Phylogenetic Analysis of Lymnaeid Snails Based on 18S rDNA Sequences

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The 18S rDNA sequences of the six most common European Lymnaeidae species (Mollusca: Gastropoda: Basommatophora) have been obtained by direct PCR cycle sequencing and silver staining methods. The sequence alignment and secondary structures of the 18S rRNA gene of Lymnaea stagnalis, L. auricularia, L. peregra, L. palustris, L. glabra, and L. truncatula are analyzed. This gene proves to be a good marker for both specific determination and supraspecific lymnaeid phylogeny. The malacological importance is evident, considering the specific determination problems of individual snails and the present systematic chaos in Lymnaeidae due to their pronounced morphoatomic uniformity, which makes a classification by traditional methods impossible. The majority (17) of the total of 43 nucleotide-substituted positions appears to be confined to a small region included in helix E1O-1 of the variable region V2, enabling species group distinction: (1) the first sequence is common to L. auricularia and L. peregra; (2) the second sequence is unique to L. truncatula; and (3) the third sequence is identical for L. glabra, L. palustris, and L. stagnalis. The other 26 nucleotide-substituted positions are dispersed over the entire gene, although four grouped nucleotide positions in helix 6 of V1 are of interest in distinguishing L. glabra from both L. palustris and L. stagnalis. The phylogenetic trees obtained by comparison with four other molluscan species (a polyplacophoran, two bivalves, and a stylommatophoran gastropod) show the presence of four well-defined subgenera among the genus Lymnaea sensu lat. (1) Lymnaea (Radix), (2) Lymnaea (Galba), (3) Lymnaea (Leptolimnaea), and (4) Lymnaea (Lymnaea). Two branches, L. auricularia-L. peregra-L. truncatula and L. glabra-L. palustris-L. stagnalis, are worth mentioning from the parasitological point of view, since the two digenean species of large medical and veterinary impact transmitted by lymnaeids, Fasciolu hepatica and F. gigantica, appear to be linked to the first branch.

Introduction

The pulmonate basommatophoran superfamilies Lymnaeacea includes several families of freshwater snails, among which is the family Lymnaeidae Rahn-esque, 1815. Molluscan species of this family are not only of malacological interest, but also of great parasitological importance because of their capacity to act as intermediate hosts of numerous trematode parasite species (Yamaguti 1975; Brown 1978), among which are several digenean species of large medical and veterinary impact, such as Fasciolu hepatica (Linneaus, 1758) and Fasciolu gigantica Cobbold, 1855 (Malek 1980; Boray 1982; Chen and Mott 1990; Mas-Coma and Bargues 1996), as well as other cestode and nematode species. Despite having been the subject of numerous studies, mainly because of the important trematode parasitic diseases they transmit, the present knowledge of the classification of this gastropod group, as well as their parasite-host interrelationships, is far from sufficient.

A good example of this insufficient knowledge is the confused state in which the systematics and taxonomy of this molluscan family are found. At the species level the problems are found mainly because of the interspecific uniformity of numerous species, concerning both shell morphology and visceral mass morphoanatomy, usually causing serious difficulties in species determination (e.g., Oviedo, Bargues, and Mas-Coma 1995). At the supraspecific (genus, subgenus) level the confusion is even more evident, with specialists considering numerous genera and subgenera (e.g., Malek 1985) and other authors only accepting the large genus Lymnaea Lamarck, 1799 sensu lat., following the old classification of Hubendick (1951, 1978).

Among lymnaeacean pulmonates, the assessment of phylogenetic relationships between families has traditionally been based on reproductive tract characteristics (Hubendick 1951, 1978, Harr 1964, Starobogatov 1967) or on outgroup analysis and component analysis (Swiderski 1990). The characteristics used, however, do not always correlate, leading to considerable doubt concerning the present taxonomic classifications. Even the nearest relatives of the lymnaeaceans cannot be identified using morphological characters because of the large amount of convergence in the order Basommatophora (Duncan 1960; Hubendick 1978; Tillier 1984).

Molecular approaches can contribute to clarifying Lymnaea evolutionary branching patterns. rRNA molecules provide a good opportunity to examine the patterns of nucleotide sequence change (Wheeler and Honeycutt 1988). The different ribosomal genes (28S, 5.8S, and 18S) have different rates of evolution and have been extensively used in phylogenetic analysis. 18S rRNA genes evolve slower than 28S rRNA genes and are thus used to construct deeper phylogenies. The efficacy of small ribosomal RNA sequences for resolving evolutionary relationships among taxa has been well demonstrated, and a large body of sequences representing diverse organisms has been compiled (De Rijk et al. 1992) and used extensively in phylogenetic studies, even for the molluscs in relation to other protostome coe- lomates (Winnepenickx, Backeljau, and De Wachter 1994).

Europe is the continent whose more common lymnaeid species show the clearest specific systematic sta-
Specimens of six European species of the family Lymnaeidae have been studied: (1) *Lymnaea (Lymnaea) stagnalis* (Linnaeus, 1758), from Munich (Germany), reared in our laboratory; (2) *Lymnaea (Radix) auricularia* (Linnaeus, 1758), from the island of Corsica (France); (3) *Lymnaea (Radix) peregra* (Müller, 1774), from the Natural Park of the Albufera (Valencia, Spain); (4) *Lymnaea (Stagnicola) palustris* (Müller, 1774), from the island of Corsica (France); (5) *Lymnaea (Leptolimnaea) glabra* (Müller, 1774), from the locality of Indre (France); and (6) *Lymnaea (Galba) truncatula* (Müller, 1774), from the island of Corsica (France), cultured in our laboratory.

Specimens of *L. (G.) truncatula* pertaining to two different morphs, I and II, and coming from two separate populations (Quiripujo and Batallas, respectively) of the Bolivian Northern Altiplano (Bolivia) (see Oviedo, Bargues, and Mas-Coma 1995; Jabbour-Zahab et al. 1996) were used for sequence comparison.

The absence of parasitation by helminth parasites was always verified prior to selecting the snail specimens to be used for molecular techniques. Taking helminth parasite microhabitats into account, the region of the foot was chosen as the only snail part to be used for DNA extraction.

**Molecular Techniques**

**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 described by Sambrook, Fritsch, and Maniatis (1989). After dissection under a microscope, a small quantity of this biological material was suspended in 400 μl of lysis buffer (10 mM Tris-Cl, pH 8.0; 100 mM EDTA; 100 mM NaCl; 1% sodium dodecyl sulfate [SDS]) containing 500 μg/ml Proteinase K (USB) and digested for 2 h at 55°C with alternate shaking every 15 min. For the extraction, three steps were followed: in the first, an equal volume of phenol was used; in the second, 200 μl of phenol and 200 μl of chloroform/isoamyl alcohol (24/1) were used; and in the third, 400 μl of chloroform/isoamyl alcohol (24/1) was used. After each extraction step, phases were separated by spinning at 12,000 g for 3 min. The aqueous phase finally obtained was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of −20°C cooled absolute ethanol and refrigerated at −20°C. The spoiled 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 (inverting the tube on a paper towel). The precipitated DNA was redissolved in a small volume (20–50 μl) of TE Buffer (10 mM Tris-Cl, pH 7.6; 1 mM EDTA).

It is worth taking into account that, when working on snails, protocols including a centrifugation step after precipitation risk incorporating a white flocculate substance (probably polysaccharides) and melanic pigments into the DNA pellet, which usually inhibits PCR or causes amplification of nonspecific products (Gasser et al. 1993; Bargues, Marquez, and Mas-Coma 1995).

**rDNA Sequence Amplification**

Five superposed partial regions of the lymnaeid 18S rRNA gene were amplified by PCR. A set of 8 conserved oligonucleotide primers (table 1) was designed from the 18S rDNA sequence of 12 eukaryote Metazoa (Turbieville, Field, and Raff 1992). PCR amplification was performed using a standard protocol (see Bargues, Marquez, and Mas-Coma 1995) on a DNA Thermocycler 9600 (Perkin Elmer) to amplify specific 18S rRNA regions. Ten microliters of each PCR product was checked by staining with ethidium bromide on 1% NuSieve® GTG agarose (FMC) gel, using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 mg DNA/ml as standard.

**Purification and Quantification of PCR Products**

Primers and nucleotides were removed from PCR products by purification on Wizard® PCR Prep DNA
Purification System (Promega) according to the manufacturer's protocol. The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

**DNA Sequencing**

The sequence of the 18S rRNA gene was obtained by direct PCR cycle sequencing and silver staining methods (Bargues, Marquez, and Mas-Coma 1995). PCR cycle sequencing was performed (Silver Sequence® DNA Sequencing System, Promega) using the same primers used in PCR amplification reactions (table 1). Sequencing reaction products were separated on 50-cm-long 6% polyacrylamide 7 M urea gels by electrophoresis in Sequi-Gen Nucleic Acid Sequencing System (BioRad). Silver nitrate staining and developing was done according to Promega protocol. A permanent record was made using EDF film (Kodak).

**Software Programs Used**

**Sequence Alignment**

Sequences were aligned using CLUSTAL-V (Higgins, Bleasby, and Fuchs 1992). The alignment was made both including only the six lymnaeid species studied and also including known sequences of other Mollusca present in GenBank–EMBL: *Limicolaria kambeul* (Gastropoda: Stylommatophora) (accession no. X66374), *Crassostrea virginica* (X60315) (Bivalvia), *Placopesten magellanicus* (X53899) (Bivalvia), and *Acanthopleura japonica* (X70210) (Polyplacophora). The partial sequence of the 18S rRNA of *Lymnaea* (Pseudosuccinea) columella (Say 1817) (Lymnaeidae) (Shubkin et al. 1992; Rognlie, Diné, and Knapp 1994; L24040) was not included because it was not complete.

**18S RNA Secondary Structure Representation**

The Mulfold v. 2.0d85 program (Zuker 1989) was used to examine potential secondary structures. The previously published secondary structure prediction for *Limicolaria kambeul* 18S rRNA (Winneppenckx et al. 1992) based on the general eukaryote 18S rRNA secondary structure (De Rijk et al. 1992) was used to predict the secondary structure of the 18S rRNA of *Lymnaea auricularia*. The sequence of *L. auricularia* was chosen to facilitate analysis because it showed the highest sequence similarity to the *Limicolaria kambeul* 18S gene. The 18S rRNA secondary structure of *L. auricularia* was afterward used as a model to obtain those of the other lymnaeid species.

**Phylogenetic Analysis**

Distance and maximum-parsimony methods were used in phylogeny reconstruction. For distance analysis, a neighbor-joining (NJ) tree (Saitou and Nei 1987) was generated from a Kimura two-parameter distance matrix (Kimura 1980) using the MEGA program (Kumar, Tamura, and Nei 1993). Maximum-parsimony analysis was performed with PAUP v3.1.1 (Swofford 1993) using exhaustive search. Support for all derived phylogenies was examined using bootstrapping (heuristic option) (Felsenstein 1985) over 1,000 replications.

**Results**

**rDNA Sequences**

All the lymnaeid 18S rDNA sequences have been deposited in the EMBL under the following accession numbers: *L. stagnalis*: Z73984; *L. auricularia*: Z73980; *L. peregrina*: Z73981; *L. palustris*: Z73983; *L. glabra*: Z73982; *L. truncatula*: Z73985. An average of 1,815 nucleotides (*L. stagnalis*: 1,815; *L. auricularia*: 1,816; *L. peregrina*: 1,818; *L. palustris*: 1,817; *L. glabra*: 1,815; *L. truncatula*: 1,809), comprising the 18S rRNA gene, have been sequenced. Base frequencies were: A: 23.7%–23.9% (mean 23.89%); T: 24.5%–24.8% (mean 24.64%); G: 27.9%–28.1% (mean 28.00%); C: 23.3%–23.5% (mean 23.46%). There were no significant differences in nucleotide composition between species, the average G+C content being 51.5%. When aligning the sequences of the six lymnaeid species, the average number of nucleotide substitutions was 0.0067 per site, of which the average number of transitions was 0.0032 and the average number of transversions was 0.0035.

A total of 43 nucleotide-substituted positions appear. Two types of nucleotide substitutions can be distinguished (table 2):

1. Seventeen nucleotide-substituted positions appear to be confined to a small region (positions 233–257).
2. The other 26 nucleotide-substituted positions are dispersed over the entire gene, except for positions 72–74 and positions 156–158.

The distinction between these two groups of nucleotide-substituted positions appears to be of great importance, allowing us to distinguish between species groups. The small 233–257 region shows only three possible different nucleotide sequences which are shared by different lymnaeid species: (1) the first sequence is common to *L. auricularia* and *L. peregrina* (group I); (2) the second sequence is unique to *L. truncatula* (group II); and (3) the third sequence is identical for *L. glabra*, *L. palustris*, and *L. stagnalis* (group III).

**Secondary Structures**

When locating the 43 positions showing nucleotide differences in the 18S rDNA secondary structure of the six European lymnaeid species according to the models proposed by De Rijk et al. (1992) and Winneppenckx et al. (1992), 31 of these positions appear to be distributed in variable areas and 12 in conserved areas (variable/conserved areas after De Rijk et al. 1992). According to the nomenclature of De Rijk et al. (1992), from the 31 variable positions located in variable areas there are 4 in helix 6 of area V1, 1 in a position which would correspond to the origin point of helix E8-1 (nonexistent in *Lymnaea* spp.) in V2, 17 in helix E10-1 of V2, 1 in helix E11 of V2, 5 in different subhelices of helix E21 of V4, 1 in helix 27 of V5, 1 in helix 41 of V7, and 1 in helix 47 of V9 (table 2). From the 12 positions located in conserved areas there are 1, 4, 1, 1, 2, 1, and 2 variable positions in helices 8, 9, 12, 13, 18, 28, and 35, respectively (table 2).

It is worth mentioning that not only the majority (17) of the variable positions, but also the whole above-
Table 2
Nucleotide Differences Found in the Whole 18S rRNA Sequence of the Six *Lymnaea* Species Studied and Their Locations in the Secondary Structure

<table>
<thead>
<tr>
<th>Variable areas:</th>
<th>1111</th>
<th>1111</th>
<th>1111</th>
<th>1111</th>
</tr>
</thead>
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<tr>
<td>Helix:</td>
<td>2222222222</td>
<td>31568901356780137</td>
<td>3333344444444445555</td>
<td>897713834349028</td>
</tr>
<tr>
<td>Position:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. auricularia</td>
<td>GTG-CAAT</td>
<td>GTG-TCA-T</td>
<td>GTG-TCA-T</td>
<td>GTG-TCA-T</td>
</tr>
<tr>
<td>L. peregra</td>
<td>GTG-TCATTT</td>
<td>GTG-TGCTTGCTTCA</td>
<td>GTG-TGCTTGCTTCA</td>
<td>GTG-TGCTTGCTTCA</td>
</tr>
<tr>
<td>L. truncatula</td>
<td>GTG-TAA-T</td>
<td>T---CTTF-CTTG--T</td>
<td>C-T-T-GCCACTG-GG</td>
<td>C-T-T-GCCACTG-GG</td>
</tr>
<tr>
<td>L. glabra</td>
<td>TAAAT---T</td>
<td>CGTGCGGGGACTCTGCC</td>
<td>CGTGCGGGGACTCTGCC</td>
<td>CGTGCGGGGACTCTGCC</td>
</tr>
<tr>
<td>L. palustris</td>
<td>GTG-TCA-T</td>
<td>C-TTT-CGCACCG-CA</td>
<td>C-TTT-CGCACCG-CA</td>
<td>C-TTT-CGCACCG-CA</td>
</tr>
<tr>
<td>L. stagnalis</td>
<td>GIU-&quot;T&quot;--&quot;T&quot;</td>
<td>ACUUCUGGGAGUGAGACCTGGA</td>
<td>ACUUCUGGGAGUGAGACCTGGA</td>
<td>ACUUCUGGGAGUGAGACCTGGA</td>
</tr>
<tr>
<td>Difference no.</td>
<td>12345678910</td>
<td>12345678901234567890123</td>
<td>12345678901234567890123</td>
<td>12345678901234567890123</td>
</tr>
</tbody>
</table>

Note.—Position numbers (to be read in vertical) refer to positions obtained in the alignment made with CLUSTAL-V, including only the six Lymnaeidae. Areas and helices code numbers (to be read in vertical) are according to the secondary structure proposed for the gastropod *Lonicera kambeul* by Winnepennickx et al. (1992) and based on the model and nomenclature of De Rijk et al. (1992). Lymnaeidae species group differences in positions 233–257 correspond to helix E1O-1 of the variable area V2.

mentioned small 233–257 region, enabling species group distinction (groups I, II, and III), are entirely included in helix E1O-1 of the variable region V2 (fig. 1). Secondly, helix 6 of V1 is of interest because it includes the four main nucleotide positions (69, 72, 73, and 74) distinguishing *L. glabra* from both *L. palustris* and *L. stagnalis*.

Phylogenetic Analysis

The data set can be reduced to 321 variable positions of the 1,869 positions in the aligned sequences of the 10 molluscan species considered, of which 159 were phylogenetically informative positions.

The NJ tree (fig. 2) shows that there were seven clearly defined clades when *Acanthopleura* was used as outgroup, owing to the clear separation of the class Polyplacophora (included in the superclass Testaria) from the classes Gastropoda and Bivalvia (included in the subclasses Testaria and Bivalvia, respectively) (see Runnegar and Pojeta 1985; Pojeta et al. 1987; Salvini-Plawen 1985, 1990). Bivalvia and Polyplacophora units are well separated from the Pulmonate Gastropod unit (Stylom-
mauropora and Basommatophora), the lymnaeid entity (Basommatophora) appearing connected first to Limicolaria (Stylomatophora), second to Crassostrea and Placopecten (Bivalvia), and, finally, to Acanthopleura (Polyplacophora). Among lymnaeids two branches can be distinguished, one for *L. auricularia*, *L. peregra*, and *L. truncatula* and the other for *L. glabra*, *L. palustris*, and *L. stagnalis*. The first branch includes the grouping of *L. auricularia* and *L. peregra*, *L. truncatula* appearing alone. The second branch presents the grouping of *L. palustris* and *L. stagnalis*, with *L. glabra* isolated. The high bootstrap values (between 82% and 97%) are worth noting (bootstrap values below 70% do not reach the appropriate level of significance—see Hillis and Bull 1993) (fig. 2). The genetic distances between the 10 molluscan species analyzed (table 3) are greatest between the six lymnaeid species and the bivalves *C. virginica* and *P. magellanicus* and between lymnaeids and the polyplacophoran *A. japonica*. The genetic distances are relatively small between lymnaeids and the stylomatophoran gastropod *L. kambeul* and very small among the six lymnaeids.

 Parsimony analysis of the aligned sequences using the exhaustive option yielded a single most-parsimonious tree, 421 steps long (fig. 3). For the tree construction, four taxa (*L. kambeul*, *C. virginica*, *P. magellanicus*, and *A. japonica*) were transferred to outgroups. It is worth mentioning that an identical topology was obtained by the NJ method and parsimony analysis.

**Discussion**

**Differentiation at Species Level**

Each lymnaeid species studied has a unique 18S rDNA sequence. The lack of intraspecific interpopulational variability in the 18S rDNA sequence was confirmed by studying individuals from different geographically separated populations of the species *L. truncatula*. The whole gene sequences of individuals of two different extreme morphs of *L. truncatula* from two different populations of the Bolivian Northern Altiplano (the fascioliasis endemic zone presenting the highest human prevalences known by *Fasciola hepatica*—Hillyer et al. 1992; Mas-Coma et al. 1995), as an example of intraspecific morphological differences, extreme environment conditions, and extreme geographical distance (Oviedo, Bargues, and Mas-Coma 1995; Jabbour-Zahab et al. 1996), proved to be identical to the sequence obtained in European *L. truncatula*.

It can be concluded that the 18S rDNA sequence is an excellent marker for species determination and differentiation among Lymnaeidae. This fact contrasts with the value generally ascribed to this conserved gene as a marker for high-level taxons, although recent studies have demonstrated that it has different values in different eukaryotic groups. Thus, different situations can be found, from protozoans of the genus *Cryptosporidium*

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**Table 3**

Pairwise Comparisons of Nucleotide Divergences and Standard Errors Estimated According to Kimura's (1980) Two-Parameter Model for the Whole Set of 18S rDNA Sequences from the 10 Molluscan Species Analyzed

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>A. japonica</em></td>
<td>0.0096</td>
<td>0.092</td>
<td>0.099</td>
<td>0.095</td>
<td>0.096</td>
<td>0.094</td>
<td>0.072</td>
<td>0.095</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>2. <em>L. auricularia</em></td>
<td>0.0086</td>
<td>0.039</td>
<td>0.116</td>
<td>0.010</td>
<td>0.010</td>
<td>0.002</td>
<td>0.010</td>
<td>0.010</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>3. <em>L. kambeul</em></td>
<td>0.008</td>
<td>0.005</td>
<td>0.117</td>
<td>0.041</td>
<td>0.041</td>
<td>0.039</td>
<td>0.100</td>
<td>0.040</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>4. <em>C. virginica</em></td>
<td>0.008</td>
<td>0.009</td>
<td>0.099</td>
<td>0.115</td>
<td>0.112</td>
<td>0.115</td>
<td>0.080</td>
<td>0.111</td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>5. <em>L. glabra</em></td>
<td>0.008</td>
<td>0.002</td>
<td>0.003</td>
<td>0.009</td>
<td>0.003</td>
<td>0.010</td>
<td>0.102</td>
<td>0.003</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>6. <em>L. palustris</em></td>
<td>0.008</td>
<td>0.002</td>
<td>0.005</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.102</td>
<td>0.001</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>7. <em>L. peregra</em></td>
<td>0.008</td>
<td>0.001</td>
<td>0.005</td>
<td>0.009</td>
<td>0.002</td>
<td>0.002</td>
<td>0.103</td>
<td>0.009</td>
<td>0.004</td>
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<td>8. <em>P. magellanicus</em></td>
<td>0.007</td>
<td>0.008</td>
<td>0.008</td>
<td>0.007</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.101</td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td>9. <em>L. stagnalis</em></td>
<td>0.008</td>
<td>0.002</td>
<td>0.005</td>
<td>0.008</td>
<td>0.001</td>
<td>0.000</td>
<td>0.002</td>
<td>0.008</td>
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<tr>
<td>10. <em>L. truncatula</em></td>
<td>0.008</td>
<td>0.002</td>
<td>0.005</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.001</td>
<td>0.008</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide divergences.
* Standard errors.
(Apicomplexa: Cryptosporidiidae), in which this gene shows interpopulational variability within a given species (Kilani and Wenman 1994), to platyhelminths or arthropods of the tick family Ixodidae (Acari), in which this gene serves as a marker of subfamily and family distinction (Lumb, Bray, and Rollinson 1993; Blair et al. 1996; Black, Klompen, and Keirans 1996), through species and genus distinction in protozoan Plasmodiidae (Waters and McCutchan 1990; Escalante and Ayala 1994) or metazoan trematodes (Johnston, Kane, and Rollinson 1993). The results of the present paper suggest the value of studying the 18S rDNA sequence in parasitic diseases. For instance, Planorbidae, transmitters of schistosomiasis, are closely related to Lymnaeidae, also placed among the superfamily Lymnaeacea (Brown 1978).

The number of nucleotide-substituted positions (43) detected in the six lymnaeid species from only one continent, as well as the relatively high proportion of base differences in positions located in conserved areas of the secondary structure (12 among 43: 27.9%) must be stressed in that we are dealing with a markedly uniform organism group of free-living (=nonparasitic) eukaryotic metazoans and not with protozoans. The question arises of whether this finding could be linked to given evolutionary characteristics mainly related to their self-fertilization capacity (Hubendick 1951; Duncan 1975; Jarne and Delay 1991; Jarne, Vianey-Liaud, and Delay 1993), selfing having a depressing effect on the neutral variability maintained within populations (see recent reviews by Charlesworth, Morgan, and Charlesworth 1993 and Jarne 1995 for a detailed list of reasons). Studies of the 18S rDNA of other basommatophoran pulmonate gastropod families (no data were found in GenBank) presenting selfing capacity are needed to elucidate whether the 18S rDNAs of self-fertile hermaphrodite snails shows a higher number of nucleotide substitutions than those of other metazoan eukaryotes.

Differentiation at the Supraspecific Level

There is a state of chaos in the systematics of the family Lymnaeidae. The apparent morphoanatomic uniformity within Lymnaea makes a classification into sections and subgenera difficult and, indeed, often impossible. As a consequence, it becomes impossible to construct a phylogeny based on morphoanatomic characteristics (Hubendick 1951). The small region of helix E10-1 in V2 and the obtained phylogenetic cladograms clearly allow species groupings, thus distinguishing supraspecific entities and demonstrating the usefulness of the 18S rDNA as a marker for the definitive taxonomic reorganization of Lymnaeidae. The molecular classification of supraspecific units will further permit us to analyze which phenotypic characteristics are linked to each grouping and, consequently, to deduce, whenever possible, a significant taxonomic value for morphoanatomic characteristics, enabling species classification by traditional methods. Finally, supraspecific taxa will be able to be redefined, actual available descriptions being completely insufficient and inapplicable (see Hubendick 1951, 1978).

Our phylogenetic analyses show the existence of four clear species groupings: one comprises L. auricularia and L. peregra, the second contains only L. truncatula, the third contains L. glabra, and the fourth comprises L. palastris and L. stagnalis. It seems most appropriate to provisionally confer only a subgenus status for such groups within the genus Lymnaea sensu lato until a sufficient number of other lymnaeid species are studied from the point of view of the 18S rDNA. At least the type species of the most important generic taxa described by several authors some time ago, and, if possible, of lymnaeid species from continents other than Europe, need to be studied. Moreover, it is always preferable for generic taxa to be determined by traditional morphoanatomic methods. Accordingly, following the review by Hubendick (1951), these subgenera are:

1. Lymnaea (Radix) Montfort, 1810: for the species L. auricularia and L. peregra, with L. (Radix) auricularia as type species.
2. Lymnaea (Galba) Schranck, 1803: for the species L. truncatula, with L. (Galba) truncatula as type species.
3. Lymnaea (Leptolymnaea) Swainson, 1840: for the species L. glabra, with L. (Leptolymnaea) glabra as type species.
4. Lymnaea (Lymnaea) Lamarck, 1799 sensu stricto: for the species L. palastris and L. stagnalis, with L. (Lymnaea) stagnalis as type species.

According to our results, L. palastris is very close to L. stagnalis, and a separation of both species in two different subgeneric taxa therefore does not appear to be justified. Consequently, the subgenus Stagnicola Leach, 1830 (type species: L. palastris) must be synonymized with Lymnaea (Lymnaea) sensu stricto. If in the future the raising of theses subgenera to genus rank becomes justified, then the subgeneric status of Stagnicola could perhaps be maintained.

In the phylogenetic trees obtained, the presence of the two branches L. auricularia—L. peregra—L. truncatula and L. glabra—L. palastris—L. stagnalis is worth mentioning from the parasitological point of view. As is well known, significant genetic information is offered by parasite/host systems (Haftner and Nadler 1988; Humphery Smith 1989; Page 1993; etc.), as in the case of trematode species/first intermediate molluscan host species (Brown 1978). Among the latter, the fasciolid/lymnaeid models have been largely studied (see reviews by Brown 1978; Malek 1980; Boray 1982; Mas-Coma and Bargues 1996). The large knowledge on lymnaeid species specificity of the digeneans F. hepatica and F. gigantica suggests that the genus Fasciola Linnaeus, 1758, is linked to the first branch.

In Europe, F. hepatica uses L. truncatula as its fundamental intermediate host, although under special natural conditions this digenean parasite can develop in L. glabra (Bouix-Busson and Rondelaud 1985, 1986) and...
**L. palustris** (Dreyfuss et al. 1994). In the laboratory, both *L. glabra* and *L. palustris*, and even *L. stagnalis* and *L. peregra*, 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 (Brown 1978). The resistance of *L. auricularia* to the *F. hepatica* infection has been stressed by Kendall (1950) and Boray (1978). Concerning *F. gigantica*, all through its geographical distribution, this digenean species uses mainly *L. auricularia* and other non-European lymnaeid species closely related to it (the so-called *L. auricularia* superspecies) in nature (Brown 1978; Mas-Coma and Bargues 1996). Among the so-called alternate or facultative host species (=lymnaeid species used by the parasite only rarely or secondarily in nature) there are *L. peregra* in Asia and perhaps *L. truncatula* in Kenya (Mas-Coma and Bargues 1996). Under laboratory conditions, full parasite development has been experimentally obtained in young (aged up to 4 weeks) *L. peregra* and *L. stagnalis*, and even in adults of *L. truncatula*, though only 5% of the latter produced metacercariae (Brown 1978). Summing up, the two above-mentioned compatibility models of parasite specificity/host immune system, concerning data on digenean infectivity/snail susceptibility, suggest that *F. hepatica* is linked to the subgenus *Lymnaea* (*Galba*) and that *F. gigantica* is linked to *Lymnaea* (Radix). The capacity of these two fasciolid species to infect lymnaeid species of the other branch, *L. glabra*–*L. palustris*–*L. stagnalis*, in exceptional circumstances can be explained after the parasitophylectic rule of Mas-Coma (1982, 1992). According to this rule, recent parasite species presenting a low host specificity can maintain the capacity of their parasite ancestors to infect and develop in host species of older origin, but still existing, which are phylogenetically closely related (ancestors, descendants of the same ancestors) to their recent main host species. In other words, the capacity of these fasciolids to exceptionally infect *L. glabra*, *L. palustris*, or *L. stagnalis* would only be a reminiscence of fasciolid ancestors. At any rate, concerning this question, *F. gigantica* appears to be more restrained to the *L. auricularia*–*L. peregra*–*L. truncatula* branch than *F. hepatica*.

**Parasitological Interest**

The results of this study show that snail phylogeny obtained through the 18S rDNA sequences might help to interpret the pattern of parasite/host relationships and that the 18S rDNA sequences might help in the distinction between transmitter and nontransmitter snail species, as well as between infected and noninfected snail individuals, by eliminating cross-reactivity of probes to parasite rDNA (see Shubkin et al. 1992; Heussler et al. 1993; Rognlie, Dimke, and Knapp 1994; Kaplan et al. 1995).

Owing to the already-mentioned lymnaeid snail classification difficulties, the usefulness of the 18S rDNA as a marker for lymnaeid species classification is undoubtedly very great for research investigations and also for epidemiological and control studies on the digenean parasitic diseases they transmit. The pronounced similarity between several lymnaeid species (see Oviedo, Bargues, and Mas-Coma 1995) is, unfortunately, a usual problem found among different lymnaeid species able to act as first intermediate hosts of the two main fasciolid flukes. Thus, lymnaeid species transmitting the cosmopolitan parasite species *F. hepatica* appear to present a similar shell type morphology, the so-called Galba type, marked by *L. truncatula*, in any part of the world, and the lymnaeid species transmitting *F. gigantica* appear to belong to the Radix type, marked by *L. auricularia*, all over the geographic distribution of this parasite (see Hubendick 1951 concerning shell types).

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