Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fascioliasis

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Abstract
Background: Lymnaeidae snails play a prominent role in the transmission of helminths, mainly trematodes of medical and veterinary importance (e.g., Fasciola liver flukes). As this family exhibits a great diversity in shell morphology but extremely homogeneous anatomical traits, the systematics of Lymnaeidae has long been controversial. Using the most complete dataset to date, we examined phylogenetic relationships among 50 taxa of this family using a supermatrix approach (concatenation of the 16 s, ITS-1 and ITS-2 genes, representing 5054 base pairs) involving both Maximum Likelihood and Bayesian Inference.

Results: Our phylogenetic analysis demonstrates the existence of three deep clades of Lymnaeidae representing the main geographic origin of species (America, Eurasia and the Indo-Pacific region). This phylogeny allowed us to discuss on potential biological invasions and map important characters, such as, the susceptibility to infection by Fasciola hepatica and F. gigantica, and the haploid number of chromosomes (n). We found that intermediate hosts of F. gigantica cluster within one deep clade, while intermediate hosts of F. hepatica are widely spread across the phylogeny. In addition, chromosome number seems to have evolved from n = 18 to n = 17 and n = 16.

Conclusion: Our study contributes to deepen our understanding of Lymnaeidae phylogeny by both sampling at worldwide scale and combining information from various genes (supermatrix approach). This phylogeny provides insights into the evolutionary relationships among genera and species and demonstrates that the nomenclature of most genera in the Lymnaeidae does not reflect evolutionary relationships. This study highlights the importance of performing basic studies in systematics to guide epidemiological control programs.

Background
Basommatophora (Gastropoda: Pulmonata) is a suborder comprising essentially all pulmonate gastropods living in freshwater. Basommatophorans are monophyletic and encompass five families: Acroloxidae, Chilinidae, Lymnaeidae, Physidae, and Planorbidae (including the Ancylidae) [1]. The group contains ~300 species and has been extensively studied because some species have a role in transmitting parasites of human and veterinary importance (e.g., Schistosoma and Fasciola). The Lymnaeidae, Physidae and Planorbidae comprise ~90% of the Basommatophoran species [1,2]. The phylogenetic relationships within the Physidae and Planorbidae are now well established (e.g., [3-5]). However, the phylogeny of the Lymnaeidae has only been partially inferred [6-11] and we currently lack a comprehensive treatment of this family.

Lymnaeidae snails are distributed worldwide [12-14]. They are of major medical and veterinary importance since they act as vectors of parasites that severely affect human populations and livestock, and cause important economic losses [15,16]. Indeed, lymnaeids serve as intermediate hosts of at least 71 trematode species among 13 families [17,18], including some species of Schistosomatidae and Echinostomatidae, with implications for human health [19,20], and Paramphistomum daubneyi, which is of veterinary interest [21].
Undoubtedly, the most emblematic case of parasite transmitted by lymnaeids is *Fasciola hepatica* (Digenea: Fasciolidae), the agent of fascioliasis. The disease it causes is recognized as a major veterinary problem as it is responsible of the loss of productive capacity (e.g., meat, milk). Fascioliasis is also an important human disease with about 20 million cases around the world [22]. *Fasciola hepatica* presumably originates from Europe and now has a worldwide distribution. The definitive hosts of this parasite are vertebrates, usually mammals, *e.g.*, cows, sheep, goats, buffalos, and also humans. Molusks, generally lymnaeids, are required as intermediate hosts to complete the life cycle. At least 20 species of Lymnaeidae have been described as potential vectors of fascioliasis (see reviews in [17,23,24]). Due to the important role of these species as intermediate hosts of trematodes, a solid phylogenetic framework of Lymnaeidae is required. The correct identification of intermediate-host species should help characterize areas of epidemiological risk and increase our understanding of the evolution of the Lymnaeidae- *Fasciola* host-parasite interaction.

Lymnaeidae exhibit a great diversity in shell morphology which is linked to substantial eco-phenotypic plasticity (see *e.g.*, [25,26]). Hubendick [12] illustrated this point by compiling up to 1143 species names, a large number of which he synonymized. In contrast, the anatomy of their reproductive tracts (including prostate, penis and preputium) is extremely homogeneous (*e.g.*, [1,27,28]). Immunological [29], cytogenetical [30,31], enzyme electrophoresis studies [25,32,33], and DNA-based approaches [7,10] have demonstrated extensive homoplasy in anatomical characters [12,26,34-38]. The difference between patterns in shell morphology and anatomy explains why lymnaeid systematics has been controversial [6]. Today, it is accepted that the total number of species might be less than 100 [1], with most occurring in the Palearctic and Nearctic regions [2]. Although some effort has been done to resolve their phylogenetic relationships, the small number of genes or species considered (*e.g.*, a single gene in [7]) and limited geographical coverage (*e.g.*, Neotropic in [10], Australasia in [11] and ancient European lakes in [39]) represent severe limitations and biases. Furthermore, some well-recognized species have never been considered (*e.g.*, the Neotropical species *Lymnaea cousini* and *L. diaphana*). These limitations and biases have generated gaps in our understanding of their biogeographic patterns, and their epidemiology in areas of high endemicity of trematode diseases (*e.g.*, South America; [40]).

In this paper, we contribute to fill these gaps by performing a phylogenetic analysis using a supermatrix approach. We infer the most complete phylogeny to date in Lymnaeidae using sequences of 50 taxa (*i.e.*, approximately half of the supposed diversity of the family) covering most Neotropical species (including the unstudied *L. diaphana*, *L. cousini* and *Lymnaea* sp. from Colombia). Using this phylogenetic framework, we analyze how susceptibility to infection by *F. hepatica* and *F. gigantica* (the sister-species of *F. hepatica* mainly responsible for fascioliasis in Asia and Africa) has evolved. In addition, this phylogeny allows us to establish biogeographic aspects, to pinpoint potential biological invasions, and determine the evolution of chromosome numbers.

**Methods**

**Sampling**

Sequences of one to three genes among the two nuclear internal transcribed spacers of the ribosomal DNA (ITS-1 and ITS-2) and the mitochondrial 16 S ribosomal DNA of 38 lymnaeid species and two outgroups, the planorbids Bulinus forskalii and Biomphalaria tenagophila (Planorbidae), were retrieved from GenBank (Table 1). We chose these three genes because they are highly variable and have been extensively used in phylogenetic studies of Lymnaeidae. They represent most known clades of this family (*e.g.*, [6,7,9-11,41]). In addition, living individuals of 12 lymnaeid species and an additional outgroup, Physa acuta (Physidae), were collected in 13 populations sampled in a variety of countries/continents (Table 1). Sampled individuals were stored in 80% ethanol for DNA analyses.

Genus names are still fluctuating in Lymnaeidae. However, we conserved the names given in GenBank for the sequences retrieved there in order to facilitate comparisons between this and previous studies. For the species we sampled, we adopted the widely accepted genus *Lymnaea* for all species, except for *Galba truncatula*, Omphiscola glabra, Pseudosuccinea columella and Radix peregra (see Table 1).

**DNA Extraction and Polymerase Chain Reaction (PCR) Amplification**

Total DNA was isolated from the distal part of the foot. We carefully controlled for trematode presence during dissection in order to avoid exogen DNA. Extractions were performed using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturers’ instructions. The two nuclear internal transcribed spacers (ITS-1 and ITS-2) and the 16 S gene were amplified using published primers [6,42] (Table 2). PCR amplification was performed for each pair of primers in a total volume of 25 μl containing 5 μl of PCR reaction buffer 5×, 2.5 mM MgCl₂, 200 μM of each dNTP, 10 pmol of each primer, 1 U GoTaq DNA polymerase (Promega), and 2 μl of DNA template. Temperature cycling for the ITS-1 and ITS-2 was as follows: 94°C for 2 min, 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, repeated for 30
Table 1 Names and accession numbers given in GenBank for the species used in the phylogeny presented in figure 1

| Species                    | Country, locality              | ITS-1         | ITS-2         | 16S          |
|----------------------------|--------------------------------|---------------|---------------|--------------|
| Autotropelea lessoni       | Australia                      | NA            | EU536308      | EU556266     |
| Autotropelea ollula        | Philippines                    | NA            | NA            | U82067       |
| Autotropelea tomentosa     | Australia, Guyra               | NA            | EU556270      | AF485645     |
| Autotropelea viridis (= Lymnaea viridis) | Australia, Perth (Queensland) | NA            | EU556313      | AF485642     |
| Biomphalaria tenagaphila   | Brazil, Rio Grande do Sul, Goias | AY425730     | AF198655      | AY030220     |
| Bulimnea megasoma          | Canada, Manitoba               | NA            | NA            | U82069       |
| Bulimus forskalii          | Tanzania, Mafia Island, Angola, Quifangondo | AF503573     | AM921961      | AY029550     |
| Bullastra cunningiana      | Philippines, Luzon            | NA            | EU556314      | U82068       |
| Fossaria bulimoides        | USA, Oklahoma                  | NA            | NA            | AF485657     |
| Fossaria obrussa           | Canada, Ontario                | NA            | NA            | AF485658     |
| Galba truncatula           | France, Limoges                | HQ283251      | HQ283262      | HQ283236     |
| Kutikina hispida           | Australia, Franklin River       | NA            | EU556311      | EU556268     |
| Lymnaea corvus             | Austria, Wallersee;           | NA            | AJ319625      | U82079       |
| (= Stagnicola corvus)      | Bulgaria                       |               |               |             |
| Lymnaea cousini            | Venezuela, Mucubají            | HQ283255      | HQ283266      | HQ283237     |
| Lymnaea cubensis (= Bakellymnaea cubensis) | Colombia, Antioquia         | HQ283253      | HQ283264      | FN182204     |
| Lymnaea diaphana           | Argentina, Lago Escondido     | HQ283256      | HQ283260      | HQ283241     |
| Lymnaea fuscus             | Germany, Westfallen            | AJ626855      | AJ319622      | NA           |
| Lymnaea gen. sp.           | Hawaii, Waikiki                | NA            | NA            | U82070       |
| Lymnaea humilis            | USA, Charleston (South Carolina) | FN182193     | FN182191      | FN182195     |
| Lymnaea (Radix) natalensis | La Réunion, Bras de Pontho     | HQ283257      | HQ283270      | HQ283242     |
| Lymnaea neotropica         | Peru, Lima                     | AM412228      | AM412225      | NA           |
| Lymnaea occulta            | Poland                         | AJ626858      | AJ457042      | NA           |
| (= Catascopia occulta)     |                                |               |               |             |
| Lymnaea palustris (= Stagnicola palustris)  | Sweden, Umeå, | HQ283250      | HQ283267      | U82082       |
| Radix peregra              | Germany                        |               |               |             |
| Lymnaea sp. EEAR-China-2002 | France, Viols le Fort (Herault); Turkey, Söke | HQ283258     | HQ283271      | U82074       |
| Lymnaea sp. Colombia       | China, Wuhan                   | NA            | NA            | AF485643     |
| Lymnaea sp. EEAR-Hawaii-2002 | Colombia, Antioquia         | HQ283252      | HQ283263      | HQ283235     |
| Lymnaea stagnalis          | USA, Hawaii                    | NA            | NA            | AF485644     |
| Stagnicola turricula      | France, Lacépède (Lot et Garonne); Canada | NA           | HQ283268      | AF485659     |
| (= Lymnaea palustris turricula) | Austria, Wallersee         | AJ626853      | AJ319618      | NA           |
| Lymnaea viatrix            | Argentina, Rio Negro           | HQ283254      | HQ283265      | HQ283239     |
| Omphiscola glabra          | France, Limoges                | HQ283249      | HQ283269      | HQ283246     |
| Physa acuta                | Mexico, Veracruz               | HQ283259      | HQ283272      | GQ415021     |
| Pseudosuccinea columella   | Colombia, Antioquia, Australia | HQ283248      | HQ283261      | U82073       |
| Radix ampla                | Austria, Wallersee             | NA            | AJ319640      | NA           |
| Radix auriculana           | Czech Republic; Danube Delta, Romania | NA          | AJ319628      | AF485646     |
| Radix labiata              | Czech Republic; Turkey         | NA            | AJ319636      | NA           |
| Radix lagotis              | Austria, Schönau               | NA            | AJ319639      | NA           |
| Radix luteola              | Sri Lanka                      | NA            | NA            | AF485648     |
| Radix ovata                | Germany, Tubingen              | NA            | NA            | AF485647     |
| Radix quadraei             | Philippines, Luzon             | NA            | EU556315      | U82075       |
| Radix rubiginosa           | West Java, Malaysia            | NA            | EU556316      | U82076       |
| Radix sp. EEAR-Canada-2002 | Canada, Manitoba               | NA            | NA            | AF485650     |
| Radix sp. EEAR-Philippines-2002 | Philippines, Taal Lake        | NA            | NA            | AF485649     |
| Radix sp. EEAR-Romania-2002 | Romania, Razelm Lake          | NA            | NA            | AF485651     |
| Stagnicola bonnevillensis | USA, Utah                      | NA            | NA            | AF485655     |
| Stagnicola caperata        | Canada, Manitoba               | AF013140      | AF013140      | U82077       |
cycles, and final extension at 72°C for 7 min. Temperature cycling for the 16S gene was 95°C for 3 min, 50°C for 2 min, 72°C for 1.5 min, four times at 93°C for 15 sec, 50°C for 8 sec, 72°C for 1.5 min and 25 times at 93°C for 5 sec, 8 sec at 50°C, 72°C for 1 min and final extension at 72°C for 10 min. The amplified products (5 μl) were checked on 1% agarose gels in TAE buffer. DNA sequencing was performed by CoGenics Genome Express (Meylan, France) using PCR-amplified products as templates.

Sequence Alignment and Phylogenetic Analyses
We aligned ITS-1, ITS-2 and 16S sequences of 50 lymnaeid species and the three outgroups using Prank v. 100311 [43]. Prank is based on an algorithm that can distinguish insertions from deletions and avoid repeated penalization of insertions. Compared to Clustal-W and Muscle, it considerably improves the alignment quality especially in highly variable sequences such as ITSs. Alignments of individual genes were concatenated in a supermatrix with the seqCat.pl v1.0 script [44].

Two different approaches of tree reconstruction, Maximum Likelihood (ML) and Bayesian Inference (BI), were implemented. Analyses were performed by partitioning the supermatrix on the basis of individual loci. ML analyses were conducted using the best-fitting model of sequence evolution. Model selection was based on Akaike’s Information Criterion (AIC) using ModelTest 3.7 [45]. ML trees and the corresponding bootstrap supporting values (BP) of the nodes were obtained with PAUP* 4.0b10 [46] using heuristic search with neighbor-joining starting tree, tree bissection-reconnection swapping and 100 bootstrap replicates. BI analyses were performed with MrBayes 3.2 [47]. The tree space was explored using Markov Chain Monte Carlo (MCMC) analyses with random starting trees, five simultaneous, sequentially heated independent chains sampled every 500 trees during five million generations. Suboptimal trees were discarded once the “burn-in” phase was identified and a majority-rule consensus tree, with posterior probability support of nodes (PP), was constructed with the remaining trees.

Susceptibility to Infection by *Fasciola hepatica* and *F. gigantica*, and the Evolution of the Chromosome Number in Lymnaeidae
We searched available information on susceptibility to infection by *F. hepatica* and *F. gigantica*, derived from either analyses of natural populations or from experimental infections, in all species considered using the ISI Web of Science (Thompson-Reuters). We recorded the susceptible or not susceptible status for each species (Table 3). In addition, the haploid number of chromosomes (n) was obtained from previous publications [7,11,31,48].

Results
A Comprehensive Phylogeny of Lymnaeidae
The ML and BI trees inferred with the alignment of the supermatrix including 50 Lymnaeidae species and three outgroups (5054 aligned sites) were extremely similar, although some node supports varied between the two approaches. The best model describing the evolution of the supermatrix was TVM+I+G (proportion of invariable sites = 0.2298; shape parameter = 0.8159). Overall, the clades obtained here are consistent with previous results [7-9,18,39,41]. Three deeply-rooted clades (hereafter C1, C2 and C3) were detected, basically matching the geographic origin of species (Figure 1). The C1 clade (n = 18) included all American species and two
species thought to originate from Europe (Galba truncatula and Lymnaea occulta = Catascopia occulta), although the inclusion of Pseudosuccinea columella was only weakly supported (0.18 BP and 0.30 PP). Two highly supported subclades can be recognized within this clade. The first one (C1a) included the South American L. diaphana, the North American Stagnicola caperata, the European L. occulta (= C. occulta), and all other North American Stagnicola. The second subclade (C1b) grouped the South American L. cousini with the North American Fossaria bulbimoides and L. humilis, on the one hand, and the European G. truncatula, Neotropical L. cubensis (= Bakerilymnaea cubensis), L. neotropica and L. viatrix, the North American F. bulbimoides and Lymnaea sp. from Colombia, on the other hand.

The C2 clade (n = 18) consisted of exclusively Eurasian species. In this clade, Omphiscola glabra (a small-shelled, morphologically distinct species) first diverged from all other species, including most large-bodied species found in Europe (excluding Radix spp.). This was followed by divergence of L. stagnalis, L. corvus (= S. corvus), L. fuscus, Stagnicola sp. EEAR-Ukraine-2002, L. palustris (= S. palustris) and S. turricola (= L. palustris turricola). These relationships are identical to those reported in [7,8,18].

The C3 clade contained all Australasian and Radix species, including the African Lymnaea (Radix) natalensis. ML and BI analyses indicated that there were two subclades within C3. The first one (C3a) included the South American Austropeplea lessoni and Bullastra cumingiana (n = 16), A. tomentosa and Kutikina hispidina (n = 16), and a subclade grouping Austropeplea ollula and Austropeplea viridis (= L. viridis) (n = 16), on the one hand; and Lymanea sp. EEAR-China-2002 and Lymanea sp. EEAR-Hawaii-2002 (n = 18 according to [48] and [11]; n = 16 according to [7]), on the other hand. The second subclade (C3b) consisted of all Radix species (n = 17), including L. (R.) natalensis, and included two monophyletic groups. The first one was formed by Radix labiata sister to R. peregra, R. ampla and R. lagotis. Note that our samples of

| Intermediate host          | Infection by F. hepatica | Infection by F. gigantica | Refractory to infection |
|----------------------------|--------------------------|---------------------------|-------------------------|
| Austropeplea tomentosa     | [68]                     |                           |                         |
| Austropeplea (Lymnaea) viridis | [69]                   | [9]                       |                         |
| Austropeplea ollula        | [9]                      | [9]                       |                         |
| Fossaria bulbimoides       | [70]                     |                           |                         |
| Galba truncatula           | [71]                     | [9]                       |                         |
| Lymnaea cousini            | [72]                     |                           |                         |
| Lymnaea (Bakerilymnaea) cubensis | [73]              |                           |                         |
| Lymnaea diaphana           | [9]                      |                           |                         |
| Lymnaea fuscus             | [74]                     |                           |                         |
| Lymnaea humilis            | [75]                     |                           |                         |
| Lymnaea (Radix) natalensis | [76]                     | [9]                       |                         |
| Lymnaea neotropica         | [77]                     |                           |                         |
| Lymnaea (Catascopia) occulta | [9]                    |                           |                         |
| Lymnaea (Stagnicola) palustris | [71]            |                           |                         |
| Lymnaea sp. from Colombia  | [53]                     |                           |                         |
| Lymnaea stagnalis          | [78]                     |                           |                         |
| Stagnicola turricola       | [9]                      |                           |                         |
| (= Lymnaea palustris turricola) |                     |                           |                         |
| Lymnaea viatrix            | [79]                     |                           |                         |
| Omphiscola glabra          | [71]                     |                           |                         |
| Pseudosuccinea columella   | [73]                     | [9]                       | [80]                    |
| Radix peregra              | [71]                     | [9]                       |                         |
| Radix auriculana           | [76]                     | [81]                      |                         |
| Radix ovata                | [82]                     |                           |                         |
| Radix labiata              | [83]                     |                           |                         |
| Radix lagotis              | [76]                     |                           |                         |
| Radix luteola              | [9]                      |                           |                         |
| Radix rubiginosa           | [76]                     | [9]                       |                         |
| Stagnicola caperata        | [84]                     |                           |                         |
| Stagnicola elodes          | [73]                     |                           |                         |

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R. peregra from Southern France clustered within the MOTU2 clade described in [26] with no ambiguity, based on analyses of COI and ITS-1 sequences (results not shown). The second, more recently derived clade, was formed by R. auricularia and R. ovata which were sister to a clade comprising Radix sp. EEAR-Philippines-2002, Radix sp. EEAR-Canada-2002 and Radix sp. EEAR-Romania-2002, L. (R.) natalensis sister to R. luteola, and R. quadrasi sister to R. rubiginosa.

Discussion

A Comprehensive Phylogeny of Lymnaeidae

Recent studies have suggested that the Lymnaeidae contains approximately 100 species [1,2], meaning that our phylogeny represents approximately half the existing diversity of the family. The tree presented in Figure 1 indicates that species cluster by geographic origin in three deep clades. One is almost entirely composed of American species, while the two others are from the Old World. The split between the American C1 clade and Old World C2 clade probably dates back to the opening of the Atlantic Ocean 160-130 million years ago (Mya). This date is reasonable given the fossil record suggests the divergence of Physidae-Lymnaeidae 200-145 Mya; [49]).

Concerning the American clade (C1) various aspects should be highlighted. Our study is the first to include L. diaphana in a phylogenetic analysis and suggests that this species is sister to the North American Stagnicola and L. occulta (= C. occulta) (clade C1a), although with low support (0.54 BP and 0.71 PP). Baker [50] reported L. diaphana in South Dakota, consistent with a possible North American origin. However, the species was originally described from southern South America (Strait of Magellan, Chile; [51]) and is currently found in Argentina, Brazil, Chile, Peru and Uruguay. It could be either that the current distribution of L. diaphana in South America is not representative of its North American origin or that the ancestor of clade C1a was originally from South America and then migrated northwards, where it gave origin to the Stagnicola of clade C1a. A further point is that L. occulta (= C. occulta) from Poland is unambiguously grouped with this clade and agrees with [41]. This is unexpected as this species is thought to originate from Europe, and was formerly grouped, on the basis of anatomical characters, together with the European L. palustris (= S. palustris) and S. turricola (= L. palustris turricola), which appear, as expected, in a predominantly European clade (C2). We hypothesize that L. occulta was actually introduced in Europe from North American populations (see below). Finally, our results do not contradict the hypothesis that S. emarginata, S. elodes and S. catascopium are conspecifics, as suggested by [6] and [18]. If this is true, Stagnicola sp. EEAR-Manitoba-2002 should also be synonymized with these three taxa.

Species with similar shell morphology (G. truncatula-like) in the C1b clade cluster together. The only exception is L. cousini which clearly belongs to this clade (with maximal BP and PP values) but is morphologically different from all other species in terms of both shell morphology and the anatomy of reproductive organs. Its phylogenetic position suggests the ancestor of clade C1b had a G. truncatula-like morphology and the L. cousini morphology is a derived character. The inclusion of G. truncatula within this clade is unambiguous and agrees with previous results [10]. This species has always been considered a native from the Old World, occurring in Europe, Russia, and North Africa, and to have been recently introduced to South America [32,52]. Our phylogeny suggests that G. truncatula represents a branch of an American clade that reached the Old World, where it has evolved and diverged from its American sister species (see below). Interestingly, Lymnaea sp. from Colombia, previously described as G. truncatula [53], was unambiguously identified as a distinct taxonomic entity, yet to be sequenced. We currently lack sequences and population-genetic studies of several taxa described in North America presenting morphological similarities with Lymnaea sp. Therefore, we are not in position to ascertain that this is a new species and to determine whether it is endemic or has been introduced to South America. Thus, we refrain from describing this entity as a new species to avoid adding more noise to the already confusing systematics of the Lymnaeidae.

The second deep clade (C2) consists exclusively of Eurasian species, and species branching agrees [7] and [18]. It does not contradict the hypothesis that L. palustris (= S. palustris) and S. turricola (= L. palustris turricola) are synonymous, as proposed by [18]. These two taxa indeed only differ by two anatomical characters (preputium length to penis length ratio, and the relative length of the distal part of the prostate) [54].

The third deep clade (C3) includes all species inhabiting Australia and the Indo-Pacific region, as well as Radix species. According to this tree, Austropeplea is a polyphyletic genus, in agreement with results obtained by [6], [7] and [11]. Remigio [7] suggested monophyly of Austropeplea sp., Bullastra cumingiana, Lymnaea sp. EEAR-China-2002 and Lymnaea sp. EEAR-Hawaii-2002. Our results indicate that Kutikina hispida and Lymnaea gen. sp. from Hawaii are also members of this clade. On the other hand, Radix seems to be a monophyletic genus, in agreement with [8], [11], [18] and [26], but in contradiction with results of [6] and [7]. Importantly, L. (R.) natalensis, the only known endemic African species, branched unambiguously within the Radix clade, in
agreement with [39], sister to *R. luteola* from Sri Lanka. This result confirms that the name *Radix natalensis* better reflects its phyletic relationships than does *Lymannea natalensis*. Our results do not support the hypothesis that *R. peregra* and *R. ovata* are synonymous [18]. Their synonymy is indeed still a controversial matter [26,38,55]. Alternatively, *R. peregra*, *R. ampla* and *R. lagotis* are closely related taxa, in agreement with [18], and might potentially be conspecifics, although confirmation using mating experiments seems necessary.

Figure 1 Phylgeny of the Lymanneidae. The tree was obtained by concatenating the 16 S, ITS-1 and ITS-2 sequences, and includes 50 species and three outgroups. Colored branches represent geographic origin; blue = Australasian; red = Eurasia; brown = Africa and Indic ocean; ochre = North America; green = Central and South America. Species naturally or experimentally serving as intermediate hosts of Fasciola hepatica (H), F. gigantica (G) or refractory to infection (Ø) are shown. (n) is the haploid number of chromosomes. Values on nodes represent bootstrap percentages (BP) and posterior probabilities (PP, given within parentheses). Species sequenced by us are in bold characters.
In summary (and ignoring recent introductions) we have three old centers of diversification in the Lymnaeidae family: America, Eurasia and the Indo-Pacific region. In the latter, the Radix clade diversified and then expanded towards Eurasia and Africa. This is also true of G. truncatula, a branch of the American clade that invaded the Old World. In general, the Lymnaeidae morphology has evolved slowly and most species within clades are similar: small-shelled turritiform, G. truncatula-like in the American clade; large and high-spined shells in the Eurasian Lymnaea; and large, rounded or ovate shells in the Indo-Pacific clade, especially in Radix. A few branches, including O. glabra, and L. coussini have evolved distinctive morphologies that differ from all other lymnaeids. Note, however, that limpet-shaped species (e.g., Laxus) were not analyzed here, and it is not possible to discuss their phylogenetic position and morphological evolution. If they constitute a separate clade, say C4, our hypothesis of slow morphological evolution would be valid.

Concerning chromosome numbers, [31] and [56] hypothesized that evolution in Lymnaeidae proceeded from low (n = 16) to high (n = 17 and 18) values. Accordingly, Austropeplea with 16 chromosome pairs was thought to be the most “primitive” form, followed by Radix with 17 pairs and Stagnicola with 18 pairs. Our molecular phylogeny contradicts this idea. The ancestral state in Lymnaeidae seems to be n = 18, as it is in other Basommatophoran gastropods (Chilinidae, Lanciniae, Latiidae, Planorbidae and Physidae) [57]. Eighteen pairs of chromosomes is likely to be a pleomorphic character, in agreement with [6] and [7], and thus species of clades C3a (n = 16) and C3b (n = 17) would represent derived rather than ancestral states. Remigio [7] suggested that n = 16 evolved from n = 17. Our results do not contradict this idea, although it is difficult to infer the number of chromosomes for the ancestor of clade C3. In addition, if Lymnaea spp. from Hawaii and China have indeed 18 chromosome pairs, as suggested by [11], either a reversion from n = 16 to n = 18 or three independent evolutions to n = 16 would be required (Figure 1).

Nomenclature in Lymnaeidae

The nomenclature of genera has been one of the most confusing issues in the Lymnaeidae systematics. Most genus names are not fixed and are based more on phenotypic resemblances than on sound evolutionary and phylogenetic considerations. For instance, a single genus was recognized by [58], two by [12], and up to 34 genera by others [13,59-62]. Our results indicate that genera in Lymnaeidae do not reflect phylogenetic relationships, to the notable exception of Radix (including L. (R.) natalensis).

The type species of Lymnaea is L. stagnalis Linnaeus, 1758; the type species of Stagnicola Jeffreys, 1830 is S. palustris (= L. palustris); and the type species of Omphiscola Rafinesque, 1819 is O. glabra. However, it is clear from our results that these three species belong to the same clade (C2) and that Lymnaea is not a monophyletic group. We propose that species of clade C2 should all be called Lymnaea, according to the principle of priority of the International Code of Zoological Nomenclature (ICZN). By extension, Stagnicola should not be used to name species in clade C1a since the type species belongs to clade C2. Meier-Brook and Bargues [63] suggested including S. emarginata, S. elodes, S. catascopium and L. occulta within a new genus Catascopium, while S. caperata would belong to the genus Hinkleyia Baker, 1928 [8]. Our phylogeny does not conflict with this nomenclature, although it would seem preferable to identify all species of clade C1a with the same name to reflect the close evolutionary relationships among these species. Hinkleyia would be the preferable name according to the ICZN. On the other hand, at least four genera names have been used for species of clade C1b: Lymnaea Lamarck, 1799; Galba Schrank, 1803; Fossaria Westerlund, 1885; and Bakeryllymnaea. In the light of the present results, it would be preferable to unify nomenclature. According to the ICZN, Lymnaea should be the unified name, but given that the type species belongs to clade C2, Galba could be a more appropriate name. Finally, as said above, Austropeplea Cotton, 1942 is not a monophyletic group, and employing the genus Kutikina Ponder and Waterhouse, 1997 (one species: K. hispida) seems unjustified on the basis of the current phylogeny. This would also be consistent with results of [11]. It would be preferable to use Bullastra Pfeiffer, 1839 for all species of clade C3a to fit the ICZN.

Unravelling Biological Invasions of Lymnaeidae using the Phylogeny

Although the Lymnaeidae phylogeny presented in Figure 1 matches reasonably well with the geographical origin of samples, some species clustered in clades with different geographic origins stand out and potentially correspond to biological invasions of a more or less recent origin. From an epidemiological standpoint, this is a key issue because some lymnaeid species are more susceptible than others to trematode infection, hence biological invasions can help explain the broad geographic distribution of fascioliasis and in determining the risks for veterinary and public health (see e.g. [64]).

First, one of our most puzzling results concerns the origin of G. truncatula Müller, 1774, the main vector of fascioliasis in the Old World. The idea that this species is native to Europe, as it was described from Germany,
is widely accepted [65]. However, a very different picture emerges from our phylogeny: it is the only European species branching with the wholly American clade C1b. This strongly suggests that America is where *G. truncatula* originated. This is consistent with its detection in Alaska and the Yukon territory [50]. It is possible the current distribution of *G. truncatula* in North America is broader, but has remained cryptic because it has been confounded with other taxa. Indeed, Hubendick noted that, “it is a matter of some doubt whether *Lymnaea humilis* in North America is a distinct species or is specifically connected to *L. truncatula*” (= *G. truncatula*) [66]. It could be that some populations of *L. humilis* in reality correspond to *G. truncatula*. At least 10 species of Lymnaeidae from North America placed in synonymy of *L. humilis* by [12], but considered as valid by [13], present conchological similarities with *G. truncatula*: *L. galbana* Say, 1825; *L. modicella* Say, 1825; *L. obrussa* Say, 1825; *L. parva* Lea, 1841; *L. exigua* Lea, 1841; *L. rustic* Lea, 1841; *L. tazevelliana* Wolf, 1869; *L. dalli* Baker, 1906; *L. cyclostoma* Walker, 1908; and *L. peninsulae* Walker, 1908. Unfortunately, neither detailed anatomical descriptions nor molecular data are available in any of these taxa. Sampling and sequencing of morphologically similar North American taxa could shed light on this question.

Second, as mentioned above, *L. occulta* (= *C. occulta*) is the only species from Europe clustering within clade C2 and seems to correspond to a passage from North America to Europe. As stated by [8] (and references therein), *L. occulta* is distributed in eastern Germany, Poland, the former Czechoslovakia, the former Yugoslavia, Ukraine, Sweden, and some rivers in the delta of Lake Baikal. It is hypothesized that the species could reach the far east Asia where it could have been confounded with other stagnicoline species because of similarity in shell morphology [12]. In any case, it seems clear that *L. occulta* has its origins in America.

Finally, the two *Radix* sp. are likely introductions originating from the Indo-Pacific area to Canada and Romania. The fact that these two sister taxa (potentially the same species) are found in distant geographic locations is indicative of recent introductions.

The Relationships between Lymnaeidae and Fasciola hepatica and *F. gigantica*

From an epidemiological standpoint, digenetic trematodes show marked specificity for their intermediate hosts but can infect a broad spectrum of definite hosts. Usually, species are ooxenous (one parasite species: one snail species) or stenoxenous (one parasite species: a few, closely related snail species) [67]. This is because when the infectious form of the parasite (the miracidium) enters a snail, it must encounter an internal physiological and biochemical environment that supports its complete development. However, the case of *F. hepatica* seems to be different. Our results show that lymnaeid species serving as intermediate hosts of this trematode are widely distributed across the phylogeny (Figure 1). Basically, all clades contain species that have proven to be naturally or experimentally infected with this parasite. Only a few species have been shown to be resistant to infection (Table 3). In contrast, species involved in *F. gigantica* transmission are more clustered in the phylogeny (Figure 1). Although not all species are equally susceptible to infection by *F. hepatica*, the broad capacity of this parasite to infect phylogenetically distant species is remarkable. The Lymnaeidae-Fasciola system differs from the well known Planorbidae-Schistosoma system, in which each trematode species infects a narrow group of hosts that share a close phylogenetic relationship [4]. The behavior of *F. hepatica* with respect to Lymnaeidae is of paramount importance in epidemiology control programs: rather than focusing on a single, or a handful of snail species, fascioliasis control programs should cover a broader spectrum of intermediate hosts that inhabit diverse habitats and ecological conditions. Our results confirm that the presence of *F. hepatica* in all continents is strongly favored by its capacity to infect local lymnaeids. Based on these results, it seems that all geographic regions in the world are exposed to the epidemiological risk of fascioliasis.

Conclusion

At least four conclusions can be drawn from this study. First, combining information from different genes (supermatrix) is a robust approach to reconstruct the evolutionary history of the Lymnaeidae. Our results indicate that members of this family diverged in three deeply-rooted clades corresponding to the geographic origin of species (America, Eurasia and the Indo-Pacific region). Our phylogeny allowed us to pinpoint discordances between ancestral and current geographic distributions of some species, potentially indicating more or less recent biological invasions. Transfers from America to Eurasia are suggested for *G. truncatula* and *L. occulta* (= *C. occulta*), as well as passages from the Indo-Pacific to Europe and North America for *Radix* sp. However, sampling and sequencing efforts remain to be done especially in the Palaeartic and Nearctic regions in which the family diversity is thought to be the largest. This would help resolve the weakly supported relationships (e.g., *P. columella*), determine the pace of morphological evolution, establish taxonomic synonymy, and determine the phylogenetic relevance of poorly known genera (e.g., *Acella*, *Lantzia*, *Lanx* and *Myxas*). Second, with the exception of *Radix* (including the African *L. (R.) natalensis*), genus names in Lymnaeidae do not
reflect the phylogenetic relationships among species. The group taxonomy should be reconsidered to gain some biological meaning. Third, the number of chromosomes in Lymnaeidae has evolved from an ancestral state of 18 pairs to a derived 17 and 16 pairs. Finally, while the intermediate hosts of *F. gigantica* are basically restricted to clade C3, *F. hepatica* is able to infect species from all main clades (C1, C2 and C3). This suggests that the cosmopolitan distribution of *F. hepatica* is largely favored by its capacity to infect local lymnaeids, and highlights the importance of the correct identification of intermediate-host species in fascioliasis control programs.

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**Authors’ contributions**

ACC and SHB designed the study. ACC, JSE, PD, David, PJ and JP sampled snails. ACC and PD carried out experiments. ACC and JSE performed analyses. All authors discussed the results and wrote the paper.

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