeids according to their ITS-2 lengths. Moreover, both genera cluster together in the phylogenetic trees obtained by the MP and ML methods (Fig. 1A and C), which suggests an origin of the *Fasciola* genus ancestors related to the origin of this branch. Thus, the capacity of other lymnaeid species, as those belonging to taxa other than *Galba* and *Radix*, to play the role of intermediate host in given circumstances can be explained by one of Mas-Coma's specificity rules, according to which Recent parasite species may conserve the capacity of their ancestors of infecting their ancestral hosts besides recently acquired modern hosts (Mas-Coma, 1992).

4.4.2. Schistosomatids

In Europe, human dermatitis is caused by cercariae of the genera *Trichobilharzia*, *Bilharziella* and *Gigantobilharzia*, all of them parasites of birds. *Trichobilharzia* is the genus which includes more species involved in human dermatitis and all these species include lymnaeids as first intermediate hosts in their life cycles: *L. stagnalis*, *L. palustris*, *R. auricularia*, *R. peregra* and *R. ovata* are used by *T. ocellata* and *T. szidati* (however, these data are of limited value owing to trichobilharzid misclassifications time ago; formerly all cercariae were classified as *T. ocellata* or *T. szidati*); *R. auricularia* by *T. franki*; *R. peregra* and *R. ovata* by *T. regenti*; *R. peregra* by *Cercaria* sp.; and *R. peregra* by *T. salmanticensis* (Simon-Vicente, 1983; Kolárová et al., 1997, 1999; Horák et al., 1998, 1999; Simon-Martin and Simon-Vicente, 1999). Unpublished findings of *Trichobilharzia* in small planorbid snails (*Anisus leucostomus*, *Anisus vortex*, *Gyraulus albus*) by Nasincová (1992, Ph.D. thesis, Institute of Parasitology, Academy of Sciences of the Czech Republic) should be reviewed.

Thus, European *Trichobilharzia* species causing human dermatitis are transmitted only by lymnaeids of the *Radix* and *Lymnaea s. str.–Stagnicola* groups, namely those in which lymnaeid species classification problems are greatest. ITS-2 sequencing results prove that species included in these three lymnaeid groups are more numerous than believed. Compatibility differences of a given *Trichobilharzia* species detected among populations of a given lymnaeid species may thus sometimes in fact only be a marked lymnaeid species specificity. Most of the results involving *L. palustris*, *R. peregra* and *R. ovata* may need to be reinvestigated after accurate lymnaeid species classification by ITS-2 sequencing. Misclassifications in *Radix* species involved in the transmission of *T. regenti* in the Czech Republic emphasise this necessity.

ITS-2 sequences prove that the *Radix* species living in Iceland, France and Spain belong to the same species and genotype *R. peregra* (= *R. ovata*; = *R. balthica*) GT1. The definitive hosts of trichobilharzids are mainly bird species belonging to the family Anatidae, several of which migrate from Iceland to France and Spain via Ireland and UK (Cramp, 1977). All this suggests that duck species may be involved in the introduction of *Trichobilharzia* species, such as *T. salmanticensis*, from Iberia and France into Iceland, where the trichobilharzids have the chance of finding the same, man-introduced lymnaeid species necessary for their development.

4.4.3. Echinostomatids

These trematodes show increasing interest because of the recent description of several new species and their different snail host specificities (Toledo et al., 1998a,b, 1999, 2000; Kechemir et al., 2001). In Europe, several echinostomatid species use lymnaeids of the *Radix* and *Lymnaea s. str.–Stagnicola* groups. Similarly as with schistosomatids, the first intermediate lymnaeid host specificity of several of these digenean species may need to be revised after accurate lymnaeid species classification by ITS-2 sequencing. This can, moreover, allow a step forward in the appropriate rearrangement of the actual systematic confusion among echinostomatids. Classification errors made in lymnaeid species transmitting several echinostomatid species in the Albufera of Valencia (Spain) are good examples.

4.5. Final remarks

This paper opens a new and wide field of research on the morpho-anatomical differentiation of species, systematics, taxonomy and evolution of lymnaeids, as well as on intermediate lymnaeid host specificity of digeneans and molecular epidemiology of the trematode diseases they transmit. The rDNA ITS-2 marker is now at disposal for malacologists, who have to look for the necessary morpho-anatomical characteristics which may allow European lymnaeid species classification. But this is beyond the scope of the present paper. DNA sequence studies on lymnaeids from other continents already under way will further help in the understanding of the trematode–lymnaeid interactions and respective disease epidemiologies.

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