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Phylogenetic relationships and evolution of pulmonate gastropods (Mollusca): New insights from increased taxon sampling

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ABSTRACT

Phylogenetic relationships among higher clades of pulmonate gastropods are reconstructed based on a data set including one nuclear marker (complete ribosomal 18S) and two mitochondrial markers (partial ribosomal 16S and Cytochrome oxidase I) for a total of 96 species. Sequences for 66 of these species are new to science, with a special emphasis on sampling the Ellobiidae, Onchidiidae, and Veronicellidae. Important results include the monophyly of Systellommatophora (Onchidiidae and Veronicellidae) as well as the monophyly of Ellobiidae (including *Trimusculus*, *Otina*, and *Smeagol*). Relationships within Ellobiidae, Onchidiidae, and Veronicellidae are evaluated here for the first time using molecular data. Present results are compared with those from the recent literature, and the current knowledge of phylogenetic relationships among pulmonate gastropods is reviewed: despite many efforts, deep nodes are still uncertain. Identification uncertainties about early fossils of pulmonates are reviewed. Impacts of those phylogenetic and fossil record uncertainties on our understanding of the macro-evolutionary history of pulmonates, especially transitions between aquatic and terrestrial habitats, are discussed.

1. Introduction

The two most comprehensive data sets thus far for euthyneuran (opisthobranch and pulmonate) phylogenetics have been published by Grande et al. (2008), based on mitochondrial genomes, and by Klussmann-Kolb et al. (2008), based on 18S, 28S, 16S, and COI data. Dinapoli and Klussmann-Kolb (2010) also published a study focusing on early heterobranchs, i.e., the lineages that branched off just before euthyneurans.

Taxon sampling in analyses based on complete mitochondrial genomes is necessarily limited because gastropod mitochondrial genomes are still difficult to obtain. As a consequence, in the most recent analysis (Grande et al., 2008), several higher taxa (e.g., Trimusculidae, Amphiboloidea, and Veronicellidae) were not represented, while others were only represented by a single species (except for Stylommatophora represented by two species). However, this low taxon sampling was compensated by long sequence data (~14.5 kb) which tended to provide strong node support values. Some interesting, well-supported results from Grande et al. (2008) were (Fig. 1A): Siphonariidae is nested within Opisthobranchia, closely related to a shelled sacoglossan (*Ascobulla*); Stylommatophora (land snails and slugs) emerge at the base of Euthyneura; Eupulmonata (=Stylommatophora, Veronicellidae, Onchidiidae, and Ellobiidae) are polyphyletic; Pulmonata is not monophyletic; and, Pyramidellidae is nested within Euthyneura, closely related to Onchidiidae.

Klussmann-Kolb et al. (2008), who focused on both opisthobranchs and pulmonates, targeted shorter sequence data but broader taxon sampling: they presented a data set including 29 species of pulmonates (one marker is missing for nine of those 29 species, generating gaps in the data set) and 24 species of opisthobranchs, with most higher-level taxa of pulmonates and opisthobranchs represented by at least one species. Some interesting, well-supported results from Klussmann-Kolb et al. (2008) are (Fig. 1B): Pulmonata is monophyletic, although Siphonariidae may not be included within Pulmonata; Eupulmonata (Stylommatophora, Ellobiidae, Onchidiidae) is monophyletic (although veronicellids were not sampled); *Otina* and *Trimusculus* are nested within Eupulmonata (Stylommatophora, Ellobiidae, Onchidiidae), and seem to be closely related to ellobiids; the monophyly of Ellobiidae is not supported; Amphiboloidea and Pyramidellidae are sister-taxa; Hygrophila is monophyletic, including Chilinoidea (Chilinidae and Latiidae) and Lymnaeoidea. The analyses focusing

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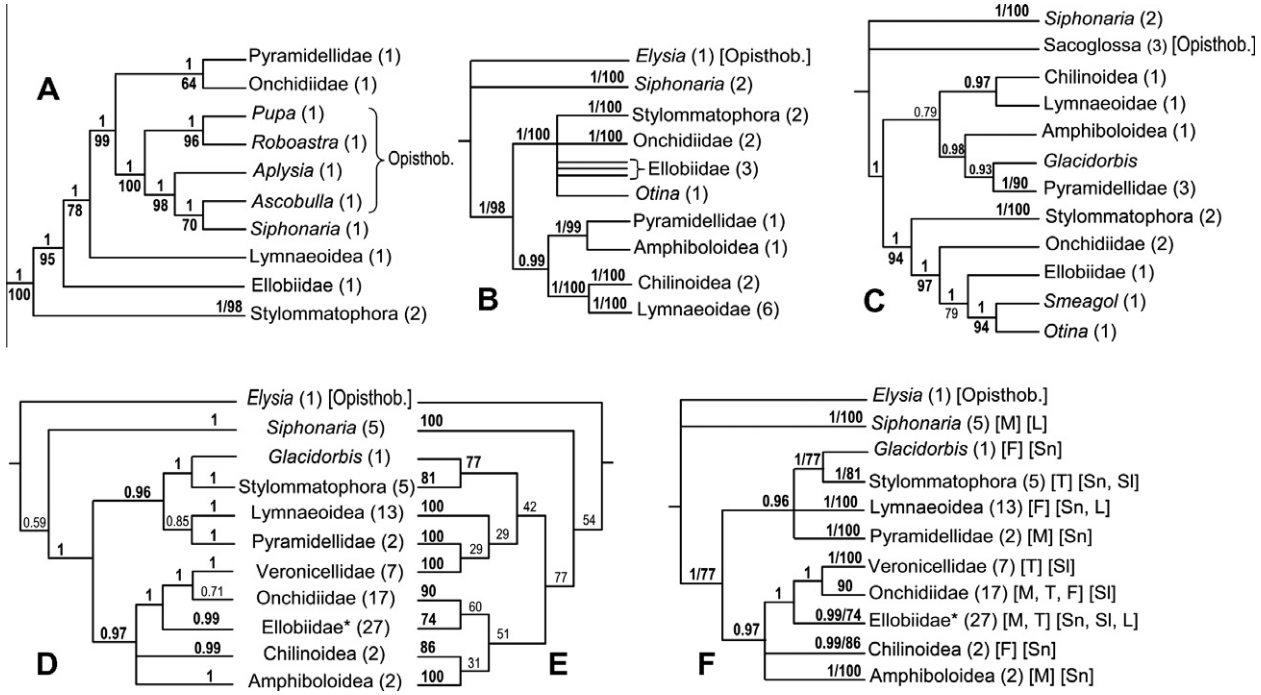

Fig. 1. Summary of phylogenetic relationships for euthyneuran (pulmonate and opisthoranch) gastropods from various past studies as well as the present study. Only BI posterior probabilities > 0.75 and ML bootstrap values > 50% are shown (except in E in which all bootstrap values are shown). Node supports are cited using the following format: "1.00/77" above a branch or next to a node means that BI posterior probability = 1.00, and that ML bootstrap value = 77%. (A) From Grande et al. (2008), based on all protein-coding genes from complete mitochondrial genomes (after Grande et al., 2008: Fig. 3). (B) From Klussmann-Kolb et al. (2008), based on complete 18S, partial 28S, 16S, and COI genes (after Klussmann-Kolb et al., 2008: Fig. 3). (C) From Dinapoli and Klussmann-Kolb (2010) based on complete 18S, partial 28S, 16S, and COI genes (after Dinapoli and Klussmann-Kolb, 2010: Fig. 2). (D) Summary of the phylogram from the present data using Bayesian Inference (see Fig. 2). (E) Summary of the phylogram obtained from the present data using Maximum Likelihood. (F) Combination of only the well-supported nodes from D and E (with BI posterior probability > 0.95 and ML bootstrap > 75); next to taxon names, letters indicate whether taxa include species that are terrestrial [T], marine [M], or freshwater [F], as well as whether animals are coiled snails [Sn], slugs [Sl], or limpets [L].

on basal heterobranchs (Dinapoli and Klussmann-Kolb, 2010) based on a subsample of pulmonate species from Klussmann-Kolb et al. (2008) yielded similar results (Fig. 1C). However, *Glacidorbis*, traditionally regarded as a basal heterobranch, is nested within pulmonates; also, *Smeagol*, a problematic pulmonate taxon, seems to be closely related to ellobiids.

Overall, the results based on complete mitochondrial genomes (Grande et al., 2008) and individual markers (Klussmann-Kolb et al., 2008) are incongruent and depict two different phylogenetic scenarios. Possible explanations for this incongruence are discussed below.

The present study provides new sequences (18S, 16S, COI) for 64 species of pulmonate gastropods, with a special focus on three taxa that thus far have remained poorly sampled, i.e., the ellobiids, veronicellids, and onchidiids: 25 ellobiids (15 genera), 16 onchidiids (five genera), seven veronicellids (five genera), six Hygrophila (six genera), two stylommatophorans (two genera), two amphiboloids (two genera), five *Siphonaria*, and one *Trimusculus*. This increase in taxon sampling was targeted in order to address a series of unresolved questions in pulmonate relationships, such as: the relationships of the veronicellid slugs, the phylogenetic status of Ellobiidae and its five traditional "subfamilies," the basal nodes within Pulmonata, especially the status of Eupulmonata (Stylommatophora, Ellobiidae, Onchidiidae, Veronicellidae), and the relationships within Ellobiidae, Onchidiidae, and Veronicellidae.

2. Materials and methods

2.1. Taxon sampling

A total of 96 species were included in this study (Table 1). Of these 96 species, 30 are represented by sequences obtained from Genbank. Sequences for the remaining 66 species are new.

The data set used by Klussmann-Kolb et al. (2008) served as a starting point for this study. However, some species from that data set were not included in the present study: seventeen species were excluded because one of the three markers used here was missing (e.g., *Siphonaria alternata*, *Amphibola crenata*); also excluded were species for which the 18S sequence was incomplete (<1200 bp) (e.g., *Siphonaria concinna*, *Chilina* sp. 1, *Trimusculus afra*); *Dendronotus dalli* (opisthobranch) and *Planorbis planorbis* (Lymnaeoidea) were excluded because their 18S and COI sequences, respectively, were difficult to align in some regions. New sequences were produced for several taxa (*Phallomedusa solida*, *Myosotella myosotis*, *Onchidium verruculatum*, *Onchidella floridana*) that were represented in the data set by Klussmann-Kolb et al. (2008). We also included sequence data of *Glacidorbis rusticus* and *Smeagol philippensis* from the study of Dinapoli and Klussmann-Kolb (2010), as they had been reported to be nested within pulmonates (Fig. 1C).

Ten additional species for which COI, 16S, and 18S sequences are available from Genbank but that were not previously used by Klussmann-Kolb et al. (2008), were also included: the neritimorph *Nerita funiculata*, two caenogastropods (*Crepidula fornicata*, *Viviparus georgianus*), the onchidiid *Onchidella celtica*, four freshwater pulmonates (*Radix auricularia*, *Biomphalaria alexandrina*, *Indoplanorbis exustus*, *Laevepex fuscus*), and the two land snails *Cepaea nemoralis* and *Deroceras reticulatum*.

Sequences for the remaining 66 species are newly produced, focusing on non-stylommatophoran pulmonates (Table 1).

2.2. Species identifications

Identifications of the species for which new DNA sequences were determined have all been confirmed by taxonomic experts (and authors of the present article): Christian Albrecht identified the freshwater snails, Benoît Dayrat the onchidiids, Rosemary Golding

Table 1
List of the species included in the present study. Locality data and museum catalogue numbers of vouchers are indicated for the material newly sequenced for this study. Institution abbreviations for the museums that house the voucher material are: Australian Museum Sydney, New South Wales (AMS), Natural History Museum, London, United Kingdom (BM), California Academy of Sciences, San Francisco, United States of America (CAS), Museu de Ciências e Tecnologia da PUCRS, Porto Alegre, Brazil (MCP), Museo de Ciencias Naturales de La Plata, Buenos Aires, Argentina (MLP), Natal Museum, Pietermaritzburg, South Africa (NM), and Florida Museum of Natural History, Gainesville, University of Florida, USA (UF). An asterisk (*) indicates that a sequence was newly obtained for the present study.

Classification, higher taxa	Species name	Locality	Voucher #	Genbank (18S)	Genbank (COI)	Genbank (16S)
Neritimorpha	*Nerita funiculata*	–	–	DQ093429	DQ093517	DQ093471
Caenogastropoda, Calyptraeidae	*Crepidula fornicata*	–	–	AY377660	AF353149	AF545973
Caenogastropoda, Cerithiidae	*Clypeomorus brevis*	Wake Island	UF 380209	HQ659928*	HQ659994*	HQ650562*
Caenogastropoda, Viviparidae	*Viviparus georgianus*	–	–	AY090794	AF120634	AY377626
Heterobranchia, Orbitestellidae	*Orbitestella* sp.	–	–	EF489352	EF489397	EF489333
Heterobranchia, Pyramidellidae	*Otopleura nodicincta*	Caroline Islands	UF 299490	HQ659929*	HQ659995*	HQ650563*
Heterobranchia, Pyramidellidae	*Turbonilla* sp.	–	–	EF489351	EF489396	EF489332
Heterobranchia, Glacidorbidae	*Glacidorbis rusticus*	–	–	FJ917211	FJ917284	FJ917264
Opithobranchia	*Pupa solidula*	–	–	AY427516	DQ238006	EF489319
Opithobranchia	*Toledonia globosa*	–	–	EF489350	EF489395	EF489327
Opithobranchia	*Haminoea hydatis*	–	–	AY427504	DQ238004	EF489323
Opithobranchia	*Aplysia californica*	–	–	AY039804	AF077759	AF192295
Opithobranchia	*Bathydoris clavigera*	–	–	AY165754	AF249808	AF249222
Opithobranchia	*Umbraculum umbraculum*	–	–	AY165753	DQ256200	EF489322
Opithobranchia	*Pleurobranchus peroni*	–	–	AY427494	DQ237993	EF489331
Opithobranchia	*Tomthompsonia antarctica*	–	–	AY427492	DQ237992	EF489330
Opithobranchia	*Elysia viridis*	–	–	AY427499	DQ237994	AJ223398
Siphonariidae	*Siphonaria normalis*	Hawaii	UF 303670	HQ659930*	HQ659996*	HQ650564*
Siphonariidae	*Siphonaria lateralis*	Argentina	MLP 13163	HQ659931*	HQ659997*	HQ650565*
Siphonariidae	*Siphonaria lessoni*	Argentina	MLP 13164	HQ659932*	HQ659998*	HQ650566*
Siphonariidae	*Siphonaris japonica*	Japan	UF 350544	HQ659933*	HQ659999*	HQ650567*
Siphonariidae	*Siphonaria pectinata*	Trinidad Island	UF 382817	HQ659934*	HQ660000*	HQ650568*
Trimusculidae	*Trimusculus reticulatus*	California	CASIZ 177988	HQ659935*	HQ660001*	HQ650569*
Amphiboloidea, Phallomedusidae	*Phallomedusa solida*	Australia, NSW	No tissue left	HQ659936*	HQ660002*	HQ650570*
Amphiboloidea, Amphibolidae	*Salinator rhamphidia*	Australia, NSW	CASIZ 180470	HQ659937*	HQ660003*	HQ650571*
Ellobiidae, Carychiinae	*Carychium minimum*	–	–	EF489341	EF489386	EF489308
Ellobiidae, Ellobiinae	*Auriculastra subula*	Hong Kong	CASIZ 180471	HQ659938*	HQ660004*	HQ659872*
Ellobiidae, Ellobiinae	*Auriculinella bidentata*	Azores	No tissue left	HQ659939*	HQ660005*	HQ659873*
Ellobiidae, Melampodinae	*Melampus bidentatus*	Jamaica	CASIZ 180472	HQ659940*	HQ660006*	HQ659874*
Ellobiidae, Melampodinae	*Melampus fasciatus*	Caroline Islands	UF 294608	HQ659941*	HQ660007*	HQ659875*
Ellobiidae, Melampodinae	*Microtralia alba*	Australia, NSW	AMS 398688	HQ659942*	HQ660008*	HQ659876*
Ellobiidae, Melampodinae	*Pseudomelampus exiguus*	Azores	CASIZ 180473	HQ659943*	HQ660009*	HQ659877*
Ellobiidae, Pedipedinae	*Marinula chathamensis*	Chatham Island	CASIZ 180474	HQ659944*	HQ660010*	HQ659878*
Ellobiidae, Pedipedinae	*Pedipes mirabilis*	Jamaica	CASIZ 180475	HQ659945*	HQ660011*	HQ659879*
Ellobiidae, Pedipedinae	*Pedipes pedipes*	Azores	CASIZ 180476	HQ659946*	HQ660012*	HQ659880*
Ellobiidae, Pythiinae	*Allochroa layardi*	United Arab Emirates	BM 20080090	HQ659947*	HQ660013*	HQ659881*
Ellobiidae, Pythiinae	*Allochroa* sp.	Tonga	UF 294620	HQ659948*	HQ660014*	HQ659882*
Ellobiidae, Pythiinae	*Cassidula angulifera*	Australia, Queensland	AMS 448376	HQ659949*	HQ660015*	HQ659883*
Ellobiidae, Pythiinae	*Cassidula* cf. *labrella*	United Arab Emirates	BM 20080095	HQ659950*	HQ660016*	HQ659884*
Ellobiidae, Pythiinae	*Laemodonta monilifera*	United Arab Emirates	BM 20080099	HQ659951*	HQ660017*	HQ659885*
Ellobiidae, Pythiinae	*Laemodonta punctostriata*	Hong Kong	CASIZ 180477	HQ659952*	HQ660018*	HQ659886*
Ellobiidae, Pythiinae	*Myosotella myosotis*	Portugal	CASIZ 180478	HQ659953*	HQ660019*	HQ659887*
Ellobiidae, Pythiinae	*Ophicardelus ornatus*	Australia, NSW	AMS 397363	HQ659954*	HQ660020*	HQ659888*
Ellobiidae, Pythiinae	*Ophicardelus sulcatus*	Australia, NSW	AMS 405360	HQ659955*	HQ660021*	HQ659889*
Ellobiidae, Pythiinae	*Ovatella firminii*	Crete	CASIZ 180479	HQ659956*	HQ660022*	HQ659890*
Ellobiidae, Pythiinae	*Ovatella vulcani*	Azores	CASIZ180480	HQ659957*	HQ660023*	HQ659891*
Ellobiidae, Pythiinae	*Pleuroloba quoyi*	Australia, NSW	AMS 397375	HQ659958*	HQ660024*	HQ659892*
Ellobiidae, Pythiinae	*Pythia cecillei*	Papua New Guinea	UF 339082	HQ659959*	HQ660025*	HQ659893*
Ellobiidae, Pythiinae	*Pythia fimbriosa*	Papua New Guinea	UF 339086	HQ659960*	HQ660026*	HQ659894*
Ellobiidae, Pythiinae	*Pythia scarabeus*	Papua New Guinea	UF 366491	HQ659961*	HQ660027*	HQ659895*
Ellobiidae, Pythiinae	*Pythia* sp.	Christmas Island	UF 296120	HQ659962*	HQ660028*	HQ659896*
Otinidae	*Otina ovata*	–	–	EF489344	EF489389	EF489310
Smeagolidae	*Smeagol philippensis*	–	–	FJ917210	FJ917283	FJ917263
Chilinoidea, Chilinidae	*Chilina* sp.	Chile	CASIZ 180481	HQ659964*	HQ660030*	HQ659898*
Chilinoidea, Latiidae	*Latia neritoides*	–	–	EF489339	EF489384	EF489307
Lymnaeoidea, Acroloxidae	*Acroloxus lacustris*	–	–	AY282592	AY282581	EF489311
Lymnaeoidea, Acroloxidae	*Acroloxus* cf. *oblongus*	Turkey	No tissue left	HQ659963*	HQ660029*	HQ659897*
Lymnaeoidea, Lymnaeidae	*Galba truncatula*	Ethiopia	CASIZ 180482	HQ659965*	HQ660031*	HQ659899*
Lymnaeoidea, Lymnaeidae	*Lymnaea palustris*	France	CASIZ 180483	HQ659966*	HQ660032*	HQ659900*
Lymnaeoidea, Lymnaeidae	*Lymnaea stagnalis*	–	–	EF489345	EF489390	EF489314
Lymnaeoidea, Lymnaeidae	*Radix auricularia*	–	–	Z73980	EU818827	AF485646
Lymnaeoidea, Physidae	*Physa acuta*	–	–	AY282600	AY282589	AY651241
Lymnaeoidea, Physidae	*Physa gyrina*	California	CASIZ 180484	HQ659967*	HQ660033*	HQ659901*
Lymnaeoidea, Planorbidae	*Biomphalaria alexandrina*	–	–	U65225	DQ084825	DQ084847
Lymnaeoidea, Planorbidae	*Helisoma anceps*	California	CASIZ 180485	HQ659968*	HQ660034*	HQ659902*
Lymnaeoidea, Planorbidae	*Indoplanorbis exustus*	–	–	AY282598	AY282587	AY577471
Lymnaeoidea, Planorbidae	*Ancylus fluviatilis*	–	–	AY282593	AY282582	EF489312
Lymnaeoidea, Planorbidae	*Laevapex fuscus*	–	–	AY282599	AY 282588	EU 038346
Onchidiidae	*Onchidella celtica*	–	–	X70211	AY345048	AY345048
Onchidiidae	*Onchidella floridana*	Tobago	UF 382844	HQ659969*	HQ660035*	HQ659903*

(*continued on next page*)

Table 1 (*continued*)

Classification, higher taxa	Species name	Locality	Voucher #	Genbank (18S)	Genbank (COI)	Genbank (16S)
Onchidiidae	*Onchidella hildae*	Panama	UF 372677	HQ659970*	HQ660036*	HQ659904*
Onchidiidae	*Onchidium* cf. *tumidum*	Australia, NSW	UF 395149	HQ659971*	HQ660037*	HQ659905*
Onchidiidae	*Onchidium* cf. *tumidum*	Australia, Queensland	UF 458136	HQ659973*	HQ660039*	HQ659907*
Onchidiidae	*Onchidium vaigiense*	Papua New Guinea	UF 366435	HQ659974*	HQ660040*	HQ659908*
Onchidiidae	*Peronia peronii*	Guam	CASIZ 180486	HQ659975*	HQ660041*	HQ659909*
Onchidiidae	*Peronia* cf. *peronii*	Mozambique	BM 20060414	HQ659976*	HQ660042*	HQ659910*
Onchidiidae	*Peronia* cf. *verruculata*	Okinawa	UF 352288	HQ659977*	HQ660043*	HQ659911*
Onchidiidae	*Peronia* sp. 1	Hawaii	UF 303653	HQ659972*	HQ660038*	HQ659906*
Onchidiidae	*Peronia* sp. 2	Oman	UF 332088	HQ659978*	HQ660044*	HQ659912*
Onchidiidae	*Peronia* sp. 3	Australia, Queensland	AMS 459511	HQ659982*	HQ660048*	HQ659916*
Onchidiidae	*Peronia* sp. 4	Mozambique	BM 20080190	HQ659979*	HQ660045*	HQ659913*
Onchidiidae	*Peronia* sp. 5	Mozambique	BM 20060257	HQ659981*	HQ660047*	HQ659915*
Onchidiidae	*Peronia* sp. 6	Indonesia, Sulawesi	BM 20050628	HQ659980*	HQ660046*	HQ659914*
Onchidiidae	*Platevindex* cf. *coriaceus*	Mozambique	BM 20060274	HQ659983*	HQ660049*	HQ659917*
Onchidiidae	*Scaphis* sp.	Philippines	UF 368518	HQ659984*	HQ660050*	HQ659918*
Veronicellidae	*Laevicaulis natalensis*	South Africa	NM-W1444	HQ659985*	HQ660051*	HQ659919*
Veronicellidae	*Laevicaulis* sp.	South Africa	NM-W4061	HQ659986*	HQ660052*	HQ659920*
Veronicellidae	*Phyllocaulis tuberculosus*	Brazil	MCP 8857	HQ659987*	HQ660053*	HQ659921*
Veronicellidae	*Phyllocaulis variegatus*	Brazil	CASIZ 180487	HQ659988*	HQ660054*	HQ659922*
Veronicellidae	*Sarasinula linguaeformis*	Brazil	CASIZ 180488	HQ659989*	HQ660055*	HQ659923*
Veronicellidae	*Vaginulus taunaisii*	Brazil	MCP 8858	HQ659990*	HQ660056*	HQ659924*
Veronicellidae	*Veronicella cubensis*	Hawaii	CASIZ 180489	HQ659991*	HQ660057*	HQ659925*
Stylommatophora	*Arion ater*	France	CASIZ 180490	HQ659992*	HQ660058*	HQ659926*
Stylommatophora	*Arion sylvaticus*	–	–	AY145365	AY987918	AY947380
Stylommatophora	*Cepaea nemoralis*	–	–	AJ224921	CMU23045	CMU23045
Stylommatophora	*Deroceras reticulatum*	–	–	AY145373	AF239734	AF238045
Stylommatophora	*Succinea putris*	France	CASIZ 180491	HQ659993*	HQ660059*	HQ659927*

the amphiboloids, Suzete R. Gomes the veronicellids, Antonio M. de Frias Martins the ellobiids, and Tracy White the *Siphonaria*.

2.3. Voucher specimens

Voucher specimens of all the 66 species for which new sequences were obtained have been deposited in museum collections (Table 1). For each of these species, all sequences (18S, 16S, COI) were obtained from a single individual. In most cases, that individual is included as part of the lot deposited as the voucher. However, in some rare cases, small specimens were destroyed to obtain DNA. In these cases, the voucher lot contains other individuals from the same population.

2.4. DNA extraction

All DNA extractions were performed under sterile conditions (i.e., using sterilized equipment). For slugs, a small piece of the dorsal notum or foot was sampled (in many onchidiids, however, DNA had to be extracted from the gonad because pieces of the mantle originally yielded protist sequences). For snails, a small piece of the foot was cut; or, if not easily accessible, then part of the shell was broken to access soft tissues.

DNA extractions were performed using a CTAB DNA extraction method. Each sample was placed into a tube containing 50 μl of CTAB (Cetyl Trimethyl Ammonium Bromide) solution, with the following final concentrations: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris–HCl (pH 8.0), and 2% β-mercaptoethanol. After grinding the tissue with a pestle, 550 μl more of CTAB solution was added while rinsing the pestle of any tissue adhered to it. Then, 20 μl of Proteinase K (final concentration of 100 μg/ml) was added to each sample, vortexed and incubated for about 2 h at 65 °C. During incubation, tube contents were re-suspended via vortexing every 10 min. After centrifugation at 13,000 rpm for 15 min, the upper phase was transferred into a new tube; then, 600 μl of chloroform was added to the tube and gently mixed. In order to precipitate the DNA, after a centrifugation period of 15 min. at 13,000 rpm, the upper phase was transferred into a new tube containing 750 μl of cold isopropanol and placed in the freezer overnight. The following day, the precipitate was made into a pellet by centrifugation and washed with 70% ethanol and then re-suspended with 30 μl–100 μl of DNA re-suspension buffer (Teknova).

2.5. PCR amplification and DNA sequencing

For each gene or gene fragment, amplification was initially attempted with a single pair of standard primers that are routinely used in gastropod systematics (indicated in bold in Table 2). If samples did not successfully amplify, alternate pairs of primers were used (Tables 2 and 3). In order to sequence 18S, a series of eight internal primers were used in addition to the primers used for amplification. In the rare event that 18S amplification was not successful, amplification was carried out using internal individual-specific primers. Amplified products were then sent out individually for sequencing and subsequently assembled. Sequenced fragments represented ~680 bp of COI, ~530 bp of 16S, and the complete 18S (~1850 bp).

2.6. Phylogenetic analyses

Alignments were obtained using Clustal W in MEGA 4 (Tamura et al., 2007) and refined manually to increase positional homology. Gaps and ambiguous positions were removed from alignments prior to phylogenetic analyses. Following alignment, chromatograms of newly analyzed sequences were consulted to resolve rare ambiguous base calls.

The COI alignment was guided by translated amino acid sequences; the ends were trimmed; also, a few positions for which a nucleotide was present in only one (Genbank) sequence, disrupting the reading frame of that sequence and thus likely due to a sequencing error, were removed, yielding an alignment of 590 sites. The original 16S alignment contained a few regions with ambiguous positions that could not be aligned properly as well as gaps due to inserts in one sequence. Regions with ambiguous positions that could not be aligned were difficult to identify manually and were removed using Gblocks (Castresana, 2000), with the

Table 2
List of primers used in the present study. Primers indicated in bold are standard primers commonly used in gastropod systematics (e.g., Klussmann-Kolb et al., 2008). Alternate primers (not in bold) were used in the few cases in which PCRs were not successful with standard primers.

Primer name	Primer sequence (5′–3′)
COIH	TAA ACT TCA GGG TGA CCA AAR AAY CA
COIL	GGT CAA CAA ATC ATA AAG ATA TTG G
COI 14F	WYT CNA CDA AYC AYA AAG AYA TTG G
COI 698R	TAD ACY TCN GGR TGH CCR AAR AAY CA
COI 839R	AAY ATR TGH GCY CAN ACA ATA AAW CC
16S-R	CCG GTC TGA ACT CAG ATC ACG T
16Sar	CGC CTG TTT ATC AAA AAC AT
16s F	CGG CCG CCT GTT TAT CAA AAA CAT
16s R	GGA GCT CCG GTT TGA ACT CAG ATC
16S 437F	CRN CTG TTT ANC AAA AAC AT
16S 972R	CCG GTC TGA ACT CAG ATC ATG T
18S A1	CTG GTT GAT CCT GCC AGT CAT ATG C
18S 1800	GAT CCT TCC GAC GGT TCA CCT ACG
18S 400F	ACG GGT AAC GGG GAA TCA GGG
18S 400R	CCC TGA TTC CCC GTT ACC CGT
18S 700F	GTC TGG TGC CAG CAG CCG CG
18S 700R	CGC GGC TGC TGG CAC CAG AC
18S 1155F	CTG AAA CTT AAA GGA ATT GAC GG
18S 1155R	CCG TCA ATT CCT TTA AGT TTC AG
18S 1500R	CAT CTA GGG CAT CAC AGA CC
18S 1600F	CGT CCC TGC CCT TTG TAC ACA CC

following parameters (#1: 51; #2: 83; #3: 30; #4: 4; #5: with half) which removed 321 out of 783 (40%) positions from the original alignment. In the 18S alignment, gaps (due to inserts in one sequence) and ambiguous regions (with positions that could not be aligned properly) were easily identified. A total of 609 positions (mostly gaps) out of the 2343 original positions (long insertions in nudipleuran sequences considerably lengthened the alignment) were removed at the following sites of the original alignment: 19, 37, 95, 102–104, 165–176, 182, 206, 211–262, 268–84, 298, 325–328, 366, 382, 397, 421, 530, 551, 741–1008, 1022–1023, 1045, 1121–1124, 1128, 1170–1171, 1189–1191, 1293, 1395, 1719–1923, 2254–2264, 2272–2273, 2284–2286.

Substitution saturation was measured using Xia's test (Xia et al., 2003; Xia and Lemey, 2009) implemented in DAMBE (Xia and Xie, 2001). No saturation was detected in the 16S alignment (321 sites) from which gaps and ambiguous regions had been removed (Iss significantly < Iss.c). However, third codon positions were removed from the COI alignment due to substitution saturation. After removal of the third positions (which yielded a reduced COI alignment of 394 sites), no saturation was detected. Overall, our concatenated alignment included 2449 sites (1734 for 18S, 321 for 16S, and 394 for COI).

Prior to phylogenetic analyses, the best-fitting evolutionary model was selected independently for each partition using Modeltest 3.7 (Posada and Crandall, 1998) and the Model Selection option from Topali v2.5 (Milne et al., 2004). A GTR + I + G model was selected for all three markers.

Maximum Likelihood analyses were performed using both RaxML (Stamatakis, 2006) and PhyML (Guindon and Gascuel, 2003) as implemented in Topali v2.5. Node support was evaluated using bootstrapping with 1000 replicates. For the maximum Likelihood analyses, four out-groups were selected: *N. funiculata*, *C. fornicata*, *Clypeomorus brevis*, and *V. georgianus*. Bayesian analyses were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) with four simultaneous runs of 10^6 generations each, sample frequency of 100, and burn in of 25%. *N. funiculata* was selected as the outgroup for the Bayesian analyses. Posterior probabilities (PP) were calculated to evaluate node support. Bayesian posterior probabilities (PP) measure different types of confidence in node support than bootstrap values (e.g., Alfaro et al., 2003; Douady et al., 2003). However, it is usually estimated that Bayesian PP > 0.95 are an indication of a good support, i.e., an indication that a node can be given serious consideration.

3. Results

3.1. General remarks on tree topologies

The phylogram obtained from BI analyses is shown in Fig. 2. Analyses based on Maximum Likelihood (ML) and Bayesian Inference (BI) yielded trees differing in the position of Veronicellidae. Indeed, if Veronicellidae were to be removed, then the trees would be identical. However, this difference in the position of Veronicellidae is not regarded as an issue here because the deep nodes in the ML analyses are poorly supported (Fig. 1D–F). Thus, the difference in position of the Veronicellidae is not viewed here as an incongruence. Throughout the paper, node supports are cited following the same format (Fig. 2): (1.00/77) means that BI posterior probability = 1.00 and ML bootstrap value = 77. In addition, trees from ML and BI differ in minor details due to very poorly-supported nodes (ML bootstrap < 50%, and PP < 0.75).

Deep nodes among major clades of pulmonates are poorly supported in Maximum Likelihood analyses (Fig. 1E). All bootstrap values are less than 75% (Fig. 1E), with two exceptions: the monophyly of the clade including all pulmonates without *Siphonaria* (1.00/77), and the close relationship between *Glacidorbis* and Stylommatophora (1.00/77). Two additional nodes are supported by bootstrap values of 60% (Onchidiidae and Ellobiidae) and 51% (Eupulmonata without Veronicellidae). However, the relationships among major clades are well supported in Bayesian Inference analyses, with most PP superior to 0.95 (Fig. 1D). Thus, although the deep nodes between ML and BI trees are incongruent, this incongruence is not regarded as an issue here because the nodes in ML are very weakly supported. As a result, the well-supported nodes can be easily combined by hand together and shown on a tree (Fig. 1F).

3.2. Basal branches

The Heterobranchia corresponds to the ingroup taxa (the four basal out-groups are *Nerita*, *Crepidula*, *Clypeomorus*, and *Viviparus*). Within Heterobranchia, the monophyly of Euthyneura (1/100) is strongly supported, including all the taxa sampled here except for *Orbitestella* (traditionally regarded as a lower heterobranch) and the four out-groups. Within Euthyneura (Pulmonata and Opisthobranchia), the most basal branch is *Pupa* (Acteonoidea, Opistobranchia), and the clade including all other euthyneu-

Table 3
PCR conditions with corresponding primers used in the present study.

PCR programs	Primers
94° 5 min, 30 × (94° 40 s, 46° 1 min, 72° 1 min), 72° 10 min, 4.0° hold	COIH, COIL, 16S-R, 16Sar, 16s F, 16s R
94° 2 min, 5 × (94° 40 s, 40° 45 s, 72° 1 min), 30 × (94° 40 s, 50° 40 s, 72° 1 min), 72° 10 min, 4.0° hold	COI 14F, COI 698R, COI 839R, 16S 437F, 16S 972R, 18S 400F&R, 18S 700F&R, 18S 1155F&R, 18S 1500R, 1800 1600F.
95° 1 min, 30 × (95° 30 s, 52.5° 30 s, 72° 30 s), 72° 3 min, 4.0° hold	18S A1, 18S 1800

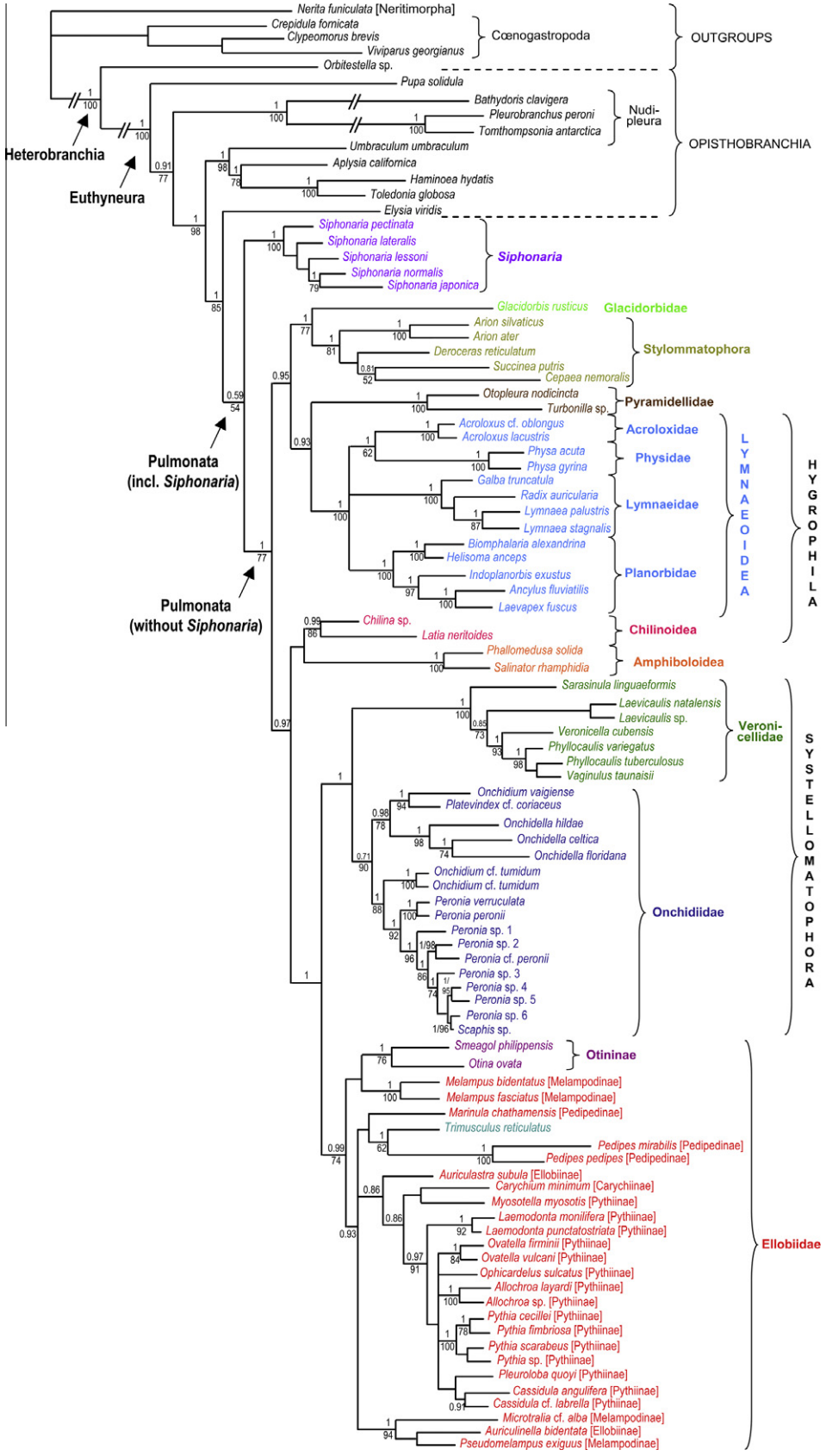

Fig. 2. Phylogram obtained through Bayesian Inference. Posterior probabilities (BI) and bootstrap values (ML) are indicated above and below the nodes, respectively. Weak support values are not indicated (ML bootstrap < 50%, and PP < 0.75), which explains why only one value or even no value is indicated for some nodes. Polytomies are due to the cutoff value specified for the consensus tree (50% used as the default value in MrBayes).

rans is moderately supported (0.91/77). The opisthobranch clade Nudipleura (*Bathydoris*, *Pleurobranchus*, *Tomthompsonia*) forms the second most basal lineage just after *Pupa*, and the clade including all other euthyneurans is well supported (1/98); the clade Nudipleura itself, represented here by three species, is strongly-supported (1.00/100). The third most basal clade, which includes various shelled opisthobranchs (*Umbraculum*, *Aplysia*, *Haminoea*, *Toledonia*), is strongly-supported (1.00/98); the next node, including *Elysia* (Sacoglossa, Opisthobranchia) and all pulmonates, is also well supported (1.00/85). This result falsifies the monophyly of Opisthobranchia. The monophyly of Pulmonata is poorly supported (0.59/54). Pulmonata includes here all the taxa traditionally regarded as pulmonates as well as Pyramidellidae. The latter, traditionally regarded as basal, non-euthyneuran heterobranchs, emerge unambiguously within Pulmonata in all analyses. *Siphonaria* is the most basal branch within Pulmonata, and the monophyly of the clade including all pulmonates without *Siphonaria* is fairly well supported (1.00/77).

3.3. Major clades of Pulmonata

Within Pulmonata, all major clades are recovered, in most cases with strong support. The strongly-supported major clades are: *Siphonaria* (1.00/100); Veronicellidae (1.00/100); Lymnaeoidea (1.00/100), which includes all freshwater snails (Hygrophila) except for Chilinoidea (*Chilina* and *Latia*); Chilinoidea (0.99/86); Pyramidellidae (1.00/100); and Stylommatophora (1.00/81). The monophyly of Onchidiidae (0.71/90) and the monophyly of Ellobiidae (0.99/74) are less strongly supported but are recovered in all analyses. Also, the false limpet *Trimusculus*, the tiny limpet *Otina*, and the slug *Smeagol*, are all nested within Ellobiidae (see below).

Besides the basal and weakly-supported position of *Siphonaria*, the data suggest the existence of two additional major clades within Pulmonata (Fig. 1F): one clade includes Lymnaeoidea, Pyramidellidae, Stylommatophora and *Glacidorbis* (the two latter being more closely related); the other clade includes Chilinoidea and Amphiboloidea as two basal branches, and Ellobiidae as sister-taxon to Systellommatophora (Veronicellidae and Onchidiidae).

3.4. Ellobiidae

Within Ellobiidae, which is moderately supported (0.99/74), are found all the taxa traditionally regarded as ellobiids (Martins, 2007), as well as three taxa that have not been traditionally regarded as ellobiids: the false limpet *Trimusculus*, the tiny limpet *Otina*, and the slug *Smeagol*. The exact position of *Trimusculus* within ellobiids is unclear because of low support, but the present data suggest that it might be more closely related to *Pedipes* (Pedipedinae). *Otina* and *Smeagol* appear to be closely related to each other (1.00/76), although their relationships with other ellobiids are unclear.

The sixteen genera sampled here include representatives of each of the five subfamilies traditionally accepted in Ellobiidae (Martins, 2007): Carychiinae (one genus represented here, out of two: 1/2), Ellobiinae (2/5), Melampodinae (3/5), Pedipedinae (2/4), and Pythiinae (8/8). The monophyly of Carychiinae is not tested here. The monophyly of Ellobiinae (represented here by *Auriculinella* and *Auriculastra*) is not supported (because *Auriculinella* is included in a well-supported clade with *Pseudomelampus* and *Microtralia*). The monophyly of Melampodinae (as traditionally defined, and represented here by *Melampus*, *Microtralia*, and *Pseudomelampus*) is neither supported nor rejected because of low node support. However, the genera of Melampodinae cluster in two different clades: *Microtralia* and *Pseudomelampus* (and *Auriculinella*) in one clade, and *Melampus* in another clade. The monophyly of Pedipedinae (represented here by *Pedipes* and *Marinula*) is not well supported: *Pedipes* and *Marinula* form a clade but with very low node support (BI PP < 0.75; ML bootstrap < 50%). However, Pedipedinae could be regarded as monophyletic if it were to include *Trimusculus*, which is closely related to *Pedipes* (1/62). The monophyly of Pythiinae (represented here by at least one species of each of its eight genera: *Allochroa*, *Cassidula*, *Laemodonta*, *Myosotella*, *Ophicardelus*, *Ovatella*, *Pleuroloba*, and *Pythia*) is neither supported nor rejected because of low node support. However, within Pythiinae, seven out of the eight existing genera, including the type genus of the subfamily (all but *Myosotella*) form a strongly-supported clade (0.97/91), which is by far the most highly supported clade in ellobiids (besides the monophyly of the genera). Within that clade, however, relationships are poorly resolved.

Six ellobiid genera represented here by more than one species are found to be monophyletic with a strong support: *Allochroa* (1.00/100), *Laemodonta* (1.00/92), *Melampus* (1.00/100), *Ovatella* (1.00/84), *Pedipes* (1.00/100), and *Pythia* (1.00/100). The monophyly of *Cassidula* (0.91) is less strongly supported.

3.5. Veronicellidae

Within Veronicellidae, which is strongly-supported (1.00/100), the most basal taxon is *Sarasinula*. The clade including all the other veronicellids (here represented by *Laevicaulis*, *Veronicella*, *Phyllocaulis*, and *Vaginulus*) is moderately supported (0.85/73). However, two clades are strongly supported: a first clade includes *Veronicella*, *Phyllocaulis*, and *Vaginulus* (1.00/93) and a second clade includes *Phyllocaulis* and *Vaginulus* (1.00/98).

3.6. Onchidiidae

The monophyly of the Onchidiidae, comprised only of taxa that have traditionally been included in the family (Dayrat, 2009), is well supported in ML analyses (0.71/90). The monophyly of *Onchidella*, represented here by three species, is strongly-supported (1.00/98). A *Peronia* clade, including all slugs with dorsal branchial plumes (gills), is also strongly-supported (1.00/92). *Scaphis*, which also bears dorsal gills, is nested within *Peronia*. Several nodes within the *Peronia* clade are also strongly supported. The genus *Onchidium*, however, is polyphyletic, with *Onchidium vaigiense* being sister-taxon to *Platevindex* cf. *coriaceus* (1.00/94), and *Onchidium* cf. *tumidum* being sister-taxon to the *Peronia* clade (1.00/88). At the base of the onchidiid tree, there is a split between two well-supported clades: the first clade (0.98/78) includes *Onchidium vaigiense*, *Platevindex*, and *Onchidella*; the second clade (1.00/88) includes *Onchidium* cf. *tumidum* and *Peronia*. Strong support for many nodes within Onchidiidae suggests that the markers used here could efficiently help resolve onchidiid relationships.

3.7. Hygrophila, Siphonaria, Stylommatophora, Amphiboloidea, Pyramidellidae, and Glacidorbis

The monophyly of Hygrophila (Chilinoidea + Lymnaeoidea), is refuted by the present data. However, the monophyly of both Chilinoidea (0.99/86) and Lymnaeoidea (1.00/100) is strongly supported. Within Lymnaeoidea, the four taxa traditionally recognized are recovered with very high support: Planorbidae (1.00/100), including former ancylids (*Laevepex* and *Ancylus*) and planorbids; Physidae (1.00/100); Acroloxidae (1.00/100); and Lymnaeidae (1.00/100). The monophyly of *Siphonaria* is strongly-supported (1.00/100), as is that of Stylommatophora (1.00/81), Amphiboloidea (1.00/100), and Pyramidellidae (1.00/100). *Glacidorbis*,

traditionally regarded as a basal heterobranch, is found here to be closely related to Stylommatophora (1.00/77).

4. Discussion

4.1. Evolution of Ellobiidae (including Otina, Smeagol, Trimusculus)

Klussmann-Kolb et al. (2008) included four species of ellobiids in their study, representing two subfamilies (Carychiinae and Pythiinae). Here, representatives of all five subfamilies recognized by Martins (2007) are included. The monophyly of these subfamilies (Carychiinae, Ellobiinae, Melampodinae, Pedipedinae, and Pythiinae) is neither rejected nor supported, with the exception of the Pythiinae (except *Myosotella*), of which the monophyly is strongly supported. *Myosotella* is the most basal lineage of Pythiinae, but within the remaining clade (Pythiinae without *Myosotella*), relationships are poorly resolved.

The close relationship of *Auriculinella* with part of the Melampodinae (*Pseudomelampus* and *Microtralia*) indicated by molecular data is not supported by anatomical data. Several features used to characterize Ellobiinae (e.g., short right parieto-visceral connective, the gradual transition of lateral to marginal teeth), are shared by *Auriculinella* (Martins, 2007).

The monophyly of Pythiinae is weakly supported by morphology (Martins, 2007). Morphological characters that are potentially diagnostic of Pythiinae include: a long, right parieto-visceral connective; a closed last whorl (inner walls), although shell resorption occurs in various degrees in all subfamilies; and a penial papilla of pilaster origin (also occurs in *Microtralia* and *Leuconopsis*, likely through convergence). The pallial gland is a poorly-understood feature that is only found in Pythiinae (Hyman et al., 2005). Data suggest that a pallial gland might have been gained once and lost secondarily in *Cassidula* and *Pleuroloba* which also both share a distinctive, digitate proximal hermaphroditic duct (also found in *Laemodonta*). Hyman et al. (2005) mentioned that *Allochroa* and *Ophicardelus* may form a natural group, but this hypothesis is not supported here (nor is it rejected).

Inclusion of *Otina* and *Trimusculus* within Ellobiidae (Klussmann-Kolb et al., 2008) and the close relationship of *Smeagol* to *Otina* (Dinapoli and Klussmann-Kolb, 2010) are confirmed here with a broader taxon sampling. However, the relationships of *Trimusculus*, *Otina* and *Smeagol* with respect to other ellobiids are still unclear, although *Trimusculus* could be closely related to *Pedipes*.

Several hypotheses have been proposed for the affinities of the tiny limpet *Otina otis*, the unique member of Otinidae. The close relationship of *Otina* to Ellobiidae was accepted for many years (e.g., Thiele, 1931; Morton, 1955; Hubendick, 1978; Tillier, 1984). More recently, *Otina* was considered to be closely related to onchidiid and veronicellid slugs (Haszprunar and Huber, 1990) or stylommatophorans (Tillier and Ponder, 1992). Dayrat and Tillier (2002) showed that morphological data fail to resolve the relationships of *Otina*. The opening of the membrane gland into the carrefour (Dayrat and Tillier, 2002), which is found only in some (but not all) ellobiids and *Otina*, is a potential synapomorphy for that clade. *Otina* and ellobiids also share a gizzard-like structure in the stomach (also found in Hygrophila, in which it was likely gained independently); *Trimusculus*, which lacks this stomach structure, may have lost it secondarily.

The close relationship between the false limpet *Otina* and the *Smeagol* slugs (known from less than ten species from Australia and New Zealand) was suggested based on features not found in other pulmonates, such as the foot divided in a propodium and a metapodium (Tillier, 1984; Tillier and Ponder, 1992). Tillier (1984) classified Otinidae (*Otina* and *Smeagol*) along with Onchidiidae and Ellobiidae in the Ellobioidea. Tillier and Ponder (1992) classified *Smeagol* in the monotypic Smeagolidae, and the latter in Otinoidea along with Otinidae. According to Haszprunar and Huber (1990), *Smeagol* is more closely related to onchidiids than ellobiids, based on features of the nervous system which might just be related to limacization (Tillier, 1984).

Van Mol (1967) described the presence of small cells in the procerebrum of *Otina*, Ellobiidae, *Trimusculus*, Stylommatophora, Veronicellidae, and Onchidiidae (large cells are found in all other pulmonates). The present topology unfortunately does not help determine whether small cells are primitive (Van Mol, 1967) or advanced (Haszprunar and Huber, 1990).

In any case, the clade Ellobiidae needs to be broadened to include *Smeagol*, *Otina*, and *Trimusculus*. *Smeagol* and *Otina* could form the clade Otininae, as one of the 'subfamilies' of Ellobiidae. *Trimusculus* could temporarily be located in Pedipedinae or as an *incertae sedis* within Ellobiidae. Ellobiidae now include limpets (*Trimusculus* and *Otina*), as well as slugs (*Smeagol*), in addition to coiled snails (ellobiids, as traditionally defined).

4.2. Evolution of Onchidiidae

The monophyly of Onchidiidae has never been questioned (Dayrat, 2009). Additional taxon sampling is needed to more accurately define relationships, but preliminary comments can be provided. Labbé (1934) divided all onchidiids in Dendrobranchiatæ (with dorsal gills) and Abranchiatæ (without dorsal gills). The *Peronia* clade (*Scaphis* nested within *Peronia*) includes all species with dorsal gills, suggesting that they are an advanced feature and a potential synapomorphy. The absence of gills (here in *Platevindex*, *Onchidium*, *Onchidella*) seems to be a symplesiomorphy. It is confirmed here that *Onchidella* is monophyletic, although *Hoffmannola* (not sampled here) could also be nested within *Onchidella*. Finally, *Onchidium* has always been the default genus for species that could not confidently be placed in *Platevindex*, *Peronia*, or *Onchidella*. Therefore, the failure of species attributed to *Onchidium* to form a monophyletic taxon is not surprising.

Except for *Onchidella* and *Hoffmannola*, all onchidiids live in the tropical Indo-West Pacific. The present data (*Onchidella* is not basal) suggest that onchidiids might have originated in tropical, warm waters, such as the former Tethys Ocean (formed during the Triassic, 250 Mya), assuming that early onchidiids had similar habitat requirements. Under that scenario, *Onchidella* could have diversified through migrating away from that center of origin and invading new coastlines (*Hoffmannola* could either be an offshoot or the result of an independent migration).

4.3. Evolution of Veronicellidae

Veronicellids have been poorly represented in prior studies (Winnepenninckx et al., 1998; Yoon and Kim, 2000; Dayrat et al., 2001; Klussmann-Kolb et al., 2008). Our data show that DNA sequences hold great promise for reconstructing veronicellid relationships (although additional sampling is needed) and our results agree with morphology (Gomes et al., in prep). Morphological data indicate that *Veronicella*, *Phyllocaulis*, and *Vaginulus* belong to an unnamed, crown clade corresponding to a large radiation in South and Central America (they share anatomical features not found in other veronicellids, such as penial gland tubules differentiated in two groups).

Our data indicate that *Sarasinula* and *Laevicaulis* are basal with respect to the clade described above. Morphology supports a basal position for *Laevicaulis* relative to *Sarasinula*, which belongs to a clade including all American genera (that all share several features such as an anal opening covered by an opercular membrane). Our data do not reject such relationships. Nor do they support them.

The monophyly of Veronicellidae, highly supported by our data, has also been supported by several morphological characteristics, such as a distinctive penial apparatus (with conspicuous papilla gland and tubules) and a female pore on the right hyponotum (Gomes et al., in prep). Finally, although no rathouisiid slugs are included here, it is generally accepted that they are closely related to veronicellids with which they share several features, such as inferior tentacles with bifid extremity (Gomes et al., in prep.).

4.4. Evolution of Systellomatophora

The monophyly of Systellomatophora (here represented by Veronicellidae and Onchidiidae) is strongly supported by Bayesian Inference. Salvini-Plawen (1970) created the Gymnomorpha to include Veronicellidae, Rathouisiidae, Onchidiidae, and Rhodopidae, a small group of marine slugs which are now thought to be opisthobranchs (Haszprunar, 1997). Solem (1978) classified Onchidiidae, Veronicellidae, and Rathouisiidae in the Systellommatophora. Dayrat and Tillier (2002) could not find synapomorphies to support the monophyly of Systellommatophora. The pedal gland at the bottom of the anterior visceral cavity (exclusively found in onchidiids and veronicellids) could be a diagnostic synapomorphy of Systellommatophora; also, the presence of eyes at the tip of the cephalic tentacles could have been acquired twice independently (once in the common ancestral lineage to Systellommatophora, and once in the common ancestral lineage to Stylommatophora).

4.5. Evolution of Hygrophila

Most morphological studies have agreed that Hygrophila was monophyletic and included all freshwater pulmonates (e.g., Thiele, 1931; Hubendick, 1978; Tillier, 1984; Salvini-Plawen and Steiner, 1996), although it is difficult to find anatomical synapomorphies (Dayrat and Tillier, 2002, 2003). Early molecular data supported a close relationship between *Chilina* and *Lymnaea* (Dayrat et al., 2001), and a more extensive sampling supported the monophyly of Hygrophila (Klussmann-Kolb et al., 2008). However, the present data do not confirm that Chilinoidea (*Chilina* and *Latia*) are sister-taxon to Lymnaeoidea. Rather, Chilinoidea is found to be closely related to Amphiboloidea, although this result is not well supported. Hubendick (1945) mentioned several features shared by *Amphibola* and *Chilina*, especially in the nervous and genital systems.

The close relationship between *Latia* and *Chilina* (Klussmann-Kolb et al., 2008), confirmed here with new *Chilina* sequences, was suggested by early anatomists (e.g., Pelseneer, 1901). Hubendick (1978) thought that chilinids were the most basal lineage of Hygrophila (because of their long visceral loop) and closely related to Latiidae and Acroloxidae.

That former ancylids (here *Laevepex* and *Ancylus*) are nested within Planorbidae was suggested long ago by Pelseneer (1897) and has been documented by extensive molecular data (Morgan et al., 2002; Jørgensen et al., 2004; Walther et al., 2006; Albrecht et al., 2007). The monophyly of Physidae (e.g., Wethington and Lydeard, 2007), Lymnaeidae (e.g., Remigio and Blair, 1997; Pusledniket al., 2009), Acroloxidae (e.g., Walther et al., 2006) is recovered here with the highest support. However, new markers are needed to determine the deep relationships among the major clades of freshwater pulmonates.

4.6. Pulmonate higher relationships

Recent studies have suggested that *Siphonaria* might be separated from other pulmonates, and, in the case of studies based on mitochondrial genomes, might even belong to opistobranchs (Fig. 1). Such hypotheses are not contradicted by morphological data. Indeed, the gills of *Siphonaria* and cephalaspideans (specially shelled sacoglossans) are anatomically similar (Dayrat and Tillier, 2002, 2003). Although they have been interpreted as resulting from convergent evolution, they may share the same ancestry. Also, the "pneumostome" of *Siphonaria* is not contractile (it is contractile in all pulmonates), and the nesting of *Siphonaria* within opisthobranchs suggests that its "pneumostome" may have been acquired independently. Should subsequent studies confirm that *Siphonaria* is more closely related to opisthobranchs than to pulmonates, many aspects of its biology and ecology will have to be re-evaluated. Given the position of *Siphonaria*, one could restrict Pulmonata not to exclude *Siphonaria*. Alternatively, Sacoglossa (and possibly Acochlidiacea, see Jörger et al., 2010) could be included in an broadened Pulmonata clade, together with *Siphonaria*.

Present data reject the Geophila hypothesis (Stylommatophora and Systellommatophora being closely related), supported by the position of the eyes at the tip of cephalic tentacles. Instead, it seems that eyes have evolved from a basal to an apical position twice independently (Fig. 1F). Present data also reject the Eupulmonata hypothesis (*sensu* Morton, 1955, i.e., including Geophila and Ellobiidae) because Stylommatophora and Systellommatophora are in two distinct clades. Although pyramidellids seem to belong to pulmonates, their exact relationships are unclear (Figs. 1 and 2). Present data also confirm that amphiboloids are not particularly 'basal' with respect to other pulmonates, although their exact relationships are still unclear: the close relationship between Pyramidellidae and Amphiboloidea is not confirmed here (Figs. 1 and 2). Finally, the pulmonate affinity of the freshwater snail *Glacidorbis*, originally suggested by Ponder (1986), is confirmed here. However, its exact position remains unclear (Figs. 1 and 2).

4.7. On the lack of markers for molluscan phylogenetics

The present study is based on a much broader taxon sampling (79 pulmonate species) than all previous studies (Fig. 1). In particular, recent studies did not include any terrestrial veronicellid slugs, and very few onchidiids and ellobiids. Naturally, this increase in taxon sampling deeply affects phylogenetic relationships (e.g., Heath et al., 2008), which probably accounts for many of the differences between our tree topology and the topologies proposed recently (Fig. 1). Our study also differs with respect to the markers used, which might also participate in generating different topologies.

However, the major differences observed in high-level pulmonate phylogenies reveal a deeper issue, namely the lack of a large number of readily-available markers. In comparison to other taxa such as arthropods, plants, and vertebrates, molluscan phylogenetics is based on few markers. For instance, even complete mitochondrial genomes (~14.5 kb), which in mollusks require months of work, look like a small data set compared to the 62 genes and ~41 kb of sequence data used in arthropod phylogenetics (Regier et al., 2008).

Thus, the differences we observe in euthyneuran phylogenies are likely due to the fact that we do not have enough markers to resolve relationships with reliable accuracy and robustness. Adding in the future a few more markers (such as partial 28S, 12S, H3, which would only add up to about 1.5 kb) for our large data set might definitely be informative, but, unfortunately, might not radically change the current situation. Several laboratories have attempted to explore new, nuclear protein-encoding genes, but the fact that the molluscan phylogenetic literature has mainly been based on COI, 12S, 16S, 18S, 28S, and H3, speaks for itself: getting more markers to work is challenging. Although we all do our best to gather more representative taxon samplings and increase the length of sequence data, it may take years before we can reach a reliable consensus on deep relationships of pulmonates.

4.8. Pulmonate macro-evolution: uncertainties in the earliest fossil record

Obviously, uncertainties about pulmonate high-level phylogenetic relationships constitute a major obstacle to understanding the macro-evolutionary history of pulmonates, and especially the pattern of transitions among marine, terrestrial and freshwater habitats. However, another major obstacle is that the identification of most of the earliest fossils—from Upper Carboniferous (300 Ma) to Early Cretaceous (140 Ma)—is highly controversial, which greatly jeopardizes the estimation of first appearances.

The most controversial pulmonate fossils are undoubtedly the terrestrial shells from the Paleozoic (Fig. 3). Solem and Yochelson (1979) recognized ten valid species of terrestrial Paleozoic (Upper Carboniferous) gastropods for northern America, and four additional species from the Paleozoic of the Old World. Authors agreed that those gastropods were terrestrial but classified them in very different taxa (Fig. 3): Stylommatophora, Ellobiidae, and even outside euthyneurans (as Helicinidae, Neritacea, or Cyclophoridae).

Solem and Yochelson (1979) argued that all those terrestrial gastropods (except for *Dowsonella* which they placed in Helicinidae) are stylommatophoran pulmonates because they could not be operculate. They considered that the ridges on the interior of the columella of *Dendropupa* were incompatible with an operculum. They also considered that the two apertural barriers in *Anthracopupa* could not coexist with an operculum. Both arguments are problematic, however: the presence of apertural teeth does not exclude prosobranch affinities because some terrestrial prosobranchs (e.g., *Proserpina*) have aperturial teeth and no operculum; and the presence of teeth is not a synapomorphy of Stylommatophora. Solem and Yochelson (1979) rejected that *Anthracopupa* nor *Dendropupa* could be ellobiids because they show no resorption of the columella, although some extant ellobiids (e.g., *Pedipes*) have a full columella.

Regardless of whether they are identified as stylommatophorans or ellobiids, those earliest terrestrial Paleozoic fossils reveal very long gaps in fossil records (Fig. 3): The next oldest stylommatophorans are from the Upper Cretaceous (85 Ma), although Bandel (1991) described one stylommatophoran species from the upper Jurassic (160 Ma); the first unquestionable pulmonates appear in the upper Jurassic (85 Ma). Even as prosobranchs, those Paleozoic terrestrial shells remain controversial: the next oldest helicinids and cyclophorids are only known from the Cretaceous (Tracey et al., 1993).

The identification of those Paleozoic terrestrial fossils has remained controversial because no reliable shell-based synapomorphies are available for higher clades. It cannot be excluded that some of those early fossils could simply not be pulmonates, but rather belong to prosobranch taxa, such as Neritopsina, known from terrestrial shells from the late Carboniferous (Kano et al., 2002). They could belong to extinct taxa (a hypothesis that has surprisingly never been considered). In any case, it seems that a new investigation of those earliest fossils is needed to determine whether they could be regarded as pulmonates (and, if so, which ones) or not, as the results have major implications on the pulmonate fossil record and, thus, on the origin of pulmonate higher clades.

There is no known fossil record for *Otina*. However, Yen (1952) described a freshwater species of *Limnopsis*, which he classified in Otinidae. This identification is problematic because the shell of *Limnopsis* is very different from *Otina*, which also is clearly a marine, coastal group, not freshwater.

As for the false limpets (Fig. 3), earliest records for *Trimusculus* are from the Oligocene or possibly the Paleocene, for *Williamia* from the Eocene, and for *Siphonaria* from the Upper Cretaceous (Zilch, 1959). Older occurrences of Siphonariidae (e.g., *Berleria* and *Rhytidopilus* from Upper Jurassic) are problematic: Zilch (1959) and Tracey et al. (1993) accepted them, but Sepkoski (2002) rejected them. The two monotypic genera of Acroreiidae (Fig. 3) might constitute two related or independent extinct lineages of patelliform pulmonates.

The fossil record of the amphiboloids is quite young, which seems to contradict the traditional idea of their being the most primitive pulmonates (e.g., Hubendick, 1978). *Salinator* has no known fossil record, and *Amphibola* has been first recorded from the Pliocene, late Tertiary (5 Ma). However, their recent appearance may be due to the fact that they live —at least the current *Amphibola* in New Zealand— in mudflats, where preservation is difficult.

Ellobiids were undoubtedly present in the Tertiary, and all seem to be marine species (Fig. 3). Older occurences are also known from the Upper Cretaceous: *Rhytophorus*, *Melampoides*, and *Melampus*, all regarded as non-marine shells by Henderson (1935). Records of ellobiids from the Purbeck beds (Upper Jurassic) of Europe are

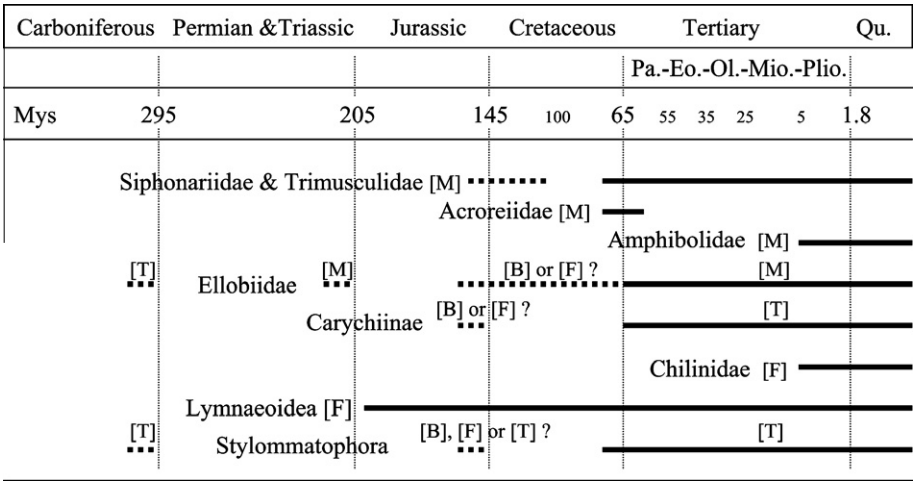

Fig. 3. Fossil record of major taxa of Pulmonata, distinguishing well-supported (black continuous lines) and questionable (dotted lines) identifications. Letters indicate marine [M], brackish [B], freshwater [F] and terrestrial [T] habitats. Taxa with no known fossil record, such as true slugs (onchidiids, veronicellids, *Smeagol*) are not shown. Based on data from: Bradley, 1870; Pilsbry, 1926; White, 1895; Henderson, 1935; MacNeil, 1939; Arkell, 1941; Yen, 1946a,b, 1947, 1949, 1951a,b, 1952; Yen and Reeside, 1946a,b; Zilch, 1959; Knight et al., 1960; LaRoque, 1960; Baker, 1963; Solem and Yochelson, 1979; Gray, 1988; Bandel, 1991, 1994, 1996, 1997; Tracey et al., 1993; Sepkoski, 2002. For more detailed references, see Section 4.

more problematic because the Purbeck beds were interpreted as a brackish (Arkell, 1941) or freshwater (Yen, 1952) habitat. None of those shells bear aperturial barriers, but their inner lip looks quite similar to that of some ellobiids. Carychiinae is represented by only two extant genera of terrestrial ellobiids: *Zospeum*, from Europe, with no known fossil record, and *Carychium*, which is holarctic. Some shells from the Purbeck beds, Upper Jurassic, have also been identified as *Carychium* (Fig. 3). The oldest records of ellobiids seem to be freshwater (Jurassic) or even terrestrial (Upper Carboniferous) instead of marine, even though there are no extant freshwater ellobiids (Fig. 3). Freshwater ellobiids are potentially known exclusively from the Late Jurassic to the Upper Cretaceous. All records of ellobiids between the Tertiary and present are from marine habitats. If all those identifications were to be correct, the evolutionary history of Ellobiidae could be quite complex (see Section 4.9).

Although chilinids have been traditionally regarded as "primitive" pulmonates, their fossil record is relatively young (Fig. 3). No fossil record is known for the Latiidae. The records of *Physa prisca* considered to be from the Upper Carboniferous are actually from the Lower Cretaceous (MacNeil, 1939). However, lymnaeoids seem to be the only pulmonates that were undoubtedly present from the late Jurassic (Fig. 3).

4.9. Macro-evolutionary transitions between aquatic and terrestrial habitats

Addressing macro-evolutionary transitions between aquatic and terrestrial habitats requires a full range of data (Vermeij and Dudley, 2000): a phylogenetic pattern of relationships; the study of physiological and morphological constraints and adaptations to new habitats; the biological context in which transitions occurred (e.g., temporal and geographical dimensions, and competitions between invaders and incumbents).

Recent species of Pulmonata are represented in marine, freshwater and terrestrial habitats (Figs. 1 and 3), unlike their closest relatives, the opisthobranchs, which almost exclusively include marine species. Terrestrial pulmonates are found in five lineages: Stylommatophora, by far the most successful terrestrial radiation of gastropods (~30,000 species), Veronicellidae (~200 species), Carychiinae (~40 species), and, *Pythia* (Ellobiidae) and *Semperoncis* (Onchidiidae) which both include a few terrestrial species (Martins, 1995; Dayrat, 2010). Carychiinae, Veronicellidae, and Stylommatophora are fully terrestrial and live their entire life cycle on land. Freshwater pulmonates are represented by three clades: Lymnaeoidea (~1000 species), Chilinoidea (~25 species), and Glacidorbidae (~15 species). Lymnaeoidea and Chilinoidea, however, may be sister-taxa (as Hygrophila). All the other pulmonates live along the coastline, including rocky intertidal, salt marsh, and mangrove habitats.

Plate (1894) first proposed a tempting scenario of evolution from "primitive" marine pulmonates to "evolved" freshwater and terrestrial pulmonates involving several direct transitions from the sea to the land and fresh water. Alternative hypotheses exist (e.g., Solem and Yochelson, 1979; Solem, 1985): freshwater pulmonates secondarily evolved from terrestrial lineages; the first pulmonates were terrestrial and then gave rise to freshwater and marine lineages.

Uncertain higher-level relationships of pulmonates constitute a major obstacle to understanding their macro-evolutionary transitions between habitats (Figs. 1 and 3). However, generally speaking, all topologies are compatible with the idea of several independent transitions from the sea to the land and fresh water, although the number and order of those transitions is unclear. The idea that pulmonates originated on land and that some lineages became marine secondarily is difficult to conceive because of developmental constraints. Indeed, data suggest that living species with fully direct development cannot transition "back" to a developmental mode with a free veliger stage (e.g., Collin, 2004). That stylommatophorans were possibly the first pulmonates to emerge during the Upper Carboniferous (300 Ma) seems to be supported by phylogenetic analyses based on mitochondrial genomes, which place stylommatophorans at the base of the tree (Fig. 1A). If pulmonates first appear in the Late Jurassic (Fig. 3), then the earliest group of pulmonates would be the lymnaeoids, which is also congruent with phylogenies based on mitochondrial genomes (Fig. 1A).

The fact that glacidorbids are pulmonates increases the number of transitions to fresh water to two (Hygrophila and Glacidorbidae) or three (Lymnaeoidae, Chilinoidea, and Glacidorbidae). Given that freshwater snails are characterized by direct development lacking a veliger stage and that they all breathe air through a lung, it is conceivable that they (or at least some) evolved from terrestrial lineages, especially considering that close relationships between lymnaeoids and stylommatophorans are suggested by some data (Fig. 1).

Uncertainties in the fossil record bring additional complexity. All fossils older than the Late Jurassic (~150 Ma) are highly controversial in terms of their identification and with respect to their habitat. Many alternatives arise when one considers all possible identifications and habitats for earliest fossil pulmonates (Fig. 3; and see above Section 4.8).

Another reason why macro-evolutionary transitions between habitats are poorly understood is that it is unclear how difficult it was for individuals of extinct species to survive in a new habitat. In that regard, the natural history of living species is highly instructive because it might inform us of the pressures that may have existed on extinct species. The onchidiid *Semperoncis montana* and the ellobiid *Pythia colmani* are particularly interesting: both species can live at high elevation (as long as they stay in the rain forest): up to 1850 m for *S. montana* (Dayrat, 2010), and up to 850 m elevation for *P. colmani* (specimens from New Britain currently studied by the first author). Although those cases are exceptional, they show that it is possible for species that belong to marine groups to survive on land. It is possible that both *Pythia colmani* and *Semperoncis montana* reproduce independently from the sea, by simply brooding their eggs, as it seems difficult to conceive that populations could migrate up and down between sea level and such high altitudes. However, their reproduction and development are unfortunately unknown. Interestingly, none of the truly terrestrial pulmonates (Stylommatophora, Veronicellidae, Carychiinae) is known to be able to survive in the sea (or freshwater for that matter), also suggesting that it might be easier for gastropods (at least extant ones) able to breathe air to invade land from the sea, than for gastropods whose development is terrestrial, i.e., independent from the sea, to invade the sea from the land. Under this scenario, ability to breathe air was acquired first, and development later became independent from the sea in at least three lineages (Stylommatophora, Veronicellidae, and Carychiinae).

All marine pulmonates (with the exception of *Williamia*) die if they are submerged for too long, although their embryological development takes place in the sea. Marine pulmonates are intertidal more so than truly marine organisms. The intertidal zone is characterized by wide ranges of variations in physical factors and requires organisms to be adapted to changing conditions. Naturally, it seems easier to invade the land for intertidal animals adapted to breathe air than for fully-marine organisms. Maybe, the fact that some lineages of pulmonates have invaded the land and fresh water partly comes from the fact "marine" pulmonates are air-breathing, intertidal animals, unlike the opisthobranchs which all must remain submerged.

In that sense, brackish habitats from the Upper Jurassic (Fig. 3) could represent well the kind of habitats where pulmonates lived

and evolved (i.e., habitats that were not typically marine or freshwater), and from which transitions towards a more specialized terrestrial or freshwater habitat were more easily conceivable.

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