DNA multigene sequencing of topotypic specimens of the fascioliasis vector *Lymnaea diaphana* and phylogenetic analysis of the genus *Pectinidens* (Gastropoda)

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Freshwater lymnaeid snails are crucial in defining transmission and epidemiology of fascioliasis. In South America, human endemic areas are related to high altitudes in Andean regions. The species Lymnaea diaphana *has, however, been involved in low altitude areas of Chile, Argentina and Peru where human infection also occurs. Complete nuclear ribosomal DNA 18S, internal transcribed spacer (ITS)-2 and ITS-1 and fragments of mitochondrial DNA 16S and* cytochrome c oxidase *(*cox*)1 genes of* L. diaphana *specimens from its type locality offered 1,848, 495, 520, 424 and 672 bp long sequences. Comparisons with New and Old World* Galba/Fossaria*, Palaearctic stagnicolines, Nearctic stagnicolines, Old World* Radix *and* Pseudosuccinea *allowed to conclude that (i)* L. diaphana *shows sequences very different from all other lymnaeids, (ii) each marker allows its differentiation, except* cox*1 amino acid sequence, and (iii)* L. diaphana *is not a fossarine lymnaeid, but rather an archaic relict form derived from the oldest North American stagnicoline ancestors. Phylogeny and large genetic distances support the genus* Pectinidens *as the first stagnicoline representative in the southern hemisphere, including colonization of extreme world regions, as most southern Patagonia, long time ago. The phylogenetic link of* L. diaphana *with the stagnicoline group may give light to the aforementioned peculiar low altitude epidemiological scenario of fascioliasis.*

Key words: *Lymnaea diaphana* - Lymnaeidae - nuclear rDNA - mtDNA - phylogeny - fascioliasis vectors

Freshwater snails of the family Lymnaeidae are of great importance in public health due to their capacity to transmit fascioliasis, a parasitic disease caused by the two liver fluke species *Fasciola hepatica* and *Fasciola gigantica* (Mas-Coma et al. 2009a). Whereas the consequences of fascioliasis are the cause of concern in livestock husbandry since long ago (Spithill et al. 1999, Torgerson & Claxton 1999), its impact on human communities has shown to progressively increase from an amount of 2,500 human cases in 1990 (Chen & Mott 1990) to a global estimation of 17 million people affected which may be even worse if the lack of knowledge in many regions of Africa and Asia are considered (Mas-Coma et al. 2009a). This recent emergence appears to be at least in part related to climate change (Mas-Coma et al. 2008, 2009b). General concern about fascioliasis has moreover risen due to the large long-term pathogenicity of fasciolid flukes (Valero et al. 2003, 2006, 2008) and their immunosuppression effect (Gironés et al. 2007) recently demonstrated in the advanced chronic stage of the disease, which appears to be the usual situation of infected subjects in the human endemic areas.

Within the several human fascioliasis hotspot regions known, South America is characterized by the so-called Andean transmission pattern, including the Altiplano subpattern and Valley subpattern. Both subpatterns are characterized by high altitude endemic areas, including high prevalences and intensities in humans caused by *F. hepatica*, such as in Bolivia (Hillyer et al. 1992, Esteban et al. 1997a, b, 1999) and Peru (Esteban et al. 2002, Gonzalez et al. 2011). In Argentina the human fascioliasis situation, although underestimated, also shows a link to altitude areas (Mera y Sierra et al. 2011). However, human infection in South America has also been described to be relatively frequent in given low altitude areas, such as in Arequipa region, Peru and southern Chile, where the species *Lymnaea diaphana* King, 1830 has been noted to be directly involved in the transmission (Cordova et al. 1961, Tantalean et al. 1974, Larrea et al. 1994) or known to be present in the transmission area (Sielfeld 2001, Valdovinos 2006), respectively. Additionally, *L. diaphana* is known to inhabit the southernmost areas of South America (Hubendick 1951) where animal fascioliasis has been described, in both Chile (Alcaino & Apt 1989, Morales et al. 2000) and Argentina (Olaechea 1994).

In spite of their applied interest, our knowledge on lymnaeid snails is far from sufficient regarding both their genetics and their vector role. This situation is well illustrated by the systematic-taxonomic controversy in which this molluscan family is immersed (see review in

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Bargues et al. 2001). At lymnaeid species level, the problems are mainly due to the interspecific morphological and anatomic uniformity numerous species show, usually presenting serious difficulties in specimen classification, sometimes even impeding it. Moreover, intraspecific variation of shell shape is particularly well marked within lymnaeids depending on environmental conditions, although a genetic component in shell shape has been shown at least in some lymnaeid populations (Samadi et al. 2000). In the Americas, there are many specimen classification problems, mainly concerning fascioliasis vector species of the so-called "fossarine" or *Galba*/*Fossaria* group (Bargues et al. 2007), which is characterized by a shell shape and size range within which *L. diaphana* fits (Paraense 1984).

Recent studies have shown that nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) sequences furnish appropriate markers to clarify the systematics of this snail group as well as lymnaeid specimen classification even in particularly confusing lymnaeid groups (Bargues & Mas-Coma 1997, 2005, Bargues et al. 1997, 2001, 2003, 2006, 2007, Remigio & Blair 1997a, b, Remigio 2002, Remigio & Hebert 2003). Additionally, analyses have recently shown that the only way to perform a correct, definitive species ascription to a DNA sequence is by comparing with the sequence of the same marker obtained in specimens collected in the type locality of the species (Bargues et al. 2011a), as already followed concerning shell and morphoanatomical characteristics by Paraense (1976, 1984).

The current trend in molecular population genetics is to use increasing numbers of genes in the analysis. Here we describe a multigenic sequence analyses of *L. diaphana* thanks to the attainment of complete sequences of the 18S gene and the first and second internal transcribed spacers (ITS), ITS-1 and ITS-2, of the rDNA and partial sequences of the 16S gene and cytochrome c oxidase subunit I (*cox*1) coding gene. To avoid any possible doubt, the molecular characterization is based only on specimens collected in the type locality of this species. These sequences are analyzed in full detail, by means of pairwise comparisons and phylogenetic methods, with those of the same markers in (i) other American lymnaeid species of the "fossarine" or *Galba*/*Fossaria* group, most of them represented by specimens from the respective type localities of the species, (ii) the main fascioliasis vector species throughout the world *Galba truncatula*, which also belongs to the *Galba*/*Fossaria* group and (iii) morphologically close Nearctic and Palaearctic species of the "stagnicoline" group. Finally, the study is also used for the analysis of the lymnaeid genus *Pectinidens*, which was proposed with *L. diaphana* as type species long ago (Pilsbry 1911). *Pectinidens* has also been used at subgenus level to even include other *Galba*/*Fossaria* vector species such as *Lymnaea viatrix* (Alcaino & Apt 1989).

MATERIALS AND METHODS

Lymnaeid snail materials - The snail specimens studied were collected in the field, at the type locality included in the original description of the species *L. diaphana*: neighbourhood of Cape Gregory, which is on the continental side of the eastern end of the Strait of Magalhaens, province of Magallanes, Chile (King & Broderip 1832, Pilsbry 1911, Paraense 1984). For a complete description of the shell and anatomy of topotypic specimens of this species, including detailed drawings, see Paraense (1984). A typic specimen of the lymnaeid snails collected is illustrated in Fig. 1. The terra typica of this lymnaeid (S52º37'52.3" W70º15'18.0", altitude 2-5 m) is constituted by different neighbouring water collections resulting from subsoil effluences in a wide area of sheep farming (see kind of biotopes in Fig. 2A, B). Specimens were usually found inside cold water, mostly on stony waterbottom (Fig. 2C) and only very rarely outside water as on floating leafs (Fig. 2D). No other lymnaeid species was found in the water collections studied.

Molecular techniques - DNA was only isolated from the foot of each alcohol-fixed snail (Bargues et al. 1997, 2007). Total DNA was isolated according to the phenol-chloroform extraction and ethanol precipitation method. The procedure steps were performed according to methods outlined previously (Bargues & Mas-Coma 1997, Bargues et al. 2001, 2007). The pellet was dried and resuspended in 30 µL sterile tris-ethylenediamine tetraacetic acid (TE) buffer (pH 8.0). This suspension was stored at -20ºC until use.

A combined set of nuclear rDNA and mtDNA markers were polymerase chain reaction (PCR) amplified independently for each lymnaeid specimen and each PCR product was sequenced for a bona-fide haplotype characterization. The complete 18S rRNA gene was amplified using specific primers (Bargues et al. 1997, 2011a). The rDNA spacers ITS-2 and ITS-1 were amplified using primers designed in conserved positions of 5.8S and 28S rRNA genes and 18S and 5.8S rRNA genes, respectively (Bargues et al. 2001, 2006, 2007). The target 16S gene region was amplified using a set of universal primers (Simon et al. 1991). Amplification procedures and thermal cycler conditions were carried out as previously described for lymnaeids (Remigio & Blair 1997a). A *cox*1 gene fragment was amplified using other universal primers (Folmer et al. 1994). Amplifications were generated in a Mastercycle ep gradient (Eppendorf, Hamburg, Germany) using specific PCR conditions for each marker, as previously described (Bargues et al. 2011a). Ten microlitres of each PCR product were checked by

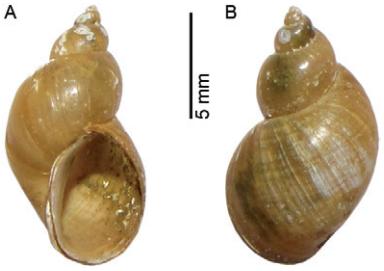

Fig. 1: specimen of *Lymnaea diaphana* collected at its type locality of Cape Gregory, Strait of Magellan, province of Magallanes. Chile. A: ventral view; B: dorsal view.

staining with ethidium bromide on 1% Nusieve® Genetic Technology Grade agarose (FMC Bioproducts) gel electrophoresis using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 µg DNA/µL as control.

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's protocol, and suspended 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.

DNA sequencing was performed on both strands by the dideoxy chain-termination method (Sanger et al. 1977). It was carried out with the Taq dye-terminator chemistry kit for ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using PCR primers. Sequences were aligned using CLUSTALW version 1.8 (Thompson et al. 1994) and MEGA 5.0 (Tamura et al. 2011). Minor corrections were manually introduced for a better fit of nucleotide correspondences in microsatellite sequence regions.

For 18S rRNA secondary structure representation, the previously published secondary structure prediction for *Limicolaria kambeul* 18S rRNA (Winnepennickx et al. 1992) based on the general eukaryote 18S rRNA secondary structure (De Rijk et al. 1992) was used and extended to encompass lymnaeid sequences.

DNA haplotype nomenclature - The codes for the sequences obtained follow the standard nomenclature proposed for lymnaeid snails previously (Bargues & Mas-Coma 2005, Bargues et al. 2006, Mas-Coma et al. 2009a). It shall be noted that haplotype codes are only definitive in the case of complete sequences. When dealing with fragments or incomplete sequences, haplotype codes are provisional.

Sequence comparisons - The following sequences from GenBank and European Molecular Biology Laboratory (EMBL) have been used for comparison and/or phylogenetic analyses: (i) 18S rRNA gene: complete sequences of *Lymnaea (Lymnaea) stagnalis* (GenBank accession Z73984), *Lymnaea (Stagnicola) palustris* (Z73983), *Omphiscola glabra* (Z73982) and *G. truncatula* (Z73985) (Bargues & Mas-Coma 1997), *Lymnaea cubensis* (Z83831) (Bargues et al. 1997, 2007), *L. viatrix* and *Lymnaea neotropica* (both species with the same sequence AM412222) (Bargues et al. 2007), *Lymnaea humilis* (FN182190) (Bargues et al. 2011a) and *Pseudosuccinea columella* (FN598152) (Bargues et al. 2011b), *Radix auricularia* (Z73980) and *Radix balthica* (Z73981) (Bargues & Mas-Coma 1997, Bargues et al. 1997). Other lymnaeid incomplete sequences available in the GenBank have not been used to avoid problems in comparative sequence analyses. The planorbids *Biomphalaria glabrata*

Fig. 2: type locality of *Lymnaea diaphana* at San Gregorio, Strait of Magellan, province of Magallanes. Chile: A: water collection inhabited by the species in a view to the Strait (see sea on the background); B: another water collection inhabited by the species in a view to the inland; C: specimens rarely found out of water, on a floating leaf; D: stony water ground on which specimens were usually found.

(Hanelt et al. 1997) and *Bulinus truncatus* (Jorgensen et al. 2011) were included for comparison purposes; (ii) rDNA ITS-2: *L.* (*S.*) *palustris palustris* (AJ319620), *Lymnaea* (*Stagnicola*) *fuscus* (AJ319621) and *Catascopia occulta* (AJ319642) (Bargues et al. 2001, 2003), *Catascopia catascopium* (AF013143), *Catascopia emarginata* (AF013141, AF013142), *Catascopia elodes* (AF013138) and *Hinkleyia caperata* (AF013139) (Remigio & Blair 1997b), *G. truncatula* H1 (AJ296271) (Bargues et al. 2001, Mas-Coma et al. 2001), *L. cubensis* H1 (AM412223) and H2 (FN182200) (Bargues et al. 2007, 2011a), *L. viatrix* H1 (AM412224) and *L. neotropica* H1 (AM412225) (Bargues et al. 2007), *L. humilis* H1 (FN182191) (Bargues et al. 2011a), *P. columella* H1 (FN598155) (Bargues et al. 2011b), *R. auricularia* (AJ319628) and *R. balthica* (AJ319633) (Bargues et al. 2001); (iii) rDNA ITS-1: *L.* (*S.*) *palustris palustris* (AJ626849), *L.* (*S.*) *fuscus* (AJ626856) and *C. occulta* (AJ626858) (Bargues et al. 2006), *C. catascopium* (AF013143), *C. emarginata* (AF013142), *C. elodes* (AF013138) and *H. caperata* (AF013139) (Remigio & Blair 1997b), *G. truncatula* HA (AJ243018) (Mas-Coma et al. 2001), *L. cubensis* HA (AM412226) and HB (FN182202) (Bargues et al. 2007, 2011a), *L. viatrix* HA (AM412227) and *L. neotropica* HA (AM412228) (Bargues et al. 2007), *L. humilis* HA (FN182193) (Bargues et al. 2011a), *P. columella* HA (FN598160) (Bargues et al. 2011b); *R. auricularia* (JF922878) and *R. balthica* (JF922879) (Bargues et al. 2011b); (iv) 16S rRNA gene of the mtDNA: *Fossaria bulimoides* (AF485657), *Fossaria obrussa* (AF485658) and *Stagnicola bonnevillensis* (AF485655) (Remigio 2002), *F. bulimoides* (EU038315) and *Stagnicola elodes* isolate 44106 (EU038305) (Wethington & Lydeard 2007), *L. humilis* (FN182195) and *L. cubensis* (FN182204) (Bargues et al. 2011a), *P. columella* (PCU82073) (Remigio & Blair 1997a); (v) mtDNA *cox*1 gene: *S. elodes* (AY227368), *F. bulimoides* (AY227367) and *Austropeplea tomentosa* (AY227365) (Remigio & Hebert 2003), *G. truncatula* from Spain (AM494011) (Bargues et al. 2007) and Germany (EU818799) (Albrecht et al. 2008), *L. cubensis cox*1a (AM494009) and *cox*1b (FN182205), *L. neotropica cox*1a (AM494008) and *cox*1b (FN356741), *L. humilis cox*1a, *cox*1b and *cox*1c (FN182197-9) (Bargues et al. 2007, 2011a, Mera y Sierra et al. 2009), *P. columella* (FN598165) (Bargues et al. 2011b) and *P. columella* (AY227366) (Remigio & Hebert 2003), *Radix rubiginosa* (GU451737) (Liu et al. 2010).

Phylogenetic inference - Phylogenetic analysis of ITS-1 and ITS-2 combined haplotypes was firstly performed with a maximum likelihood (ML) approach using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 (Swofford 2002) and the PhyML program version 3.0 aLRT. ML parameters and the evolutionary model best fitting our dataset were determined using Akaike and Bayesian information criteria (Akaike 1974, Posada & Buckley 2004), implemented in jModeltest version 0.1.1 (Posada 2008). Starting branch lengths were obtained using the least-squares method with ML distances. The intergenic region sequence (AY030361) (De Jong et al. 2001) including both ITSs of a planorbid species, *Biomphalaria pfeifferi*, was used as outgroup.

To provide an assessment of the reliability of the nodes in the ML tree, four methods were used. First, a distance-based phylogeny using the neighbour-joining (NJ) algorithm (Saitou & Nei 1987) with the ML pairwise distances was obtained and statistical support for the nodes was evaluated with 1,000 bootstrap replicates, with and without removal of gapped positions, in PAUP. Second, a bootstrap analysis using 1,000 replicates was made by using branch-swapping algorithm (tree-bisection-reconnection) with full heuristic search in PAUP. Third, a Bayesian phylogeny reconstruction procedure was applied to obtain posterior probabilities (BPP) for the nodes in the ML tree, by using the same evolutionary model as above, implemented in MrBayes 3.1 (Ronquist & Huelsenbeck 2003) with four chains during 1,000,000 generations and trees being sampled every 100 generations. The first 1,000 trees sampled were discarded ("burn-in") and clade posterior probabilities were computed from the remaining trees. Fourth, reliability for internal branch was assessed with a fast method using the aLRT test (SH-like) implemented in PhyML for final comparison purposes.

RESULTS

DNA sequences - Nuclear rDNA 18S, ITS-2 and ITS-1 and mtDNA 16S and *cox*1 nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DNA Data Bank of Japan databases under the accessions noted in Table I.

18S rRNA gene - The 18S rDNA sequences obtained in the *L. diaphana* specimens analyzed from the type locality are identical base to base, with a length of 1,848 bp and a guanine-cytosine (GC) content of 51.68% (Table I).

A multiple sequence alignment of 11 different 18S sequences, including several *Galba*/*Fossaria* vector species such as *L. cubensis*, *L. viatrix* (with 18S sequence identical to that of *L. neotropica*), *G. truncatula* and *L. humilis*, the peculiar species *P. columella,* three representatives of stagnicolines such as *L. (L.) stagnalis*, *L. (S.) palustris* and *O. glabra* and two species of the *Radix* group such as *R. auricularia* and *R. balthica*, was 1,867 bp long, show-

TABLE I

Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) barcode haplotype identification and respective GenBank accessions for the lymnaeid species *Lymnaea diaphana* from its type locality

DNA marker	Haplotype code	Accession
18S rRNA	L.dia 18S-H1	JF909497
rDNA ITS-2	L.dia ITS2-H1	JF909498
rDNA ITS-1	L.dia ITS2-HA	JF909499
mtDNA 16S	L.dia 16S-HA[a]	JF909500
mtDNA *cox*1	L.dia *cox*1-Ha[a]	JF909501

a: mtDNA haplotype codes only preliminary due to incomplete gene sequence. H: haplotypes.

ing a total of 62 variable positions (3.32% nucleotide divergence). Thirty of these 62 polymorphic sites appear grouped in the short sequence between positions 233 and 266, which corresponds to the helix E10-1 of the variable area V2 of the secondary structure (Supplementary data). Pairwise nucleotide differences at the level of the 18S between *L. diaphana* and the other 10 aforementioned lymnaeid species, as well as with representatives of Planorbidae to comparatively assess distances at family level, are shown in Table II.

rDNA ITS-2 - Specimens of *L. diaphana* from the type locality present a 495 bp-long ITS-2, with a 56.97% GC content, which has been ascribed to *L. diaphana* haplotype 1 (L.dia ITS2-H1) (Table I).

Worth mentioning is the very high number of nucleotide differences detected in the pairwise comparisons of this ITS-2 sequence of *L. diaphana* with the ITS-2 of other European and American *Galba*/*Fossaria*, stagnicolines and Pseudosuccinea species available in GenBank. The ITS-2 dataset distance matrix obtained with PAUP shows that the number of total and mean character differences between *L. diaphana* and the other species considered are considerably high in all cases (Table III). These total differences ranged between 68-116 (average 88.6), when comparing with *Galba*/*Fossaria* species, and between 105-108 (average 106.5) and 66-80 (71.5), when *L. diaphana* is compared with European and American stagnicolines, respectively. Regarding *P. columella*, the differences are 81 (Table IV). According to these ITS-2 pairwise distance results, the group of species appearing to be more close to *L. diaphana* is the one of the American stagnicolines. Genetic distances between *L. diaphana* and its molecularly closest lymnaeid species according to PAUP (*L. neotropica*, *C. catascopium*, *C. elodes* and *H. caperata*) are shown in Table V.

rDNA ITS-1 - *L. diaphana* specimens collected in the type locality present an ITS-1 with a length of 520 bp and a GC content of 54.05%. This ITS-1 sequence has been ascribed to *L. diaphana* haplotype A (L.dia ITS1-HA) (Table I).

Similarly as in the case of ITS-2, a very high number of nucleotide differences appear in the pairwise comparisons of this ITS-1 sequence of *L. diaphana* with the ITS-1 of species of *Galba*/*Fossaria* and stagnicolines, as well as to *P. columella* available in GenBank. The ITS-1 dataset distance matrix obtained with PAUP shows that the number of total and mean character differences between *L. diaphana* and the other species considered are considerably high in all cases (Table VI). These total differences ranged between 73-95 (average 85.6) when comparing with *Galba*/*Fossaria* species and between 100-101 (100.5) and 76-93 (86.7) when *L. diaphana* is compared with European and American stagnicolines, respectively. Regarding *P. columella*, a total of 105 differences appear (Table IV). According to these ITS-1 pairwise distance results and similarly as detected in the ITS-2 analysis, the group of lymnaeids appearing to be more close to *L. diaphana* is the one of the American stagnicolines. Genetic distances between *L. diaphana* and its molecularly closest lymnaeid species according to PAUP (*G. truncatula* and *H. caperata*) are shown in Table V.

16S mtDNA - Snail specimens from the type locality furnished a 16S fragment sequence of a length of 424 bp characterized by a considerable adenine-thymine (AT) biased average nucleotide composition of 70.75%. The provisional code L.dia 16S-HA has been assigned for this fragment (Table I).

Estimates of evolutionary divergence and base composition bias differences in the 16S sequence alignment including *L. diaphana* and other species available in GenBank demonstrate that *L. diaphana* is different from any other species of *Galba*/*Fossaria* and stagnicolines, as well as from *P. columella*, at the level of this mtDNA gene (Table VII, Supplementary data). In pairwise comparisons, minimum differences were 31 mutations when *L. diaphana* is compared with *S. bonnevillensis* and a maximum of 46 mutations appeared with *F. bulimoides*. Nucleotide differences were numerous and very similar when comparing *L. diaphana* with *Galba*/*Fossaria* (41-46, average 42.0), with stagnicolines (31-41, 36.0) and with *P. columella* (42) (Table VII). The eight species 445-bp-long alignment shows a total of 108 polymorphic sites, including 89 variable positions (20%), of which 43 were parsimony informative (p-info), 46 were singleton sites and 19 gapped or ambiguous sites (Supplementary data). These variable positions do not appear regularly distributed throughout the 16S fragment and show evident concentrations in given hot spot regions (Supplementary data).

mtDNA cox1 - The code L.dia *cox*1-Ha has been ascribed for the provisional haplotype obtained for this fragment. The sequence in question is 672-bp long and shows a high AT-biased composition of 69.40% (Table I).

In a multiple 672-bp-long sequence alignment restricted to 17 sequences similar in nucleotide length, a total of 473 positions were conserved and 199 variable, comprising 166 p-info and 33 singleton sites (alignment not shown). When comparing the *L. diaphana* *cox*1 sequence with these other proximal lymnaeid species, including species of the *Galba*/*Fossaria* group, stagnicolines such as *S. elodes*, *P. columella* and also species of the *Radix* group such as *A. tomentosa* and *R. rubiginosa*, available in GenBank, the high number of nucleotide differences appears evident in a pairwise *cox*1 distance matrix (Table VIII).

The code L.dia COX1-HI has been assigned to the provisional haplotype represented by the 224-aa-long protein sequence of that *cox*1 gene fragment (Table I). In the protein alignment comprising *L. diaphana* and the aforementioned species, a total of 213 positions appeared to be conserved and 11 were variable, including five p-info and six singleton sites. A pairwise comparison showed a 100% identity between this *L. diaphana* haplotype *cox*1-a and *L. neotropica cox*1-b form Argentina and only one amino acid change (S/G) when compared with *F. bulimoides, G. truncatula, L. cubensis* and *L. neotropica* haplotype *cox*1-a (Table IX).

Phylogenetic analysis - The combination of the two ITS in a single data-set generated a robust tree, indicating phylogenetic accordance between the two spacers. The ML model best fitting this data-set was HKY85+G+I, using a ts/tv ratio of 1.32 (kappa = 2.5975285), base fre-

TABLE II

Pairwise distances between ribosomal DNA 18S nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Lymnaea) stagnalis*	-	0.00108	0.00325	0.00813	0.00705	0.00761	0.00704	0.00923	0.00758	0.00597	0.00923	0.03168	0.03444
2 *Lymnaea (Stagnicola) palustris*	2	-	0.00325	0.00813	0.00650	0.00760	0.00811	0.01030	0.00865	0.00651	0.01030	0.03273	0.03559
3 *Omphiscola glabra*	6	6	-	0.00814	0.00813	0.00870	0.00813	0.01032	0.00867	0.00706	0.01032	0.03169	0.03387
4 *Radix auricularia*	15	15	15	-	0.00216	0.00489	0.00812	0.00869	0.00866	0.00651	0.00706	0.02837	0.03084
5 *Radix balthica*	13	12	15	4	-	0.00380	0.00812	0.00869	0.00866	0.00651	0.00706	0.02946	0.03203
6 *Galba truncatula*	14	14	16	9	7	-	0.00597	0.00706	0.00597	0.00706	0.00544	0.02787	0.02973
7 *Lymnaea viatrix*	13	15	15	15	15	11	-	0.00271	0.00215	0.00433	0.00595	0.03050	0.03254
8 *Lymnaea humilis*	17	19	19	16	16	13	5	-	0.00271	0.00541	0.00596	0.02999	0.03201
9 *Lymnaea cubensis*	14	16	16	16	16	11	4	5	-	0.00433	0.00595	0.03106	0.03316
10 *Lymnaea diaphana*	11	12	13	12	12	13	8	10	8	-	0.00434	0.02944	0.03142
11 *Pseudosuccinea columella*	17	19	19	13	13	10	11	11	11	8	-	0.02840	0.03147
12 *Biomphalaria glabrata*	58	60	58	52	54	51	56	55	57	54	52	-	0.01480
13 *Bulinus truncatus*	58	60	57	52	54	50	55	54	56	53	53	25	-

the planorbids *B. glabrata* and *B. truncatus* included for comparison purposes of family distances. Sequence correspondences detailed in Materials and Methods section. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

TABLE III

Pairwise distances between ribosomal DNA internal transcribed spacer nucleotide-2 sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Stagnicola) palustris*-H1	-	0.02998	0.24737	0.29398	0.28362	0.25693	0.23200	0.23174	0.22922	0.21782	0.22879	0.25899	0.20460
2 *Lymnaea (Stagnicola)*fuscus-H2	14	-	0.25397	0.28467	0.28889	0.26209	0.23720	0.22251	0.21995	0.21197	0.22798	0.25547	0.20308
3 *Galba truncatula*-H1	94	96	-	0.14767	0.09819	0.07752	0.07937	0.16992	0.16992	0.17080	0.18732	0.20968	0.20000
4 *Lymnaea humilis*-H1	122	117	57	-	0.20920	0.18313	0.16203	0.23940	0.24190	0.25776	0.26799	0.27751	0.27632
5 *Lymnaea cubensis*-H2	116	117	38	91	-	0.04556	0.04380	0.21120	0.21628	0.20603	0.21558	0.24010	0.25000
6 *Lymnaea viatrix*-H1	102	103	30	76	20	-	0.02651	0.17016	0.17016	0.17708	0.17935	0.21429	0.22841
7 *Lymnaea neotropica*-H1	87	88	30	64	18	11	-	0.15342	0.15342	0.16120	0.17280	0.18329	0.20178
8 *Catascopia catascopium*	92	87	61	96	83	65	56	-	0.00676	0.05301	0.10579	0.15603	0.17297
9 *Stagnicola elodes*	91	86	61	97	85	65	56	3	-	0.05301	0.10327	0.15603	0.17838
10 *Catascopia occulta*-H1	88	85	62	108	82	68	59	22	22	-	0.13382	0.18476	0.18684
11 *Hinkleyia caperata*	89	88	65	108	83	66	61	42	41	55	-	0.17831	0.17204
12 *Lymnaea diaphana*-H1	108	105	78	116	97	84	68	66	66	80	74	-	0.20876
13 *Pseudosuccinea columella*-H1	80	79	67	105	93	82	68	64	66	71	64	81	-

sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

quencies for A, C, G and T of 0.22830, 0.26280, 0.22550 and 0.28340, respectively, gamma (continuous) with shape parameter alpha of 1.07 and a proportion of invariable sites equal to 0.121 (-ln L 9275.68103).

In the ML tree obtained (Fig. 3), the species of the *Radix* group appear clearly independent from all other lymnaeids, this independence always showing the highest support values. Worth emphasizing is also the presence of *P. columella* located basal to the branch, including species of both the *Galba*/*Fossaria* group and the stagnicolines, although such a location is not supported in the Heuristic analysis in which *P. columella* appears in a paraphyly regarding the fossarines and all stagnicolines nor in the Bayesian phylogeny reconstruction in which it appears basal to the *Galba*/*Fossaria* group (trees not shown).

In all trees obtained, European stagnicolines appear clustering together with the *Galba*/*Fossaria* group, whereas American stagnicolines (*Hinkleyia* and *Catascopia*, the latter also including the Palaearctic species *C. occulta*) appear separately, grouped within the same branch. *L. diaphana* appears basal to that clade comprising the American stagnicolines, with a bootstrap support of 74%, 76% and 88% with NJ, Heuristic and BPP algorithms, respectively. However, in the ML tree obtained with PhyML (tree not shown), the reliability for this *L. diaphana* - American stagnicolines cluster appear with a lower support of only 45% when applying the aLRT test.

DISCUSSION

Molecular characterization of L. diaphana - Phenotypically, this species was well described at the levels of both shell features and anatomical characteristics from specimens collected in the same type locality time ago (Paraense 1984).

Recently, broad analyses on the usefulness of the molecular markers offered by DNA in different organism groups have shown that nuclear rDNA correlates with the phenotype (shape, size, anatomy), while mtDNA does not (Mas-Coma & Bargues 2009, Mas-Coma et al. 2009a), and that mtDNA poses many problems causing erroneous results when used to compare genetically distant taxa as distant species within the same genus or different genera (Lin & Danforth 2004, Ballard & Rand 2005, Mas-Coma & Bargues 2009). This has obvious implications on the usefulness of these markers, as in fact traditional systematics and taxonomy, as those always applied to snails in malacology have fundamentally relied on morphology (Bargues et al. 2011a). Therefore, in Lymnaeidae it has been more recently concluded that (i) rDNA markers are the appropriate targets when dealing

TABLE IV

Total character differences (extreme and average values of nucleotide differences) at ribosomal DNA internal transcribed spacer (ITS)-2 and ITS-1 sequences according to Phylogenetic Analysis Using Parsimony in the pairwise distance comparisons between *Lymnaea diaphana* and the different lymnaeid groups studied

DNA marker	*Galba*/*Fossaria* group	European stagnicolines	American stagnicolines	*Pseudosuccinea columella*
ITS-2	68-116 (88.6)	105-108 (106.5)	66-80 (71.5)	81 (81)
ITS-1	73-95 (85.6)	100-101 (100.5)	76-93 (86.7)	105 (105)

data set contains 736 and 765 characters for ITS-2 and ITS-1, respectively.

TABLE V

Genetic distances in internal transcribed spacer (ITS)-2 and ITS-1 detected in pairwise comparisons of *Lymnaea diaphana* with its molecularly closest lymnaeid species

Pairwise sequence comparisons	Alignment length (bp long)	Conserved positions (n)	Nucleotide differences n (%)	Mutations (transitions + transversions) n (%)	Insertions + deletions (indels) n (%)
ITS-2					
L. diaphana vs. *Lymnaea neotropica*	512	323	189 (36.91)	77 (15.04)	112 (21.87)
L. diaphana vs. *Catascopia catascopium*	511	361	150 (29.35)	67 (13.11)	83 (16.24)
L. diaphana vs. *Catascopia elodes*	499	340	159 (31.86)	104 (20.84)	55 (11.02)
L. diaphana vs. *Hinkleyia caperata*	502	331	171 (34.06)	96 (19.12)	75 (14.94)
ITS-1					
L. diaphana vs. *Galba truncatula*	536	399	137 (25.56)	89 (16.60)	48 (8.95)
L. diaphana vs. *Hinkleyia caperata*	591	427	164 (27.75)	89 (15.01)	75 (12.69)

TABLE VI

Pairwise distances between ribosomal DNA internal transcribed spacer (ITS)-1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Stagnicola) palustris*-HA	-	0.01512	0.21258	0.21849	0.20211	0.21268	0.21118	0.23409	0.24280	0.23984	0.23320	0.20739	0.25319
2 *Lymnaea (Stagnicola)fuscus*-HA	8	-	0.20950	0.21757	0.19874	0.21341	0.21237	0.23770	0.24486	0.24646	0.23123	0.20534	0.25319
3 *Galba truncatula*-HA	98	97	-	0.15208	0.12000	0.14286	0.14639	0.22009	0.22698	0.21097	0.18298	0.16115	0.24890
4 *Lymnaea humilis*-HA	104	104	73	-	0.14868	0.15551	0.15569	0.25859	0.26316	0.26000	0.23846	0.20085	0.25652
5 *Lymnaea cubensis*-HA	96	95	57	73	-	0.04864	0.05952	0.23061	0.23950	0.21946	0.18672	0.17570	0.25217
6 *Lymnaea viatrix*-HA	104	105	71	79	25	-	0.05642	0.23374	0.24033	0.22088	0.20160	0.18737	0.25934
7 *Lymnaea neotropica*-HA	102	103	71	78	30	29	-	0.24746	0.25000	0.23695	0.19960	0.19108	0.24034
8 *Catascopia catascopium*	114	116	103	128	110	115	122	-	0.00741	0.07129	0.22543	0.18699	0.25684
9 *Stagnicola elodes*	118	119	106	130	114	118	123	4	-	0.07910	0.22929	0.18941	0.25367
10 *Catascopia occulta*-HA	118	122	100	130	106	110	118	38	42	-	0.20992	0.17304	0.25156
11 *Hinkleyia caperata*	118	117	86	124	90	101	99	117	119	110	-	0.14729	0.23701
12 *Lymnaea diaphana*-HA	101	100	73	95	81	89	90	92	93	86	76	-	0.22532
13 *Pseudosuccinea columella*-HA	119	119	113	118	116	125	112	122	121	121	114	105	-

sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

with systematic-taxonomic and phylogenetic aspects, as well as for molecular characterization of species by haplotyping, (ii) mtDNA markers are more convenient for population and intraspecific variability studies and (iii) both rDNA and mtDNA markers may be used for the classification of specimens (Bargues et al. 2011a).

The 18S sequence of *L. diaphana* (1848 bp) is slightly longer than that of *G. truncatula* (1,843 bp) (Bargues et al. 1997), equally long than that of *L. humilis* (1,848 bp) (Bargues et al. 2011a), similar to that of the European stagnicolines *L. stagnalis*, *O. glabra* and *L.* (*S.*) *palustris*, the radicines *R. auricularia* and *R. balthica,* as well as to *P. columella* (ranging between 1,849-1,852 bp) (Bargues et al. 1997, 2011b), but pronouncedly shorter than that of *L. cubensis*, *L. viatrix* and *L. neotropica* (all three 1,860-bp long) (Bargues et al. 2007). This suggests that *L. diaphana* may be considered an old species within the family Lymnaeidae, according to their phylogeny in which the oldest lymnaeid fossil known is *Galba* from the Jurassic (Zilch 1959-1960, Inaba 1969), a shorter sequence would be the plesiomorphic condition and an increase in sequence length would have occurred during lymnaeid evolution (Bargues et al. 2001).

Pairwise nucleotide comparisons in a slowly evolving gene as the rRNA small subunit (Table II) show *L. diaphana* to be at a genetic distance from Old World radicines similar to that from Palaearctic stagnicolines, as well as from *Galba*. Only New World fossarines and Pseudosuccinea appear somewhat closer, suggesting a biogeographic background for such a relationship. Unfortunately, the 18S sequence is not known for any Nearctic stagnicoline so far, in the way to verify an additional support for such an assumption.

With regard to the ITS-2, *L. diaphana* presents a sequence whose 495-bp length fits within the group of lymnaeids having the longest ITS-2 sequences (Bargues et al. 2001). This might be interpreted as a species having long time derived from the old form suggested by the 18S. In this context, *L. diaphana* shows evolutionary characteristics similar to the Nearctic species *L. humilis* (Bargues et al. 2011a). Additionally, the very high number of nucleotide differences it shows when compared to all other lymnaeid species (Table III) and groups (Table IV) is surprising. Moreover, contrary to what was expected, the distances regarding Nearctic stagnicolines appear to be lower than those regarding Holarctic *Galba*/*Fossaria* species (Table IV). This indicates that *L. diaphana* should not be included in the *Galba*/*Fossaria* group as its shell and anatomic characteristics suggest (Paraense 1984).

Such a relationship with American stagnicolines does, however, not appear so clear at ITS-1 level, a marker in which the lower *L. diaphana* differences appear with Old World originary *G. truncatula* (73) and North American *H. caperata* (76) (Table VI). Interestingly, the length of ITS-1 in *L. diaphana* is the shortest hitherto known in Lymnaeidae (520 bp), only surpassed by *G. truncatula* (504 bp).

Particular aspects of the results obtained in mtDNA 16S sequences should be highlighted: (i) AT composition appears to be pronouncedly biased, which should be taken into account when analyzing the significance of the

information this marker offers, (ii) variable positions do not appear regularly distributed throughout the sequence, but concentrated in hot spot regions (Supplementary data), which indicates that the information furnished by the fragment may not appropriately reflect whole gene evolution, as already seen in other organisms (Mas-Coma & Bargues 2009), and (iii) nucleotide differences appear to be less in number than those logically expected from mtDNA (Table VII), which suggests a low mutation rate indeed only apparent, as a consequence of an evolutionary parallelism of its rRNA gene function inside the mitochondrial genome with fast evolving mtDNA coding genes giving rise to position saturation, as already seen in lymnaeids and other freshwater molluscs (Bargues et al. 2011a). Thus, the somewhat closer 16S sequence of *L. diaphana* to that of American stagnicolines, represented by *S. bonnevillensis*, may be considered with great caution.

Nucleotide saturation and biased composition may also pose a significance question mark on the potential relationships of *L. diaphana* with the different lymnaeid groups, as suggested by the high number of nucleotide differences in the mtDNA *cox*1 gene (Table VIII). This mtDNA saturation problem has already been highlighted in lymnaeids very recently (Bargues et al. 2011a). Moreover, the very few amino acid differences in the protein sequence (Table IX) indicate that most of the nucleotide differences are silent. Additionally, there is unfortunately only one stagnicoline from which the *cox*1 fragment in question is available (*S. elodes*), so that no conclusions may be obtained from that comparison.

Summing up, the following conclusions may be obtained from the sequence analyses of nuclear rDNA and mtDNA markers: (i) *L. diaphana* shows sequences very different from all hitherto lymnaeid sequences available at the level of both nuclear rDNA and mtDNA markers, (ii) each one of the five markers analyzed, including rDNA 18S, ITS-2 and ITS-1, as well as mtDNA 16S and *cox*1, allow the differentiation of *L. diaphana* specimens from all other lymnaeids; only the COX1 amino acid sequence does not, (iii) nuclear rDNA suggest that *L. diaphana* is not a fossarine lymnaeid, but rather a relict form related to ancestral stagnicolines and (iv) mtDNA markers do not furnish genetic distance information useful for the analysis of the relationships of *L. diaphana* with the different lymnaeid groups.

Phylogenetic relationships and Pectinidens genus assessment - *L. diaphana* was selected as type species of the new section *Pectinidens* within the genus *Lymnaea* Lamarck by Pilsbry (1911). The erection of *Pectinidens* was justified on the characteristics of the radular teeth of *L. diaphana*, noted to have peculiarities different from all other lymnaeids known at that time. *Pectinidens* was considered at genus level until its synonymization with *Lymnaea* by Hubendick (1951). From that moment, it disappeared from the lymnaeid literature, although a few authors still sporadically referred to it, whether at genus level (Inaba 1969) or at subgenus level (Alcaino & Apt 1989).

In the phylogenetic reconstructions performed, *L. diaphana* does not cluster together with other morphologically similar fossarine lymnaeids as the New World *L. cubensis*, *L. viatrix* and *L. neotropica*, the Nearctic *L. humilis*, or the Old World *G. truncatula*. Contrary to what would be phenotypically expected, *L. diaphana* appears basal to the Nearctic stagnicolines (Fig. 3). Values supporting such a phylogenetic relationship were higher than 70% in most of the node reliability assessment methods. Although it may be argued that higher node supports would be better as to conclude that this result is definitive, the phylogenetic tree agrees with the very numerous nucleotide differences and very large genetic distances shown by DNA markers with verified usefulness at specific and supraspecific levels as both ITSs. Thus, in ITS-2 and ITS-1 the nucleotide differences separating *L. diaphana* from all other lymnaeids appear to be of supraspecific level in both spacers (see Table

TABLE VII

Pairwise distances between mitochondrial DNA 16S ribosomal DNA gene data set nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9
1 *Lymnaea diaphana*	-	0.09762	0.09739	0.10072	0.09785	0.09927	0.09716	0.11005	0.07329
2 *Lymnaea humilis*	41	-	0.04276	0.10526	0.00000	0.00241	0.04976	0.08115	0.08789
3 *Lymnaea cubensis*	41	18	-	0.10048	0.04265	0.04589	0.01174	0.07619	0.08706
4 *Pseudosuccinea columella*	42	44	42	-	0.10526	0.10706	0.10263	0.10287	0.10476
5 *Stagnicola elodes*	41	0	18	44	-	0.00242	0.04965	0.07889	0.08768
6 *Fossaria obrussa*	41	1	19	44	1	-	0.05301	0.08495	0.09179
7 *Fossaria bulimoides*[a]	41	21	5	43	21	22	-	0.07601	0.08216
8 *Fossaria bulimoides*[b]	46	34	32	43	34	35	32	-	0.10214
9 *Stagnicola bonnevillensis*	31	37	37	44	37	38	35	43	-

a: AF485657; *b*: EU038315. Sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

TABLE VIII

Pairwise distances between mitochondrial DNA cytochrome c oxidase (*cox*)1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *Lymnaea diaphana cox*1-a	-	0.11737	0.13889	0.13426	0.13542	0.13889	0.13839	0.14333	0.11905	0.12054	0.11161	0.11012	0.11756	0.13542	0.13690	0.13839	0.15696
2 *Fossaria bulimoides*	75	-	0.14867	0.13302	0.14867	0.14867	0.10798	0.10829	0.03443	0.03286	0.02191	0.02347	0.05008	0.10955	0.10955	0.11111	0.14950
3 *Stagnicola elodes*	90	95	-	0.13889	0.14815	0.14815	0.15432	0.16000	0.14660	0.14815	0.14815	0.14969	0.14043	0.13735	0.13735	0.13889	0.16694
4 *Austropeplea tomentosa*	87	85	90	-	0.14352	0.14352	0.13117	0.13833	0.13426	0.13580	0.12500	0.12654	0.13117	0.13735	0.13889	0.14043	0.14894
5 *Pseudosuccinea columella*[a]	91	95	96	93	-	0.00000	0.14286	0.15000	0.14583	0.14435	0.14435	0.14583	0.14881	0.13542	0.13542	0.13690	0.14725
6 *Pseudosuccinea columella*[b]	90	95	96	93	0	-	0.14815	0.15000	0.15123	0.14969	0.14969	0.15123	0.15278	0.14043	0.14043	0.14198	0.14894
7 *Galba truncatula*[c]	93	69	100	85	96	96	-	0.00333	0.11161	0.11012	0.09970	0.10119	0.10119	0.08929	0.08780	0.08929	0.15372
8 *Galba truncatula*[d]	86	64	96	83	90	90	2	-	0.12000	0.11833	0.10667	0.10833	0.10500	0.09667	0.09500	0.09667	0.15878
9 *Lymnaea cubensis cox*1-a	80	22	95	87	98	98	75	72	-	0.00149	0.02083	0.02232	0.05655	0.10714	0.10714	0.10565	0.15372
10 *Lymnaea cubensis cox*1-b	81	21	96	88	97	97	74	71	1	-	0.01935	0.02083	0.05804	0.10565	0.10565	0.10417	0.15210
11 *Lymnaea neotropica cox*1-a	75	14	96	81	97	97	67	64	14	13	-	0.00149	0.04315	0.10119	0.09821	0.09970	0.14725
12 *Lymnaea neotropica cox*1-b	74	15	97	82	98	98	68	65	15	14	1	-	0.04464	0.10268	0.09970	0.10119	0.14887
13 *Lymnaea viatrix cox*1-a	79	32	91	85	100	99	68	63	38	39	29	30	-	0.10714	0.11012	0.11161	0.15372
14 *Lymnaea humilis cox*1-a	91	70	89	89	91	91	60	58	72	71	68	69	72	-	0.00744	0.00595	0.14401
15 *Lymnaea humilis cox*1-b	92	70	89	90	91	91	59	57	72	71	66	67	74	5	-	0.00149	0.14563
16 *Lymnaea humilis cox*1-c	93	71	90	91	92	92	60	58	71	70	67	68	75	4	1	-	0.14401
17 *Radix rubiginosa*	97	90	102	91	91	91	95	94	95	94	91	92	95	89	90	89	-

a: FN598165; *b*: AY227366; *c*: AM494011; *d*: EU818799. Haplotype codes only provisional due to incomplete sequences of the gene. Sequence correspondences detailed in Materials and Methods section. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

IV for p-info positions). A deep analysis of genetic distances between *L. diaphana* and its molecularly closest lymnaeid species belonging to the Nearctic stagnicoline group and the *Galba*/*Fossaria* group according to PAUP (Tables III, VI) shows very high values (ITS-2: 29.35-36.91%; ITS-1: 25.56-27.75%) of unquestionable genus level (Table V) when compared with known genetic distances between lymnaeid genera (Bargues et al. 2001, 2006). Consequently, taxonomic validity should be restored to the genus *Pectinidens*.

It should be added that, in the literature, *L. diaphana* has usually been ascribed to King, 1830. In the original article including the description of this species, both in the second page of the contents index and in page 332 at the beginning of the article 47, it is noted "By Captain Phillip P. King, R.N., F.R.S., & c. assisted by W.J. Broderip, Esq. F.R.S., & c". Moreover, although the issue 19 of The Zoological Journal corresponds, as clearly stated in the issue front cover, to the period of July 1830-September 1831, at the bottom of this front cover it is also noted "London: printed by… and Published by… 1832". Finally, different misunderstandings have appeared in the literature with regard to the original reference; the correct one should include Year 1832, Volume 5 (from 1832-1834), Issue 19 (July 1830-September 1831), article 47, species described within article list under 43 in page 344. Therefore, according to present rules, the correct type species taxon of *Pectinidens* would thus become *Pectinidens diaphana* (King & Broderip, 1832) Pilsbry, 1911.

Stagnicolines are generally characterized by its elongate and pointed shell form, relatively long size, very numerous in species number, hitherto known to be restricted to the northern hemisphere and mostly lymnaeids adapted to live in cold waters (Bargues et al. 2003). The smaller size of only up to 14.6 mm long and 8.8 mm wide of five-whorl *L. diaphana* specimens (Paraense 1984) may be interpreted as not being sufficient as to manifest the typical stagnicoline elongate trend, similarly as has been recently shown in *L. humilis* (Bargues et al. 2011a). This would explain the usual including of *L. diaphana* within the *Galba*/*Fossaria* group. Inaba (1969) even considered *Pectinidens* as a form presumably derived at the beginning of the Pleistocene from *Fossaria* species characterized by harbouring 18 chromosomes. Accepting the ascription of *L. diaphana* to the stagnicoline group would, thus, represents not only its first representative in the southern hemisphere, but also the colonization of extreme world regions, as it is the case of the most southern Patagonia, by stagnicolines long time ago.

Whether such an old southward spreading phenomenon only concerned *L. diaphana* or additionally other lymnaeid species still remains an open question. In fact, the present molecular study furnishes the baseline on which to clarify the systematic/taxonomic validity of numerous lymnaeid species described in the southernmost mainland areas and islands of South America, in Chile as well as in Argentina. All of these species are very similar to *L. diaphana* and several of them have already been proposed to be synonyms of *L. diaphana*, although the opinion about the validity of particular species differ according to authors: *Lymnaea lebruni* Mabille 1883, *Lymnaea falklandiana* Smith, 1884, *Lymnaea pictonica* Rochebrune and

Mabille, 1889, *Lymnaea patagonica* Strebel, 1907, *Lymnaea brunneoflavida* Preston, 1910, *Lymnaea andeana* Pilsbry, 1911, *Lymnaea inelegans* Pilsbry 1911, *Lymnaea riochicoensis* Pilsbry, 1911 and *Lymnaea plicata* Hylton Scott, 1954 (Rochebrune & Mabille 1885, Pilsbry 1911, Hubendick 1951, Hylton Scott 1954, Malek 1985). Indeed, many of these lymnaeid species were already included within *Pectinidens* by Pilsbry (1911).

L. diaphana and fascioliasis transmission - With regard to epidemiological characteristics and transmission patterns in human fascioliasis endemic areas, its zoonotic aspect seems to have only a relative influence due mainly to the scarce differences related to different livestock species playing a role of reservoir, given the similar infectivity of the metacercarial stage from different livestock species isolates (Valero & Mas-Coma 2000, Valero et al. 2001). On the contrary, its vector-borne aspect has proved to have a pronounced influence on human infection. The different fascioliasis patterns appear related to the different lymnaeid species, their ecology, type of water bodies they inhabit, their anthropophilic preferences, population dynamics, climatic links and transmission capacity (Bargues & Mas-Coma 2005, Mas-Coma et al. 2009a).

Fascioliasis endemic areas inhabited by *L. diaphana* are low altitude areas (Cordova et al. 1961, Tantalean et al. 1974, Larrea et al. 1994, Sielfeld 2001, Valdovinos 2006), which do not appear to fit the hitherto two main disease transmission scenarios where human infection epidemiology has been characterized: the Altiplano subpattern and Valley subpattern, both in high altitude endemic zones (Mas-Coma 2005, Mas-Coma et al. 2009a).

The phylogenetic link of *L. diaphana* with the stagnicoline group may give light to the aforementioned peculiar low altitude epidemiological scenario. Indeed, stagnicolines are only considered secondary vectors of fascioliasis when compared to the main vector species of *F. hepatica* included in the *Galba*/*Fossaria* group (Bargues et al. 2001) and to play an important transmission role only rarely in particular places where no other lymnaeid vector is present or under special natural conditions (Czapski 1962, 1997, Bouix-Busson & Rondelaud 1985, 1986, Dreyfuss et al. 1994, 2000). The existence of lymnaeid species of high transmission capacity, belonging to the *Galba*/*Fossaria* group, has already been molecularly confirmed in low altitude endemic areas of Peru (Bargues et al. 2007). The participation of *L. diaphana* in disease transmission in the southernmost fascioliasis endemic areas of South America, in both Chile (Alcaino & Apt 1989, Morales et

TABLE IX

Cytochrome c oxidase (*cox*)1 amino acid sequence differences detected in pairwise comparisons between haplotypes of *Lymnaea diaphana* and other proximal lymnaeid species available in GenBank

Nucleotidic haplotype *cox*1	GenBank accession	Country	Variable position									
									1	1	1	2
				1	3	9	9	9	0	2	6	7	0
			8	1	2	6	8	9	0	5	0	6	4
L.dia-*cox*1-a	JF909501	Chile	I	I	T	I	L	L	C	S	P	V	S
F. bulimoides	AY227367	Canada	.	.	.	.	.	.	.	G	.	.	.
S. elodes	AY227368	Canada	.	V	L	V	.	.	.	G	.	.	.
A. tomentosa	AY227365	Australia	.	V	M	.	.	.	?	S	I	T	
P. columella	FN598165	Venezuela	V	V	M	.	.	.	G	.	.	T	
P. columella	AY227366	Australia	V	V	M	.	.	.	G	.	.	T	
G.tru-*cox*1-a	AM494011	Spain	.	.	.	.	.	.	.	G	.	.	.
G. truncatula	EU818799	Germany	-	-	.	.	.	.	.	G	.	.	.
L.cub-*cox*1-a	AM494009	Cuba	.	.	.	.	.	.	.	G	.	.	.
L.cub-*cox*1-b	FN182205	USA	.	.	.	.	.	.	.	G	.	.	.
L.neo-*cox*1-a	AM494008	Peru	.	.	.	.	.	.	.	G	.	.	.
L.neo-*cox*1-b	FN356741	Argentina	.	.	.	.	.	.	.	.	.	.	.
L.via-*cox*1-a	AM494010	Argentina	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-a	FN182197	USA	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-b	FN182198	USA	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-c	FN182199	USA	.	V	.	.	.	.	.	G	.	.	.
R. rubiginosa	GU451737	Thailand	.	V	S	.	F	F	V	G	.	.	.

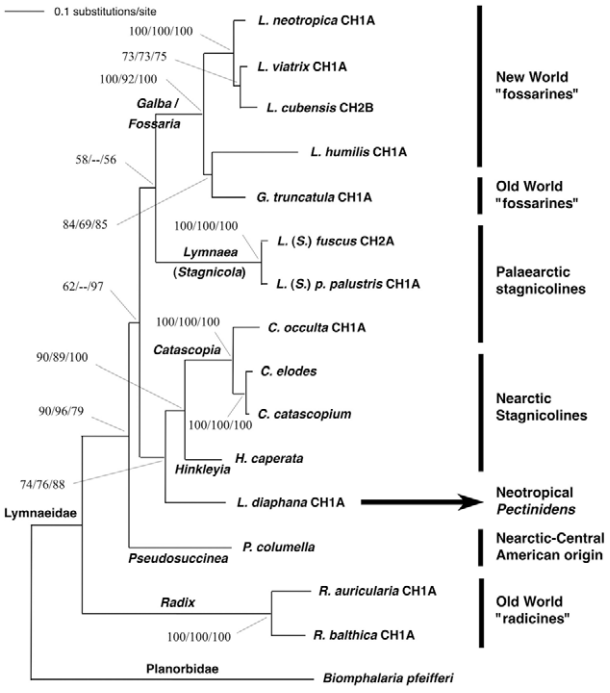

Fig. 3: phylogenetic tree of lymnaeid species studied obtained using the planorbid *Biomphalaria pfeifferi* as outgroup, based on maximum-likelihood (ML) estimates. CH: internal transcribed spacers composite haplotype. Scale bar indicates the number of substitutions per sequence position. Support for nodes a/b/c: *a*: bootstrap with neighbour-joining reconstruction using Phylogenetic Analysis Using Parsimony (PAUP) with ML distance and 1,000 replicates; *b*: bootstrap with ML reconstruction using PAUP with 1,000 heuristic replicates; *c*: Bayesian posterior probability with ML model using MrBayes.

al. 2000) and Argentina (Olaechea 1994), whether as only vector or coexisting with other lymnaeid vector species (e.g., *L. viatrix*) (Kaczorkiewick 1983, Rubel et al. 2005, Kleiman et al. 2007) should be assessed.

Molecular markers established in the present study furnish the needed baseline on which to (i) address the validity or synonymy of each one of the several aforementioned lymnaeid species cited in southern Patagonia, (ii) clarify the geographical distribution and intraspecific genetic variability of *L. diaphana* and finally (iii) assess its population dynamics correlation with fascioliasis transmission. The cold weather typical of such extreme latitudes suggest, in the southern low altitude Patagonian plains, a marked transmission seasonality restriction due to the minimum temperature 9-10ºC threshold of *F. hepatica* (Fuentes et al. 1999, 2001). Such a seasonality has already been verified somewhat more northward in Andean valleys (Kleiman et al. 2007).

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Nucleotide differences found in the complete 18S ribosomal DNA (rDNA) sequence of the lymnaeid species compared and their location in the secondary structure

Variable areas	VVVVV V	VVVVVVVVVV	VVVVVVVVVV	VVVVVVVVVV	V	VV VV VV
	11111 2	2222222222	2222222222	2222222222	2	45 79 99
Helix	666668E999	EEEEEEEEEE	EEEEEEEEEE	EEEEEEEEEE	E11111511E	EEEE223344 44
	8	1111111111	1111111111	1111111111	122222 882	2222782513 47
		0000000000	0000000000	0000000000	1 1	11111
		----------	----------	----------	-	-----
		1111111111	1111111111	1111111111	1	25777
Position						111111 11
	11112	2222222222	2222222222	2222222222	3333334557	7788123347
	6777925550	3333333444	4444445555	5555556666	1999996882	4935530133 59
	9234892786	3456789012	3456790123	4567890126	2145784025	7496643763 23
Lymnaea (Lymnaea) stagnalis	GTG-T----C	CG-----TGC	CGGGGGACTC	GTGC----GC	-CGTAC-CC-	AACGT-GCTG TA
Lymnaea (Stagnicola) palustris	...-.CA--.	..------...	....	....------.	-.......--	..
Omphiscola glabra	TAAC.-----.	..------...	....	....------.	-.......--	..
Galba truncatula	...-.AA--.	T---------	.CTTT.CGAG	.-------.T	-.....-TT-	CG.A.--.C. .G
Lymnaea cubensis	...-.AA--.	T.TCGTGCCG	...T.A.GC.	...GTCGC..	-.....CTTG	CG...-T-C. CG
Lymnaea viatrix = Lymnaea neotropica	...-.AA--.	T.TGCCTCCG	...T.A.GC.	...GTCGC..	-.....CTT-	CG...CT-C. CG
Lymnaea humilis	...-.AA--T	T-------CG	...C.AGGC.	.A.G-----.	-.....CTT-	CG...CT-CC CG
Pseudosuccinea columella	...-.AA--.	T------C.G	TCCC....-G	.G..CG--.T	-.....CTT-	CG...CT-C. ..
Lymnaea diaphana	...-.AA--.	-------CCG	...CT.C---	.CCGTG---.	-.....CTT-	TG...CT-C. ..
Radix auricularia	...-CAA--.	..------TG	.TCTT.CGGG	.........T	CGTACT-TAG	CG.AC-T-C. ..
Radix balthica	...-.CATT.	..------TG	.TCTT.CGGG	.........T	CGTACT-TT-	CG.AC--.C. ..
Total variable positions (n = 62)	1	1111111111	2222222222	3333333333	4444444444	5555555555 66
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890 12

numbers (to be read in vertical) refer to positions obtained in the alignment made with MEGA 5.0. Shaded area corresponds to variable area V2 and helix E10-1 where Lymnaeidae differences in the 18S rRNA gene are concentrated. ·: identical; -: indel.

Polymorphic sites (n = 108), including parsimony informative, singleton sites and gapped or ambiguous characters, detected in the mitochondrial DNA and 16S ribosomal DNA gene sequence alignment of the lymnaeid species compared according to MEGA 5.0

a: AF485657; *b*: EU038315; ·: identical; -: indel**?**.

???