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Short Communication

Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships

Elpidio A. Remigio* and Paul D.N. Hebert

Department of Zoology, University of Guelph, 50 Stone Rd. East, Guelph, Ont., Canada N1G 2W1

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1. Introduction

Recent studies on phylogenetic relationships within the molluscan class Gastropoda have involved morphological (Kay et al., 1998), ultrastructural (Healy, 1996), and molecular (e.g., Lydeard et al., 2002; McArthur and Koop, 1999) approaches. These investigations have provided new insights into gastropod affinities and classification and have enabled a vigorous testing of taxonomic schemes for the group. The most generally accepted system of classification now partitions the Gastropoda into five subclasses (Tudge, 2000), two of which, the Heterobranchia and the Caenogastropoda, are extremely diverse. The other three subclasses (Patellogastropoda, Neritopsina, and Vetigastropoda) are much less speciose, but are thought to represent the basal lineages of the class.

Over the past decade molecular approaches have proven their value not only in resolving phylogenetic issues, but also in providing a sense of the time scales of evolutionary divergence. Because of their slow rates of evolution, nuclear rRNA genes have been widely used in studies that attempt to resolve relationships among groups that have a long history of evolutionary divergence. In contrast, the more rapidly evolving mitochondrial (mt) genes have generally been employed to infer relationships among groups with a more recent ancestry. However, it has become apparent that the latter gene regions can also provide insights concerning deeper divergences, as shown by a study that employed 16S rDNA sequences to examine the affinities of major gastropod lineages (Thollesson, 1999).

Among the 13 protein-coding genes within the mt genome, cytochrome *c* oxidase I has gained particular popularity for estimating relationships among closely allied taxa. Despite its broad usage in resolving affinities at lower taxonomic levels, COI has been little exploited to address deeper phylogenetic issues. However, in the course of studies on various molluscan genera, we observed indications of the ability of partial COI sequences to recover deeper divergences, and the present study provides a more formal test of this gene's capacity in this regard. The implications of our results for the pattern and tempo of evolutionary divergence in gastropods, specifically among the Heterobranchia, are discussed.

2. Materials and methods

This study involved an examination of COI sequences from 73 species of gastropods representing 70 genera and including members from all five gastropod subclasses (Appendix A). New sequence data were obtained from species in four freshwater families (Ancylidae, Lymnaeidae, Physidae, and Planorbidae) belonging to the order Basommatophora, as well as representatives of the morphologically and ecologically deviant pteropod gastropods including two members of the order Thecosomata and one of the order Gymnosomata.

Genomic DNA was prepared using a modified proteinase K method, which involved the exclusion of the ethanol precipitation step. This procedure yielded snail DNA suitable for PCR amplification of the COI gene region targeted by Folmer et al.'s (1994) primers and automated sequencing of purified PCR products. The algorithms used for phylogenetic analyses included maximum parsimony (MP) and maximum likelihood (ML) methods (Remigio, 2002). Phylogenetic trees were rooted using *Katharina tunicata*, a polyplacophoran, as

* Corresponding author. Fax: +519-767-1656.
E-mail address: eremigio@uoguelph.ca (E.A. Remigio).

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an outgroup. Analyses were performed using COI sequences from the entire set of taxa or a subset of taxa, i.e., excluding the pteropods and (or) patellogastropods. The latter procedure was conducted to avoid distortions in tree topology arising from the presence of these strongly rate-accelerated lineages (see Section 3).

3. Results and discussion

The aligned data matrix, which was 672 bp in length, included 455 variable sites. No length difference from the outgroup was detected among members of three subclasses, the Neritopsina, Vetigastropoda, and Caenogastropoda. However, insertion/deletion events (indels) were observed in the other two subclasses, the Heterobranchia and Patellogastropoda (Appendix B). Most of this length variation occurred in the region of the gene coding for E1, the first loop of the protein that extends into the intermembrane space (Lunt et al., 1996). For example, two closely allied freshwater planorbid genera, *Gyraulus* and *Planorbis*, showed a 12 bp insert at position 94. A 3 bp deletion in the pteropod genus *Limacina* at positions 106–108, and a second 6 bp deletion at positions 112–117 were also detected in this region. In addition to these length variants in E1, *Limacina* had a unique 3 bp deletion involving sites 367–369, a region coding for E2. These length variants do not appear to represent pseudogenes as no termination codons were found and most sequence changes occurred at third codon sites. Wollscheid-Lengeling et al. (2001) also indicated the occurrence of 3 bp inserts at positions 94–96 and 499–501 (Appendix B), but these results require confirmation as similar inserts were not detected in other studies (e.g., Medina and Walsh, 2000; unpublished data) of closely allied taxa. Indels could represent an important source of phylogenetic information that is less subject to homoplasy than nucleotide substitutions. Indel events leading to the loss of secondary structure elements in the mt 16S gene have, for example, recently been shown to be a reliable diagnostic character for delineating certain gastropod groups (Lydeard et al., 2002). Indeed, gastropods are a good target for future work because they show a much higher incidence of length variants at COI than other invertebrate groups such as insects (Hebert et al., 2003). Aside from the prevalence of indels, the present study revealed significant shifts in nucleotide composition among groups (data not shown). For example, the rate-accelerated lineages (e.g., *Limacina* and the patellogastropods; see Fig. 1) have substantially higher G + C content than most other taxa (data not shown). The functional significance of these changes in the COI of gastropods is unclear, and comprehensive analyses are needed not only to determine the mechanisms responsible for, but also the impacts of, these changes.

The ML tree derived from the entire set of taxa and the best-fitting model (GTR + G + I; $-\ln L = 21917.72$; Fig. 1) generally revealed expected associations among closely related taxa, and was largely concordant with the results of MP analyses (trees not shown). Moreover, deeper divergences were also resolved, as shown by the placement of two recognized ancestral subclasses, the Vetigastropoda (VET) and Neritopsina (NER), at the base of the tree. Admittedly, the sole neritopsine genus, *Theodoxus*, was positioned between the two vetigastropod genera, but this likely reflects limited taxon sampling. The Patellogastropoda (PAT), which are also viewed as basal, clustered instead with the pteropod genus *Clione* at the base of the Heterobranchia (HET), whereas the other pteropod genus sampled, *Limacina*, was positioned between two freshwater pulmonate families. These results are likely a consequence of long-branch attraction linked to their extreme rate acceleration (Lyons-Weiler and Hoelzer, 1997). Because of this fact, little confidence can be placed in the placement of either group in the tree, but the data do provide some insights on pteropod affinities. Based on his analyses of mt 16S rDNA data, Thollesson (1999) concluded that the pteropod genus *Clione* was a member of the subclass Caenogastropoda. This taxonomic reassignment conflicts with our reconstructed phylogeny and the results of constraint analysis, i.e., the forced union of *Clione* with the various caenogastropod lineages required the addition of at least 29 steps to the optimal tree (e.g., in analyses that excluded the patellogastropod data), and gave a significantly poorer estimate of relationships based on Shimodaira and Hasegawa's (SH) test ($P < 0.05$). Earlier work using 28S rDNA sequences also grouped pteropods among the Heterobranchia, and specifically showed their close relationship with opisthobranchs (Dayrat et al., 2001).

Subsequent analyses that excluded the rate-accelerated patellogastropod sequences identified pteropods as a monophyletic group at the base of the Heterobranchia (tree not shown), contrary to an earlier analysis that failed to group them together (e.g., Fig. 1). The ML phylogeny based on the best-fitting model (GTR + G + I; $-\ln L = 19247.48$) and excluding both the patellogastropod and pteropod sequences is shown in Fig. 2. Both exclusion analyses resolved the subclass Heterobranchia with high confidence, separating its members into two major groups, i.e., the pulmonates (Pul) and the opisthobranchs (Opi). Higher-level relationships within the opisthobranchs were not well delineated, but several lower-level assemblages were recovered. More detailed resolution was obtained for the pulmonates. The four freshwater pulmonate families [Lymnaeidae (Lym), Physidae (Phy), Planorbidae (Pla), and Ancylidae (Anc)] were resolved as monophyletic, a result that agrees with their placement in the order Basommatophora. Within this clade, there was good

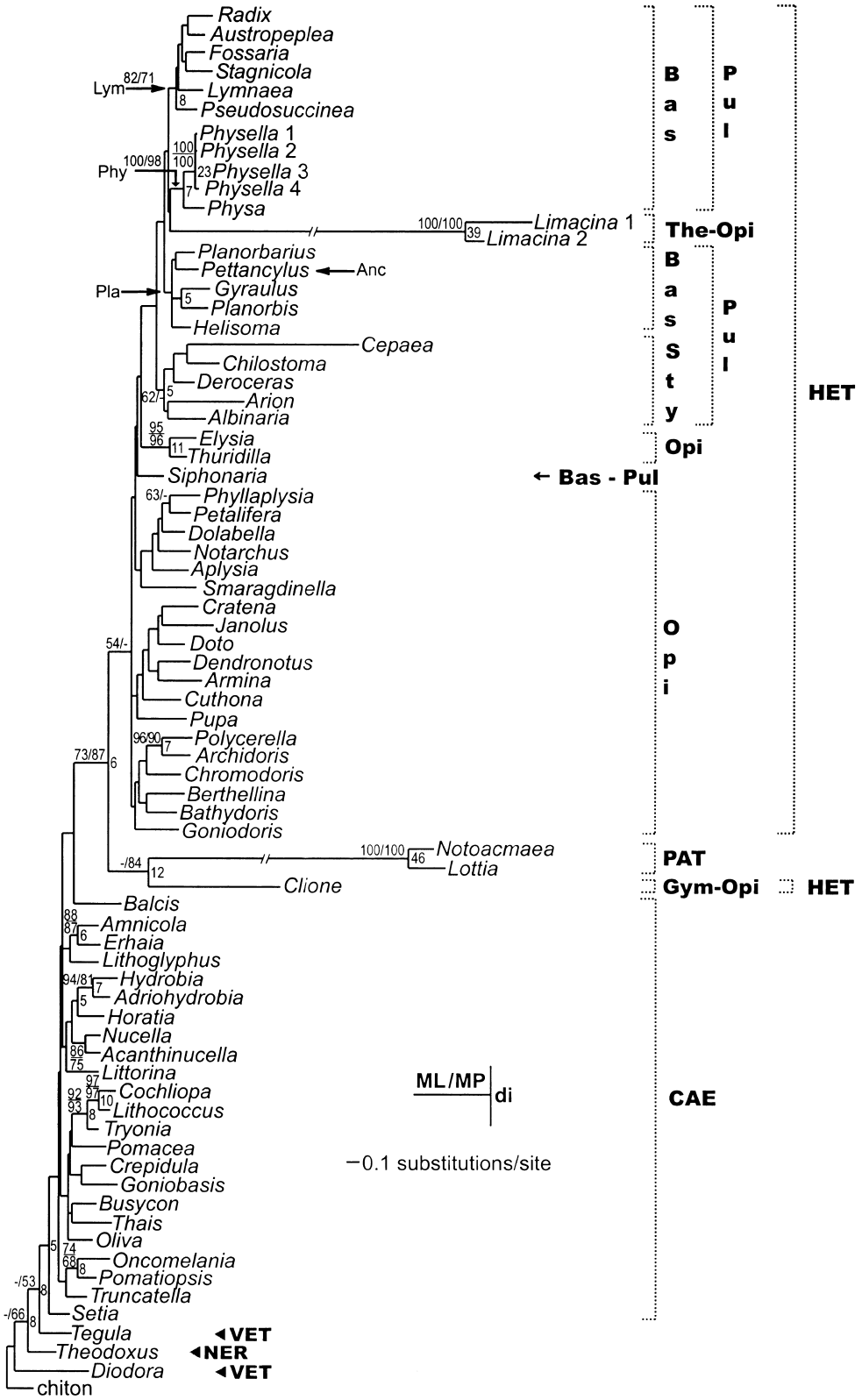

Fig. 1. COI gene tree based on analyses using the entire set of taxa. Bootstrap (bs, 100 and 1000 replicates for ML and MP methods) and Bremer (di) support values are given on the branches. Only bs and di values $\geqslant 50$ and $\geqslant 5$ are shown. Parameters used in the ML analyses are: substitution rate matrix ($r_1 = 0.57$, $r_2 = 8.96$, $r_3 = 4.72$; $r_4 = 5.26$, $r_5 = 15.14$, and $r_6 = 1.0$); $\alpha = 0.53$; $I = 0.23$; six substitution types; four rate categories; empirical base frequencies. MP analyses employed heuristic searches (100 random sequence addition, TBR branch swapping, unordered and unweighted character states; bootstrap analyses utilized full heuristic searches). The branches leading to *Limacina* and the two patellogastropods are approximately twice as long as that shown. See Appendix A for definitions of abbreviations of taxon names.

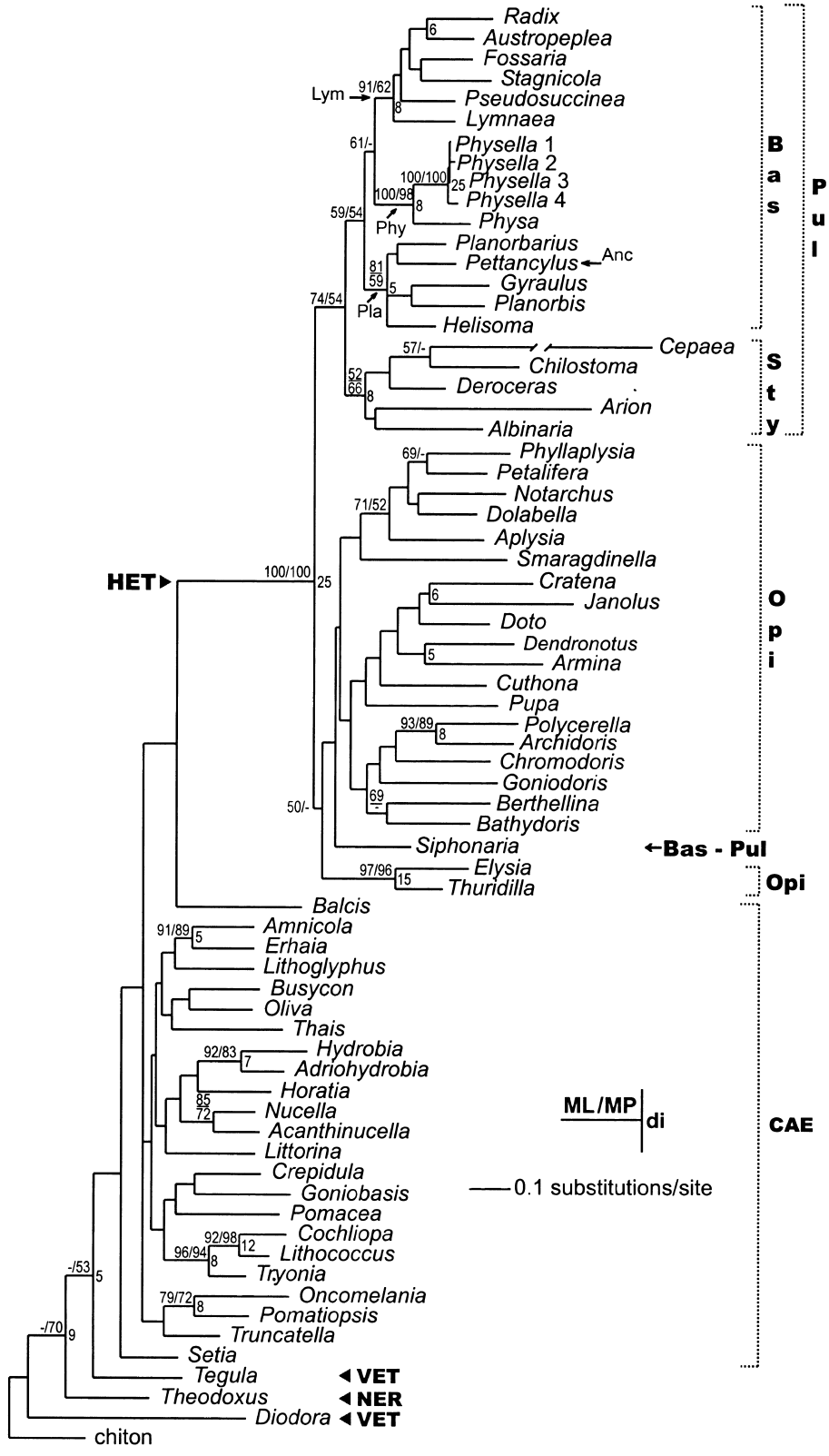

Fig. 2. COI gene tree based on analyses excluding the patellogastropod and pteropod sequences. Parameters used in the ML analyses are: substitution rate matrix ($r_1 = 0.44$, $r_2 = 9.12$, $r_3 = 4.92$; $r_4 = 4.29$, $r_5 = 15.41$, and $r_6 = 1.0$); $\alpha = 0.61$; $I = 0.31$; six substitution types; four rate categories; empirical base frequencies. The branch leading to *Cepaea* is approximately twice as long as that shown. Analytical details used in the MP analyses and for quantifying branch support are in the caption for Fig. 1. See Appendix A for definitions of abbreviations of taxon names.

support for the families Physidae and Lymnaeidae. *Pettancylus*, the lone genus of the Ancylidae examined, formed a monophyletic group with the Planorbidae, a result that appears to support the view based on morphology that these two families should be united (Hubendick, 1978). Broader taxonomic coverage for these families should clarify their relationships. Additional analyses using other outgroups (e.g., a cephalopod or bivalve) consistently identified the Heterobranchia as a distinct clade; a notable difference is that the Vetigastropoda and Neritopsina were not depicted as basal lineages in almost all analyses (trees not shown).

Siphonaria, the sole marine basommatophoran analyzed, did not group with other members of this order or for that matter even with pulmonates (Figs. 1 and 2). The phylogenetic position of this genus has long been disputed. In one classification scheme, this genus is placed in a distinct order, the Archaeopulmonata, a group viewed as ancestral to the freshwater pulmonates (Morton, 1955). Constraining *Siphonaria* to form a monophyletic group with the other pulmonates (in analyses that excluded the patellogastropod sequences) added 23–42 steps to the most parsimonious solution, which is a significantly poorer estimate of relationship based on the SH test ($P < 0.05$). It will, however, only be possible to critically assess the position of this genus by examining representatives from other presumptive basal families of the Basommatophora (e.g., Amphibolidae, Ellobiidae). Similarly, the recovery of a monophyletic clade of pulmonate land snails agrees with their placement in the order Stylommatophora, a conclusion supported by a recent molecular study (Wade et al., 2001). Prior studies (Harasewych et al., 1998; Kay et al., 1998; McArthur and Koop, 1999) have established the monophyly of the Caenogastropoda. The same result was obtained in the MP analysis (tree not shown) that excluded the patellogastropods and pteropods, but bootstrap support was <50%.

The differential success of the present analysis in resolving relationships among members of the two most taxonomically diverse subclasses, the Heterobranchia and Caenogastropoda, is intriguing. The fossil record suggests that these subclasses have similarly ancient histories, both diverging from the basal gastropod lineage approximately 360–400 mya (Tracey et al., 1993). However, the Heterobranchia was identified as a well-resolved and highly supported clade, while the Caenogastropoda was less clearly resolved. This difference was particularly evident in the ML tree (Fig. 2) that excluded the patellogastropods and pteropods, where a long branch isolated all of the heterobranchs, whereas the caenogastropods were poorly resolved. In addition, the internal branches leading to the major heterobranch lineages are short. The sharper delineation of the heterobranchs than the caenogastropods, despite their contemporaneous origins, can be reconciled in two fashions. The heterobranchs may have experienced a bottleneck in taxonomic diversity subsequent to their origin, so that the modern members of this group share a relatively recent ancestry. Alternatively, the heterobranchs may have experienced a brief episode of accelerated molecular evolution shortly after their origin. Discrimination between these hypotheses will require both broader taxon sampling and analyses of other genes.

The present study has shown the utility of COI in identifying phylogenetic affinities among many gastropod groups across a broad taxonomic range. Although support for several of the deeper branches is limited, the ability of COI to recover them is remarkable, considering that the present analysis was based on just a fragment of the gene, and because COI has generally been viewed as useful only for recovering shallow divergences. Because increased taxon (Pollock et al., 2002; Remigio, 2002) and character (Grande et al., 2002) sampling have recently been shown to improve phylogenetic accuracy, we anticipate that these approaches would provide more robust estimates of gastropod relationships.

The results of this study suggest that COI sequences are also well suited to give an indication of shifts in rates of molecular evolution (e.g., pteropods and patellogastropods) and nucleotide usage, as well as evidence of molecular diversity (e.g., planorbids). As such, COI could be employed as a 'sentinel' gene in broad surveys that seek only to identify taxa with anomalous patterns of evolution, an approach that could help clarify factors governing key aspects of molecular evolution.

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Appendix A

Gastropod taxa analyzed and their GenBank sequence accession codes. Species sequenced in the present study are marked with asterisks. **Subclass Heterobranchia (HET):** Pulmonata (Pul): Basommatophora (Bas): Family Ancylidae (Anc): **Pettancylus* sp. (AY227374); Family Lymnaeidae (Lym): **Austropeplea tomentosa* (AY227365), **Fossaria bulimoides* (AY227367), **Lymnaea stagnalis* (AY227369), **Pseudosuccinea columella* (AY227366), **Radix ovata* (AY227364), **Stagnicola elodes* (AY227368); Family Physidae (Phy): **Physella* sp. (AY227375), *Physella gyrina* (AF346744), *Physella*

johnsoni (AF346737), *Physella wrighti* (AF346745), *Physa* sp. (AF346746); Family Planorbidae (Pla): **Gyraulus deflectus* (AY227372), **Helisoma trivolvis* (AY227371), **Planorbarius* sp. (AY227370), **Planorbis* sp. (AY227373); Family Siphonariidae: *Siphonaria pectinata* (AF120638); Stylommatophora (Sty): *Albinaria caerulea* (NC001761), *Arion fasciatus* (AF239735), *Cepaea nemoralis* (NC001816), *Chilostoma trizona* (AF296998), *Deroceras reticulatum* (AF239734); Opisthobranchia (Opi): Anaspidea: *Aplysia punctata* (AF156145), *Dolabella auricularia* (AF156148), *Notarchus indicus* (AF156151), *Petalifera ramosa* (AF156153), *Phyllaplysia taylori* (AF156155); Cephalaspidea: *Pupa strigosa* (AB028237), *Smaragdinella* sp. (AF249806); Gymnosomata (Gym): **Clione limacina* (AY227377); Notaspidea: *Berthellina citrina* (AF249785); Nudibranchia: *Archidoris pseudoargus* (AJ223256), *Armina loveni* (AF249781), *Bathydoris clavigera* (AF249808), *Chromodoris luteorosa* (AJ223259), *Cratena peregrina* (AF249786), *Cuthona caerulea* (AF249807), *Dendronotus dalli* (AF249800), *Doto carinata* (AF249794), *Goniodoris nodosa* (AJ223264), *Janolus cristatus* (AF249813), *Polycerella emertoni* (AJ223273); Sacoglossa: *Elysia timida* (AF249818), *Thuridilla hopei* (AF249810); Thecosomata (The): **Limacina helicina antarctica* (AY227378), **Limacina helicina helicina* (AY227379); **Subclass Caenogastropoda (CAE):** *Acanthinucella spirata* (AY027694), *Adriohydrobia gagatinella* (AF317881), *Amnicola limosa* (AF354768), *Balcis eburnea* (AF120636), *Busycon carica* (U86323), *Cochliopa* sp. (AF354762), *Crepidula cerithicola* (AF388698), *Erhaia jianouensis* (AF213340), *Goniobasis proxima* (AY063464), *Horatia sturmi* (AF213345), *Hydrobia acuta* (AF213344), *Lithococcus* sp. (AF354763), *Lithoglyphus naticoides* (AF354770), *Littorina saxatilis* (AJ133344), *Nucella lapillus* (AF242178), *Oliva sayana* (U86333), *Oncomelania hupensis* (AF306630), *Pomacea paludosa* (AF321980), *Pomatiopsis lapidaria* (AF354774), *Setia turriculata* (AF253082), *Thais haemastoma* (U86330), *Truncatella guerinii* (AF120635), *Tryonia clathrata* (AF061767); **Subclass Neritopsina (NER):** *Theodoxus fluviatilis* (AF120663); **Subclass Patellogastropoda (PAT):** *Lottia strigella* (AF295539), *Notoacmaea fascicularis* (AF130120); **Subclass Vetigastropoda (VET):** *Diodora graeca* (AF120632), *Tegula verrucosa* (AF080668).

Appendix B

Aligned sequences for segments of the COI gene. Numbers above the alignment block are nucleotide positions. Nucleotide positions 91–105 were omitted in the analyses as positional homology at these sites is uncertain. The entire alignment is posted at the EMBL-Align database.

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                               111111111111111111111 222 333 455
                888889999999999000000000011111111112 222 666 900
                567890123456789012345678901234567890 345 789 901
Austropeplea    ATAGTA---------------TTAATTGATGAGCAT ATT CCT ---
Fossaria        ACTGTT---------------TTAATTGATGAACAT ATT CCA ---
Lymnaea         ATAGTT---------------TTAATTGATGAGCAT ATT CCT ---
Pseudosuccinea  ATAGTT---------------TTAATTGACGAGCAT ATT CCT ---
Radix           ATAGTA---------------TTAATTGATGAGCAT ATT CCT ---
Stagnicola      TTAGTT---------------CTTATTGATGAGCAT ATT CCT ---
Gyraulus        ATGGTTGATATTCCTAGTATTTTAACAAATGAACAT ATT ACA ---
Planorbis       TGAGTTTTATCTAATGACTTTATAATAGATGAGCAT ATT ACT ---
Helisoma        TTAGTT---------------TTGATAGATGAACAT ATT CCT ---
Planorbarius    TTAGTA---------------TTAATAGATGAACAT ATT CCT ---
Pettancylus     TTAGTA---------------ATTATAGATGAACAT ATT GCT ---
Physella1       TTAGTT---------------TTATTAGACGAACAT ATT CCT ---
Physella2       TTAGTT---------------TTATTAGACGAACAT ATT CCT ---
Physella3       TTAGTT---------------TTATTAGACGAACAT ATT CCT ---
Physella4       TTAGTT---------------TTATTAGACGAACAT ATT CCT ---
Physa           TTAGTT---------------TTGTTAGATGAACAT ATT CCT ---
Siphonaria      GGAGCATTT------------TTAGGTGATGATCAT ATT CCA ---
Albinaria       GGAACA---------------TTGACTGACGATCAT ATT AGT ---
Arion           GGAGTT---------------CTCACAGATGACCAA ATT ATT ---
Cepaea          GGAGTA---------------CTAACAGACGACCAT --- CTA ---
Chilostoma      GAAGTA---------------TTATCAGATAGTCAT ATT TTA ---
Deroceras       GGAGTT---------------TTACTAGATAATCAT ATT CCA ---
Clione          ACCATCATT------------CTAGGTTCTCCTCAT GTA TTA ---
Limacina1       TTTCTAAGC---------------GGT------CAA CTG --- ---
Limacina2       TTTTTAAGT---------------GGA------CAA TTA --- ---
Aplysia         GGGGCCTTT------------TTAGGGGACGATCAT ATT CCT ---
Dolabella       GGTTCTTTC------------TTAGGTGATGATCAT ATT CCA ---
Notarchus       GGTTCTTTA------------CTAGGTGACGATCAT ACT CCT ---
Petalifera      GGTTCTTTT------------TTAGGTGATGATCAT ATT CCA ---
Phyllaplysia    GGTTCTTTC------------CTAGGTGACGACCAC ATT CCA ---
Pupa            GGTGCCCTT------------TTAGGTGACGATCAT ATT GCG ---
Smaragdinella   TC???TGCTTTC---------TTAGGAGATGATCAT ATT CCT TTT
Archidoris      GGTGCTTTT------------CTAGGTGATGATCAT ATT CCT ---
Chromodoris     GGAGCATTT------------TTAGGTGATGATCAT ATT TCA ---
Goniodoris      GGGGCTTTC------------TTAGGAGACGACCAT ATT CCT ---
Polycerella     GGTGCTTTT------------TTAGGTGATGATCAC ATT CCT ---
Armina          GG???TGCTTTA---------CTAGGTGATGATCAT ATT GCT TTC
Bathydoris      GG???AGCTCTT---------TTAGGTGATGATCAT ATT ATT ATG
Cratena         GG???CGCTTTA---------TTAGGGGATGATCAT ATT GCT TTT
Cuthona         GG???AGCTCTC---------TTAGGTGATGACCAT ATT CCT TGG
Dendronotus     GG???TGCTCTA---------TTGGGTGACGATCAT ATT GCT TTA
Doto            GG???TGCCTTA---------TTAGGAGACGATCAT ATT GCT TTA
Janolus         GG???GGCTTTG---------TTAGGGGATGATCAT ATT GCT TTT
Berthellina     GG???GGCTTTC---------TTAGGTGATGATCAT ATT CCT ATG
Elysia          GG???GGCATTT---------TTAGGTGATGATCAT ATT CCT ATG
Thuridilla      GG???GGCTTTT---------CTAGGAGACGACCAC ATT CCT ATG
Amnicola        GGAGCCTTA------------TTAGGTGATGATCAA TTA AAC ---
Erhaia          GGAGCTCTT------------TTAGGAGATGATCAA TTA AAT ---
Lithoglyphus    GGAGCTCTA------------TTAGGGGATGACCAA TTA AAC ---
Balcis          GGGGCCCTT------------TTAGGAGATGATCAA CTA AAC ---
Busycon         GGAGCTTTG------------CTTGGTGATGATCAA TTA AAT ---
Oliva           GGAGCATTA------------TTAGGAGATGATCAA TTA AAT ---
Thais           GGGGCTTTA------------CTTGGAGATGATCAA TTA AAT ---
Crepidula       GGTGCTCTA------------CTAGGTGACGATCAA TTG AAT ---
Goniobasis      GGTGCCTTG------------CTTGGAGACGATCAA TTA AAT ---
Littorina       GGTGCTCTC------------TTAGGAGACGACCAG TTA AAT ---
Pomacea         GGTGCTTTA------------CTAGGGGATGATCAA TTA AAT ---
Oncomelania     GGGGCTTTG------------CTTGGGGATGATCAG CTA AAT ---
Pomatiopsis     GGTGCTCTA------------CTAGGCGATGATCAA ATC AAC ---
Truncatella     GGGGCTCTT------------TTAGGTGATGATCAA CTT AAT ---
Setia           GGAGCTTTA------------TTAGGTGATGACCAG TTA AAC ---
Acanthinucella  GGAGCTTTA------------CTCGGGGATGATCAG TTG AAC ---
Nucella         GGAGCTTTA------------CTTGGTGACGATCAG TTA AAT ---
Adriohydrobia   GGTGCACTT------------CTAGGTGACGATCAG CTA AAT ---
Hydrobia        GGTGCGCTT------------TTGGGTGATGATCAG CTT AAC ---
Horatia         GGGGCGCTC------------CTTGGGGACGATCAG TTA AAT ---
Cochliopa       GGTGCTCTT------------TTGGGTGATGATCAA TTG AAT ---
Lithococcus     GGAGCTCTT------------TTAGGTGATGATCAA TTA AAT ---
Tryonia         GGAACTCTT------------TTAGGTGATGATCAA TTA AAT ---
Diodora         GGTGCTTTG------------TTGGGGGACGATCAG TTA AAT ---
Tegula          GGTGCACTA------------TTAGGGGACGATCAG TTG AAC ---
Theodoxus       GGTGCTTTA------------TTAGGGGATGACCAG TTA AAT ---
Lottia          GGAACAGGATTT---------TTGGTCTCAGGTACC ATC TTG ---
Notoacmaea      GGGACAGGGTTT---------CTTATGAGTGGGACT ATC TTG ---
Katharina       GGGGCTTTA------------TTGGGGGATGACCAA TTA AAT ---
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