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MULTIPLE ORIGIN OF VIVIPARITY IN SOUTHEAST ASIAN GASTROPODS (CERITHIOIDEA: PACHYCHILIDAE) AND ITS EVOLUTIONARY IMPLICATIONS

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Abstract.—This study aims at a better understanding of the evolutionary significance of viviparity in some freshwater gastropods. We use a phylogeny based on partial sequences of the mitochondrial 16S gene of representatives of the limnetic and pantropical Pachychilidae to infer the relationships within this particular group of cerithioideans and the evolution of reproductive strategies. The phylogeny presented herein implies a new systematization and suggests that viviparity has appeared three times among the Pachychilidae. This is supported by the finding of very distinct reproductive morphologies in different lineages of viviparous taxa that are exclusively found in Southeast Asia. Based on the observation that oviparity is the ancestral character state in this freshwater family, we conclude that viviparity has evolved subsequent to the exploration of freshwater. We present data showing that all Pachychilidae produce considerably larger but fewer egg capsules compared to most marine snails. In other studies on freshwater gastropods, this has been discussed as an adaptation to freshwater environments. In this context we hypothesize that the increased parental investment involved in the enlargement of eggs in concert with the reduction of clutch sizes was the driving factor that ultimately lead to the evolution of viviparity in the Asian taxa. Consequently, although not directly correlated with the colonization of the new adaptive zone, viviparity is strongly favored by other consequences of this step. Hence, we hypothesize that the production of large eggs, which is necessitated by the exploration of freshwater, represents a preadaptation existing in those ancestors from which viviparous pachychilid lineages eventually evolved in Southeast Asia.

Key words.—16S rDNA, brooding, Cerithioidea, freshwater gastropods, Pachychilidae, viviparity.

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Viviparity is a common phenomenon with multiple origins in the animal kingdom; the mechanisms and causations of its development may be as plentiful. Among vertebrates alone, viviparous modes of reproduction have evolved about 150 times. Squamates account for a considerable proportion of these transitions to viviparity and in this respect these organisms represent a comparatively well-studied model group. For squamates it has been hypothesized that viviparity essentially evolved as an adaptation to cold climates (Blackburn 2000; Andrews and Mathies 2000). However, in many other organisms the evolution of viviparity remains poorly understood irrespective of the wide distribution of this phenomenon and its potentially high evolutionary significance. Molluscs might be cited as an example for such a group, which is inadequately known in regard to its diversity. In this phylum viviparous modes of reproduction have been reported from various bivalves, such as unionids, corbiculids, and sphaeriids (e.g., Heard 1997; Byrne et al. 2000; Graf and O'Foighil 2000; Korniushin and Glaubrecht 2002, 2003) but also from gastropods, such as the Viviparoidea, Cerithioidea, Rissoidea and Littorinimorpha (e.g., Calow 1978; Fretter 1984; Fretter and Graham 1994; Glaubrecht 1996). In all these taxa, viviparity represents a feature that is predominantly found in freshwater inhabitants, whereas the overwhelming majority of marine species remained oviparous. No one has yet tested hypotheses that could provide an adequate explanation for this phenomenon.

According to Andrews and Mathies (2000), a powerful tool to address the evolution of viviparity would be to focus on well-defined model groups of closely related taxa, which vary in their reproductive mode. In this context, molecular phylogenies provide an essential and reliable framework to identify and critically evaluate such taxa. However, Strathmann and Eernisse (1994) show that molecular phylogenies may fail to uncover evolutionary pathways in the development of larval forms.

In this paper we use a molecular phylogeny in conjunction with evaluation of morphological features to discuss the adaptive significance of viviparity in a certain group of freshwater gastropods. These gastropods, the Pachychilidae Troschel, 1857, are members of the superfamily Cerithioidea Férussac, 1819, which consists predominantly of marine snails but also comprises a number of freshwater families. Recent phylogenetic analyses using morphological as well as molecular genetic data have revealed that the cerithioidean freshwater lineages are not monophyletic as was formerly assumed. Rather, it is evident that freshwater environments have been colonized by several groups independently (Glaubrecht 1996, 1999; Lydeard et al. 2002; M. Glaubrecht, E. Strong, J. Healy, and W. Ponder, unpubl. data). Freshwater taxa also account for the vast majority of viviparous species among the Cerithioidea; in addition, recent studies revealed an unexpected variability in their reproductive and brooding morphologies (e.g., Glaubrecht 1996; Schütt and Glaubrecht 1999; Rintelen and Glaubrecht 1999; Köhler and Glaubrecht 2001, 2003; Strong and Glaubrecht 2002). Even in the absence of a robust phylogeny, this diversity indicates a multiple origin of viviparity in the freshwater Cerithioidea.

A number of morphological studies highlighted the Pachychilidae as a group unmatched by other cerithioidean families in the variety of realized reproductive strategies and correlated morphologies (Rintelen and Glaubrecht 1999, 2005; Köhler and Glaubrecht 2001, 2003, 2005; Glaubrecht and Rintelen 2003). Although some pachychilid taxa are

oviparous, others are viviparous, exhibiting reproductive morphologies that vary considerably in their complexity and general organization. This renders the Pachychilidae a promising focal group to address aspects related to the evolution of reproductive strategies, such as viviparity.

Although the distribution of the whole family covers the tropical regions of the Americas, Africa, Madagascar, and Asia, viviparous species are exclusively found in Asia. Among these species, three different brooding morphologies were found previously: for example, species of *Brotia* possess a subhaemocoelic brood pouch, which is situated in the neck region of the animal (Köhler and Glaubrecht 2001), *Tylomelania* and *Pseudopotamis* possess a uterine brood pouch formed by the pallial oviduct (Rintelen and Glaubrecht 1999, 2005; Glaubrecht and Rintelen 2003), and the Philippine *Jagora* broods within the mantle cavity (Köhler and Glaubrecht 2003). The different morphological origins of these structures strongly suggest that these brooding morphologies are not homologous.

Herein we use molecular data to explore whether the different modes of viviparity within the Pachychilidae truly are evolutionarily independent. Furthermore, we examine whether or not there is a correlation between the repeated development of viviparity and the colonization of freshwater in this family. Because all Pachychilidae including the oviparous taxa inhabit freshwater, the null hypothesis is that viviparity has evolved in species that already had colonized freshwater. Consequently, we test the postulate that the development of viviparity is not connected to the colonization of this new ecological zone.

Materials and Methods

Current Systematics of the Pachychilidae

Taxonomy and systematics of the pachychilid taxa treated herein has been subject to changes. Pachychilidae are freshwater snails with some shared morphological features of the radula and the operculum (Troschel 1857). However, many systematists of the 19th and early 20th century have ignored this taxon and treated its representatives as members of other cerithioidean freshwater families, that is, either of the Thiaridae or the Pleuroceridae (details in Köhler and Glaubrecht 2001, 2002). Recently, the original concept of the Pachychilidae has experienced a renaissance because cladistic analyses of morphological (Glaubrecht 1996, 1999; M. Glaubrecht, E. Strong, J. Healy, and W. Ponder, unpubl. data) and molecular data (Lydeard et al. 2002) suggest that pachychilids represent a monophylum independent of other cerithioidean groups, such as Thiaridae, Paludomidae, Melanopsidae, and Pleuroceridae. Based on shared features of the radula and the operculum, we currently affiliate 11 genera from tropical regions of the Americas, Africa, Madagascar, and Asia to this family. The current knowledge on their reproductive biology is summarized in Table 1.

Sample Collection

The specimens used in this study represent 36 species of Southeast Asian pachychilids assigned to the genera *Adamietta*, *Brotia*, *Jagora*, *Paracrostoma*, *Tylomelania*, and *Pseudopotamis* as well as four pachychilid species from outside Asia (*Pachychilus*, *Melanatria*). A sequence of *Faunus ater*, which currently is affiliated with the Melanopsidae (see Houbrick 1991), is included in the analyses because this species represents the putative sister taxon of the Pachychilidae (see Strong and Glaubrecht 2000; phylogeny in Lydeard et al. 2002). Three species of Thiaridae (*Melanoides tuberculata*, *Thiara amarula*, and *Tarebia granifera*) were chosen as outgroup representatives to root the trees. All sequences yielded were submitted to GenBank and four sequences were obtained from GenBank (Thiaridae and *Faunus ater*). An overview of the material used with GenBank accession numbers is given in the Appendix. All voucher material is deposited with the Museum of Natural History, Berlin (ZMB).

DNA Isolation and Sequencing

Total DNA was extracted by application of a modified version of the CTAB extraction protocol for molluscan tissues (Winnepenninckx et al. 1993). Tissue was taken from samples preserved in 75–90% ethanol. About 1 mm^3 of foot muscle was dried, cut into small pieces, and macerated in 300 μl of CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM tris-HCl pH 8.0, 0.2% β-mercaptoethanol) containing 20 μl of proteinase K. This solution was incubated for 2 h at 65°C. Extraction of total DNA was performed by applying two extraction steps with chloroform:isoamyl alcohol (24:1). Polymerase chain reaction (PCR) amplifications were conducted in 25-μl volumes containing 1x PCR buffer, 200 μM each dNTP, 2.0 mM $MgCl_2$, 0.5 μM each primer, 1.25 units of Taq polymerase (Invitek, Berlin) and approximately 50 ng of DNA. After an initial denaturation step of 3 min at 94°C, 35 cycles of 45 sec at 94°C, 45 sec at 50°C, and 60 sec at 72°C were performed, followed by a final extension step of 5 min at 72°C. A mitochondrial gene fragment of the 16S gene was amplified and sequenced. The primers used in PCR and sequencing were 16SF and H3059 (Wilson et al. 2004). Both strands of the amplified gene fragments were directly cycle sequenced in 10-μl volumes containing 2 μl of ABI Prism BigDye terminator cycle sequencing reaction mix (ABI Biosystems, Foster City, CA), 0.5 μM primer, 2 μl dd H_2O, and 4 μl DNA. Sequencing products were purified following the ABI standard protocol adjusted to 10-μl reaction volume by addition of dd H_2O. Sequences were obtained by running the sequencing products on a Perkin Elmer ABI 377 automated sequencer. The resulting sequence electropherograms of both strands were corrected manually for misreads.

Sequence Alignment

The 16S sequences were aligned online using the ClustalW algorithm (Thompson et al. 1994) available from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). The program was implemented in the multiple alignment routine using the default settings and the option ''accurate search.'' Alignments were conducted under application of different initial gap costs. These alignments of the 16S fragment were inspected for their accuracy. Sequences that could not be unambiguously aligned were omitted. Alignments can be obtained from the corresponding author.

TABLE 1. Genera of the Pachychilidae and their reproductive biology.

Genus	Distribution	Mode of reproduction	Incubatory structure	Species included in the analyses	Source
Pachychilus	Neotropics	oviparous bisexual?	none	3	Simone 2001
Doryssa	Neotropics	oviparous bisexual?	none	—	Simone 2001
Potadoma	Africa	oviparous bisexual?	none	—	none
Melanatria	Madagascar	oviparous bisexual	none	1	Grossmann 1967
Jagora	Philippines	viviparous bisexual	mantle cavity	2	Köhler and Glaubrecht 2003
Brotia	SE Asia	viviparous bisexual	subhaemocoelic brood pouch	18	Köhler and Glaubrecht 2001; Glaubrecht and Köhler 2004
Adamietta	SE Asia	viviparous bisexual	subhaemocoelic brood pouch	1	Brandt 1974
Paracrostoma	South India	viviparous bisexual	subhaemocoelic brood pouch	3	F. Köhler, unpubl. data
Sulcospira	Java	viviparous bisexual?	unknown	—	Köhler and Glaubrecht 2005
Tylomelania	Sulawesi	viviparous bisexual	brood pouch formed by the pallial oviduct	14	Rintelen and Glaubrecht 1999; 2005
Pseudopotamis	Torres Strait	viviparous bisexual	brood pouch formed by the pallial oviduct	2	Glaubrecht and Rintelen 2003

Phylogenetic Analyses

To explore the saturation of the sequences, transitions and transversions were plotted against sequence divergence using DAMBE (ver. 4.1.19; Xia and Xie 2001). Prior to phylogenetic reconstruction, we explored which model of DNA evolution best fits the sequence data. For that purpose, a hierarchical likelihood ratio test using log-likelihood scores was undertaken for testing the goodness-of-fit of nested substitution models employing MrModeltest (Nylander 2002). In the following analyses the substitution models and parameters were adjusted according to the estimates of MrModeltest. Phylogenetic trees were reconstructed using neighbor joining (NJ; Saitou and Nei 1987) and maximum parsimony (MP; e.g., Fitch 1971) using PAUP* version 4.0b10 (Swofford 1998) as well as by bayesian inference (BI, e.g., Yang and Rannala 1997) with MrBayes 3.0 (Huelsenbeck and Ronquist 2001) for each sequence dataset. Parsimony analyses were conducted under the option ''heuristic search'' with 10 random stepwise additions and tree bisection-reconnection branch swapping. Zero-length branches were collapsed and gaps were treated as a fifth base. Subsequently, bootstrap analyses (Felsenstein 1985) with 1000 replicates were performed for selected datasets under the option ''fast stepwise addition'' to evaluate the robustness of the trees. Neighbor-joining distance analyses were conducted using the random initial seed option to break ties and under a general time reversible model of sequence evolution (GTR; Rodriguez et al. 1990). Neighbor-joining bootstrap analyses with 1000 replicates were performed for each of the datasets. Posterior probabilities of phylogenetic trees were estimated by a Bayesian method of inference using a 750,000 generations metropolis-coupled Markov chain Monte Carlo (four chains, chain temperature = 0.2) as implemented by MrBayes with parameters estimated from the datasets. Sampling rate for trees was 100 generations. The Bayesian trees sampled for the last 2500 generations were used to construct a 50% majority rule consensus cladogram. The proportion of bifurcations found in these trees is given as posterior clade probabilities (bpp; Larget and Simon 1999).

RESULTS

Sequence Alignment

The 16S sequence dataset contained 97 sequences, which can only be aligned by inserting gaps into the alignment. The gap lengths depend on the estimates of gap costs, which are necessarily arbitrary (Wheeler 1995; Giribet and Wheeler 1999). For this reason, several sequence alignments of the 16S dataset were investigated yielded from the application of different initial gap penalties (1, 2, 5, 10, default = 16, 25, 50). Other multiple alignment parameters were left at default. Alignments yielded under application of ''extreme'' gap penalties (1, 2, 5, and 50) were immediately omitted from further analyses for the clear mismatch in many nucleotide positions (i.e., segments considered homologous were not aligned properly) or for exaggerated gap frequencies. Alignments yielded under gap costs of 10, 16, and 25 differ in their lengths and numbers of variable sites (Table 2). Pairwise transition rates (*s*) range from 0 to 0.17 and transversions (*v*) from 0 to 0.11 (for the alignment with default gap cost). Plotting the rates of transitions and transversions versus genetic distances (GTR) reveals nearly linear relationships (not shown), which indicates that the rates of substitution are not saturated.

TABLE 2. Length and proportion of constant, variable, and parsimony informative sites of the 16S alignments and the number and lengths of the maximum parsimony (MP) trees yielded in the analyses.

Alignment	Length (bp)	Constant sites	Variable sites (total)	Parsimony informative sites	Numbers of MP trees	Lengths of MP trees
gap = 10	924	334	590	73	10,663	3,534
gap = 16	915	326	589	78	31,297	3,599
gap = 25	903	319	584	69	3,650	2,008

The Molecular Phylogeny of the Pachychilidae

We analyzed alignments yielded under application of different initial gaps costs (10, 16, 25) to infer the relevance of varying gap lengths and frequencies for the phylogenetic reconstructions. Different gap costs have little impact on the topology of the phylogenetic reconstructions. Where such discrepancies emerge, this is outlined and discussed. MrModeltest supports the GTR model with six rate classes and a gamma-distributed rate heterogeneity parameter (GTR + Γ + I) as the best-fit model of DNA evolution. The parameters of the BI analyses were adjusted accordingly (gamma factor $\alpha = 0.7884$). In the following, the results of the different analyses are compared only with emphasis on the generic relationships. With one exception (*Adamietta* in the NJ trees) all trees generated from the different 16S alignments support the monophyly of the genera recognized by morphological characteristics. The monophyly of the various genera is further supported by high branch support values as shown in Fig. 1. Bayesian inference analyses result in almost identical topologies, as shown in Fig. 2. The MP strict consensus trees for the different alignments consistently reveal two large clades among the Pachychilidae, with clade 1 comprising *Adamietta* (*Paracrostoma* + *Brotia*) and clade 2 comprising *Pachychilus* (*Tylomelania* + *Pseudopotamis*; Fig. 1; the numbers and lengths of equally parsimonious trees found in the analyses are given in Table 2). These two clades are also consistently recovered by the NJ analyses. However, in contrast to the MP and BI trees, NJ trees show *Pachychilus* and not *Pseudopotamis* as the sister taxon of *Tylomelania* (not shown).

In respect to the position of *Faunus*, *Jagora*, and *Melanatria*, the analyses reveal conflicting results. Two MP trees support *Faunus* as the sister taxon of the Pachychilidae (Fig. 1 B–C). This is also suggested by all BI and NJ trees. However, the third MP tree suggests a basal position of this species within the family (Fig. 1A). This is not supported by bootstrap analyses, though. Two MP trees as well as the BI and NJ trees show *Melanatria* as the sister taxon of clade 2 mentioned above (Fig. 1 A–B). However, the MP tree for the alignment with a gap cost of 25 suggests a position basal to both major clades (Fig. 1C). Again, this is not recovered by the bootstrap analyses (i.e., bootstrap values below 50). Most ambiguous are the results concerning the position of *Jagora*. In each of the MP trees this genus occupies a different

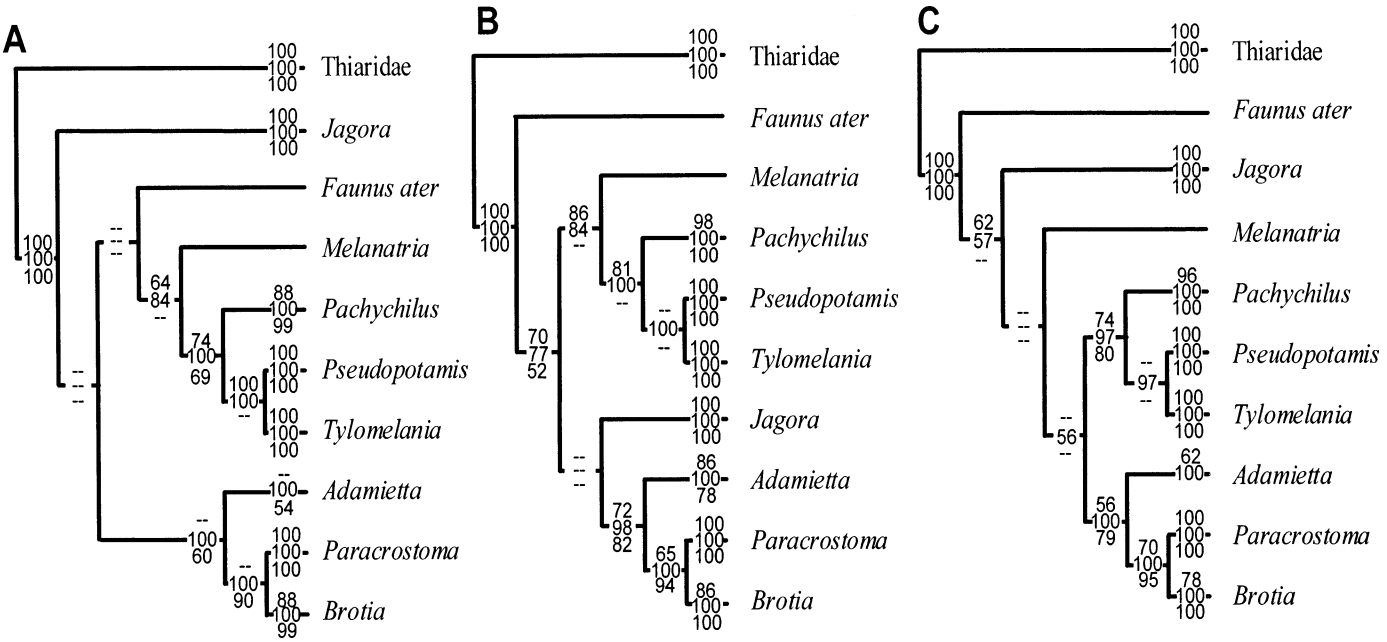

FIG. 1. Phylogenetic relationships among the pachychilid genera as revealed by maximum parsimony (MP) strict consensus cladograms. Numbers indicate support of the respective topology by MP bootstrap values (above branches), Bayesian clade probabilities (on branches), and NJ bootstrap values (below branches). Note that for clades consisting of only one species (i.e., *Melanatria*, *Faunus*) no branch support is available; dashes indicate support values ≤50. (A) MP strict consensus tree for the alignment with a gap cost of 10. (B) MP strict consensus tree for the alignment with a gap cost of 16. (C) MP strict consensus tree for the alignment with a gap cost of 25.

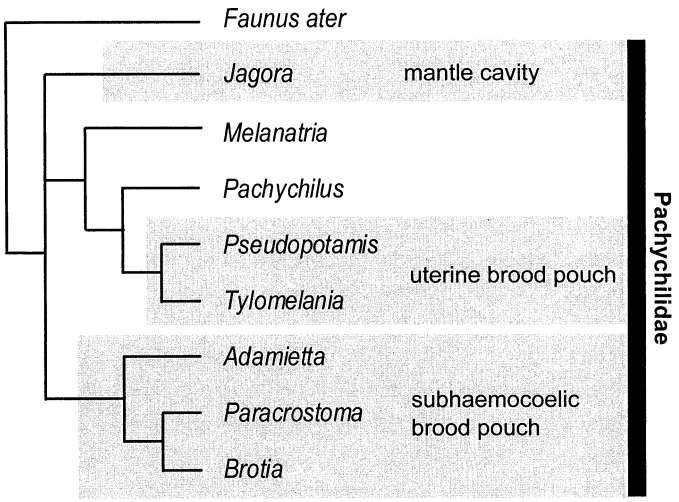

FIG. 2. Phylogenetic positions and origins of the different viviparous modes in the Pachychilidae. Viviparous taxa are shown on a gray background.

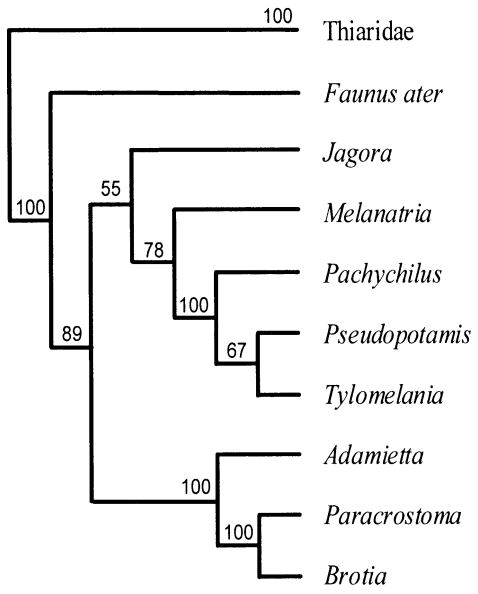

FIG. 3. Consensus cladogram of pachychilid phylogeny showing the topology that is supported by the majority of the analyses. Numbers above clades indicate the number of analyses in which this topology is found (percent of the total number of analyses conducted).

position. Two BI trees even suggest another position and show *Jagora* as the most basal branch of the family. In the BI tree for the alignment yielded under a gap penalty of 25, this relationship is shown as an unresolved and basal polytomy within the family. Neighbor-joining analyses give additional support for a basal position of *Jagora* within the Pachychilidae, also showing an unresolved polytomy between *Jagora*, clade 1, and clade 2 (not shown).

In summary, the analyses overwhelmingly support the monophyly of the Pachychilidae in regard to its putative sister taxon *Faunus ater* and deliver convincing evidence for the monophyly of all genera recognized by morphological characters. In addition, the analyses usually support a sister-group relationship of two main lineages within the family, clade 1 comprising the Asian mainland taxa *Adamietta* (*Paracrostoma* + *Brotia*) and clade 2 comprising *Melanatria* (*Pachychilus* [*Tylomelania* + *Pseudopotamis*]). This topology is observed in the majority of the trees with only a few exceptions that are not supported by bootstrap analyses. The position of *Jagora* is less clear and largely depends on the gap alignment used. In contrast to the MP trees, a position of this genus as the most basal clade within the family is suggested by the BI and NJ analyses.

As a synthesis of the different analyses we suggest a consensus cladogram as hypothesis on the phylogenetic relationships of the Pachychilidae, shown in Fig. 3. The numbers above each clade indicate the percentage of all trees (MP, NJ, BI) that support exactly the same branching pattern for each of the clades. Because the evidence for *Jagora* as the most basal clade is rather weak, in the following we show it in an unresolved basal polytomy (Fig. 2).

Much more consistent results are obtained by the various analyses for the relationships within the genera. Only minor discrepancies are observed for some single species pairs, which are not relevant for the scope of this study. For this reason, we depict only a single tree resolved to the species level (Fig. 4). This distance-based tree demonstrates that the different genera are well individualized as independent monophyla with long branches.

Among *Brotia* and *Tylomelania*, some species are observed that appear not as monophyletic lineages, as one would expect. Both are representatives of evolutionary young species flocks; that is, the *Brotia* species flock from the Kaek River in central Thailand (details in Glaubrecht and Köhler 2004) and the flock of *Tylomelania* species from the central lakes on Sulawesi (see Rintelen et al. 2005). In both cases, the mismatch observed between the mitochondrial gene tree and the expected species tree represents incomplete lineage sorting, a phenomenon that occurs when genetic distances between closely related species are minor (Page and Holmes 1998). For further discussion of this aspect see Glaubrecht and Köhler (2004) and Rintelen et al. (2005). Additionally, the clade delineated herein as *Adamietta* obviously comprises a number of species that traditionally were affiliated with *Brotia*. This hints at systematic aspects that will be revisited briefly below.

DISCUSSION

Taxonomy and Systematics of the Pachychilidae

Our data do not contradict the monophyly of the Pachychilidae with *Faunus ater* being its closest living relative. A relationship of *Faunus* and the Pachychilidae was first postulated by Strong and Glaubrecht (2000) based on morphological features and is also indicated by a molecular phylogeny of the Cerithioidea presented in Lydeard et al. (2002). In conformity with studies using morphological characters (Glaubrecht 1996, 1999; Köhler and Glaubrecht 2001, 2002), our study demonstrates that classifying pachychilid taxa under the Thiaridae (e.g. Morrison 1954; Davis 1971, 1982; Dudgeon 1982, 1989; Houbrick 1988) or Pleuroceridae

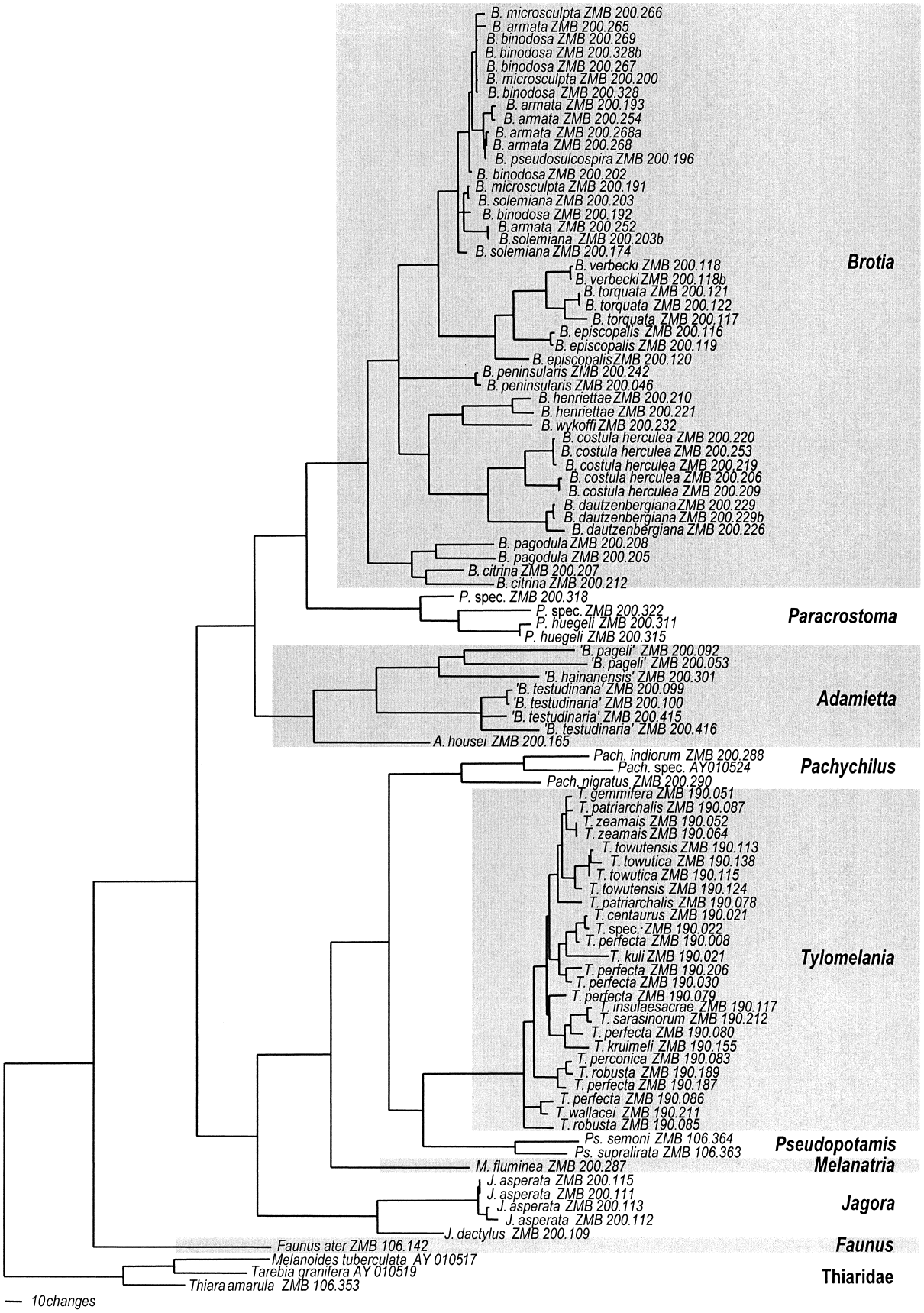

FIG. 4. Phylogenetic relationships among the Pachychilidae revealed by the analysis of the 16S sequences. Bayesian inference phylogram of the alignment with a gap cost of 25.

(Vaught 1989) is misleading. This study further helps to clarify some controversial systematic aspects especially in regard to the Southeast Asian taxa. Some major implications of the current study are summarized as follows: (1) *Brotia* as formerly circumscribed is polyphyletic and is herein restricted to those species from mainland Southeast Asia, Sumatra, and Borneo that possess a subhaemocoelic brood pouch and a wrinkled embryonic shell as described for the ''*Brotia pagodula* group'' by Köhler and Glaubrecht (2001; see Fig. 4). A formal systematic revision of this genus is needed. (2) Some species from Borneo and Java formerly assigned to *Brotia* group together with *Adamietta housei*. One morphological characteristic shared by these species, which constitute the ''*Brotia testudinaria* group'' (see Köhler and Glaubrecht 2001), is the smooth apical whorl of the juvenile. We suggest transferring those species to the genus *Adamietta*. (3) *Paracrostoma* is shown to form the sister group of *Brotia*. The systematics and taxonomy of *Paracrostoma* is controversial (summary in Köhler and Glaubrecht 2002). For the first time we provide evidence that this genus indeed represents a valid taxon; a formal systematic revision of *Paracrostoma* is pending.

The latter three genera constitute a lineage of species from Sundaland (mainland Southeast Asia including the Greater Sunda islands except for Sulawesi) and South India referred to as clade 1. They share as synapomorphy a subhaemocoelic brood pouch. In addition, two other viviparous lineages are recognized in Asia: a clade comprising *Tylomelania* endemic to Sulawesi, and *Pseudopotamis* endemic to the Torres Strait Islands. Both possess a uterine brood pouch (Glaubrecht and Rintelen 2003; Rintelen and Glaubrecht 2005). The Philippine *Jagora* broods in the mantle cavity (Köhler and Glaubrecht 2003).

Origin and Occurrence of Viviparity within the Pachychilidae

The Pachychilidae comprise viviparous as well as oviparous taxa. The presence of different viviparous strategies together with the existence of oviparous forms renders Pachychilidae the family with the largest diversity of reproductive modes among the freshwater Cerithioidea. Although viviparity is exclusively exhibited by the Asian Pachychilidae, oviparity is found in the African *Potadoma* (see Binder 1959), the Malagasy *Melanatria* (see Grossmann 1967), the Neotropical *Pachychilus* and probably also in the South American *Doryssa* (see Simone 2001; Table 1). The question therefore arises whether the reproductive mode qualifies for a synapomorphy of the viviparous Pachychilidae, and whether viviparity in the Asian taxa has a common origin. The conspicuous differences found in the morphology of the incubatory structures indicate that it does not.

The brood pouch possessed by *Adamietta*, *Paracrostoma*, and *Brotia* is a complex structure situated in the head foot and extending into the anterior visceral cavity, that is, subhaemocoel (see Köhler and Glaubrecht 2001). Although similarly complex, the incubatory structure of *Tylomelania* and *Pseudopotamis* differs completely from that of the other three. Both taxa brood in the pallial gonoduct in which fertilized eggs are retained and developing juveniles are nourished by a large albumen supply delivered with the egg capsule (Rintelen and Glaubrecht 2005). A morphologically rather simple brooding structure is found in *Jagora*, which retains egg capsules within the mantle cavity until the juveniles hatch. Nourishing structures are not found in *Jagora* and hatchlings are believed to leave the female almost immediately (Köhler and Glaubrecht 2003).

The molecular phylogeny supports the monophyly of each of the groups characterized by distinct brooding morphologies and thus points toward the homology of the respective brooding structure within each of these clades. In contrast, distinct morphological origin and strikingly different organization of the three morphologies is reliable and sufficient evidence that they are not homologous with each other. Consequently, viviparity has at least a threefold origin among the Pachychilidae; namely, in (1) *Jagora*; (2) *Tylomelania* and *Pseudopotamis*; and (3) *Brotia*, *Paracrostoma*, and *Adamietta*. The molecular phylogeny furthermore suggests that oviparity is a plesiomorphic feature within the Pachychilidae, a conclusion that is corroborated by the fact that the closest related taxon, *Faunus ater*, is also oviparous (Houbrick 1991).

Comparison with Other Freshwater Cerithioidea

Pachychilidae are not the only cerithioidean family containing viviparous species. In particular, Thiaridae are well known for their viviparity. Other viviparous taxa are known from the Paludomidae (e.g., *Tanganyicia*, *Tiphobia*, *Lavigeria*) and the Pleuroceridae (*Semisulcospira*); see Glaubrecht (1996, 1999). Subhaemocoelic brood pouches that are morphologically similar to those found in some Asian Pachychilidae are known from Thiaridae and marine Planaxidae as well (Moore 1899; Houbrick 1987; Glaubrecht 1996, 1999; Schütt and Glaubrecht 1999). Many authors assumed that these brood pouches are homologous, which has influenced systematics for almost a century (e.g. Pilsbry and Bequaert 1927; Leloup 1953; Morrison 1954; Brown and Mandahl-Barth 1987; Michel 1994; West and Michel 2000; reviewed in Glaubrecht 1999). However, recent phylogenetic data indicates that such subhaemocoelic brood pouches have developed more than once within the Cerithioidea (Lydeard et al. 2002). Only recently has it been demonstrated that the brood pouch of the paludomid *Tanganyicia rufofilosa* is mesopodial and not homologous to those in the Thiaridae, thus representing an autapomorphy of only this species (Strong and Glaubrecht 2002).

The present study demonstrates that the subhaemocoelic brood pouch of the Pachychilidae has an independent origin and thus provides additional evidence that similar brooding structures in the Planaxidae, Thiaridae, and Pachychilidae represent cases of convergence. For the Planaxidae, Houbrick (1987) has argued that their brood pouch was formed by a deep inversion of an ovipositor. We assume that the (former) presence of an egg transfer groove in the Thiaridae and Pachychilidae, which is involved in the process of egg deposition in the oviparous species, is a preadaptation for the convergent development of a subhaemocoelic brood pouch by invagination of this groove.

As stated for the subhaemocoelic brood pouch, uterine brood pouches have also evolved repeatedly among fresh-

water Cerithioidea. Such structures have a convergent origin in the Pachychilidae (*Tylomelania* and *Pseudopotamis*), Pleuroceridae (*Semisulcospira*), and Paludomidae (*Lavigeria* and *Tiphobia*). However, to brood in the mantle cavity is a feature known only in *Jagora* among Cerithioidea.

Viviparity or Ovoviviparity?

In general, viviparous modes of reproduction have been developed by originally oviparous organisms that began to retain eggs and developing embryos within their body, mostly but not exclusively in the reproductive tract. This retention necessarily is connected to the possession of appropriate morphological and physiological adaptations that otherwise are lacking in closely related oviparous species (e.g. Packard et al. 1977; Guillette 1993; Andrews and Mathies 2000). A number of definitions were suggested to differentiate among the different forms of viviparity. In a very general meaning this term indicates that an organism gives birth to more or less developed juveniles. However, more specifically some authors use this term to indicate that a direct nutrient transfer from the female to the juvenile occurs via a histotrophe (663 for gastropods: Fretter and Graham 1994), which is more specifically termed ''matrotrophic viviparity,'' and has been used for fishes by Wourms (1981), or ''eu-viviparity'' via a ''pseudo-placenta'' in Thiaridae by Glaubrecht (1996, 1999). In contrast, the simple retention of eggs without the development of nourishing structures by the female could be termed ''lecithotrophic viviparity.'' In this case, the yolk supply of the egg is the only source of embryonic nourishment. Alternative terminologies refer to ''true'' viviparity versus ovoviviparity to distinguish both modes (e.g. Tompa 1979 for pulmonates). Consequentially, lecithotrophy is also found in oviparous species.

Since morphological studies using histological methods did not reveal any nutrient transfer from the female to the developing embryo in viviparous Pachychilidae (Köhler and Glaubrecht 2001, 2003; Rintelen and Glaubrecht 2005) their mode of reproduction should be termed ovoviviparity (=lecithotrophic viviparity), irrespective of the varying structural complexity and differing parental investment involved.

Viviparity and the Colonization of Freshwater

It is striking that among cerithioideans viviparous strategies have repeatedly evolved in the freshwater lineages, for example in the Pachychilidae, the Thiaridae, some species of the Paludomidae, and in some Asian Pleuroceridae; whereas the vast majority of marine species have remained oviparous. Among marine Cerithioidea, the Planaxidae are the only family known to comprise exclusively viviparous species (Morrison 1954; Houbrick 1987; Glaubrecht 1996, 1999). It is a recurrent theme that the exploration of freshwater environments frequently is connected to shifts in life-history traits and reproductive modes (e.g., Calow 1978; Fretter 1984; Fretter and Graham 1994). In the Cerithioidea such a shift is that to viviparity. We postulate that there is an evolutionary causation explaining this phenomenon, although the mechanisms of its acquisition might not always be the same for the diversity of ecological adaptations, life-history traits, and reproductive modes involved. An analogous case is the association of brooding with small adult size in marine invertebrates. It has been shown in this respect that comparable patterns may have different causes for the varying life histories of the taxa involved (Strathmann and Strathmann 1982).

In general, freshwater caenogastropods tend to produce significantly larger eggs compared to more closely related marine taxa. This has been demonstrated for a large number of species by Fioroni and Schmekel (1976) and previously confirmed by, for example, Calow (1978), Fretter (1984), and Fretter and Graham (1994). All these authors further agree in interpreting this phenomenon as an adaptation to freshwater habitats. Accordingly, it has been stated that a free-swimming planktonic larva, present in most marine snails, would be exposed to harsher and less constant environmental conditions compared to the stable marine environment when released in freshwater, because these habitats are much smaller, more fragmented, more ephemeral, and provide less consistent food supply than the sea. In addition, these larvae were prone to dislodgment by currents in rivers and streams (Lee and Bell 1999). For this reason, telescoping all embryonic developmental stages into the egg capsule is of high adaptive significance in freshwater gastropods (Fioroni and Schmekel 1976; Calow 1978; Fioroni 1982). Fretter and Graham (1994) even argue that the acquisition of a direct development functions as a preadaptation for the colonization of freshwater.

In species with direct development, the growth of the encapsulated embryo needs to be sustained by nutrients provided by the female. In most cases this is achieved by supplying an adequate amount of yolk or albumen, which necessitates the production of enlarged egg capsules. Because the maternal capabilities to supply such nutrients are limited, the increased parental investment is strictly correlated to a decrease in the number of produced offspring (Calow 1978). This scenario holds true for most caenogastropods; alternative strategies are found in some pulmonates, however (see Aldridge 1983).

This evolutionary trend toward producing large but relatively few eggs is also observed in the Pachychilidae. Their eggs are considerably larger compared to a wide range of marine cerithioideans, which produce several thousand eggs per clutch that do not exceed 0.1 mm in diameter (e.g. Fioroni and Schmekel 1976; Calow 1978; Fretter and Graham 1994). Admittedly, available data on egg sizes and numbers of oviparous pachychilids is scarce. However, it is known that eggs of *Melanatria* are three times larger in diameter (or 25 times by their volume) than those of marine cerithioidean species. Viviparous Pachychilidae produce egg capsules a hundred times larger than the volume of marine eggs (Table 3). The number of eggs is considerably lower in the oviparous, and even more so in the viviparous, confamilial species (see Table 3). This trend continues to varying degrees in the size and number of hatchlings released from the incubatory structures of the viviparous species, which confirms the presence of slightly altered reproductive strategies among the viviparous taxa (see Dudgeon 1982, 1989; Köhler and Glaubrecht 2001, 2003).

We hypothesize that the tendency toward larger and fewer eggs that was initiated by the acquisition of direct devel-

TABLE 3. Number and size of progeny produced by pachychilids. Dash indicates data not available.

Genus	Number of species examined	Number of progeny	Size of eggs (mm diameter	Height of juvenile shells	Source
Melanatria	1	~3000	0.25–0.3	—	Grossmann 1967
Adamietta	6	≤1000	0.5–0.9	0.8–2.5 mm	Dudgeon 1982, 1989; F. Köhler, unpubl. data
Jagora	2	≤300	~1.1	1.0–2.5 mm	Köhler and Glaubrecht 2003
Brotia	10	≤150	~1.0	1.0–6.0 mm	F. Köhler, unpubl. data
Paracrostoma	1	≤30	~1.0	4.0–7.0 mm	F. Köhler, unpubl. data
Tylomelania	43	≤39	—	2.0–17.5 mm	Rintelen and Glaubrecht 2005
Pseudopotamis	2	≤6	—	~3 mm	Glaubrecht and Rintelen 2003

opment is the driving factor that ultimately lead to the repeated evolution of viviparity in the Pachychilidae. For the increased parental investment associated with increasing size, each single egg or juvenile becomes much more valuable for the female, and a loss of the brood is more detrimental. The retention of eggs and developing juveniles in the body of the female may therefore help to reduce the risk of predation and increase the fitness of the female.

To what extent is this evolutionary process correlated to the colonization of freshwater in the Pachychilidae? Based on the phylogeny presented here, it is most parsimonious to presume that oviparity is the ancestral state within the family. The closest living relative of the Pachychilidae, *Faunus ater*, is marine and produces eggs from which planktonic larvae hatch (Houbrick 1991). Hence, as is predicted from the general scheme outlined by Fioroni and Schmekel (1976) and Calow (1978), the evolution of direct development by the ancestor of Recent Pachychilidae was strictly correlated to the exploration of the limnetic milieu; in contrast, it was not necessary to become viviparous to successfully populate freshwater. To understand exactly why viviparity was a favorable step further for freshwater gastropods, we require more comparative data on the ecology of both viviparous and oviparous species. The fact that viviparity has evolved solely and repeatedly in the Asian representatives of the Pachychilidae indicates that yet unknown ecological factors might have favored viviparity. Such factors might include the presence of predators, parasites, or competitors that are present in Asia but absent from other parts of the world. Because Asia harbors the highest diversity of viviparous molluscs worldwide, uncovering these factors might help to explain the intriguing accumulation of viviparous molluscs in Asian freshwater biotopes.

Conclusions

Freshwater environments have triggered the repeated evolution of direct development with large encapsulated eggs in a number of molluscan taxa. For the increased parental investment involved in this process it is highly beneficial to protect the progeny by developing appropriate structures to retain eggs and juveniles within the body of the female. This favors evolution of a viviparous mode of reproduction.

Viviparity in pachychilids seems useful only when eggs have large reserves because the female apparently does not have the ability to provide resources other than yolk or albumen to the developing offspring. In this respect, large egg sizes can be considered a preadaptation to the evolution of viviparity in those limnetic ancestors from which the viviparous pachychilid lineages in Southeast Asia evolved. Hence, there is only an indirect correlation between the colonization of freshwater and the evolution of viviparity in the Pachychilidae. Additional, still unidentified factors may also play a role in the evolution of brooding, such as the presence of certain predators, for example. We therefore consider it important to conduct further comparative ecological research on the biology of viviparous and oviparous Pachychilidae, and also to widen the perspective toward other freshwater Cerithioidea, to illuminate the mechanisms and consequences of the evolution of different reproductive traits in concert with the colonization of freshwater.

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APPENDIX
Taxa examined in this study with GenBank accession and inventory numbers.

Genus	Species	Inventory no.	Origin	16S
Melanoides	*M. tuberculata* (Müller, 1774)	FLMNH	USA, Florida	AY 010517
Tarebia	*T. granifera* (Lamarck, 1822)	FLMNH	USA, Florida	AY 010519
Thiara	*T. amarula* (Linné, 1758)	ZMB 106.353	Australia, Queensland	AY 010520
Faunus	*F. ater* (Linné, 1758)	ZMB 106.142	Indonesia, Sulawesi	AY 010526
Melanatria	*M. fluminea* (Gmelin, 1791)	ZMB 200.287	Madagascar	AY 311946
Pachychilus	*P. indiorum* (Morelet, 1849)	ZMB 200.288	Mexico, Palenque	AY 311948
	P. sp.	ZMB 200.290	Mexico	AY 311947
	P. sp.	FLMNH	Mexico	AY 010524
Adamietta	*A. housei* (I. Lea, 1856)	ZMB 200.165	Thailand	AY 330774
Brotia	*B. armata* (Brandt, 1968)	ZMB 200.193	Thailand	AY 330810
		ZMB 200.252	Thailand	AY 330809
		ZMB 200.254	Thailand	AY 330808
		ZMB 200.265	Thailand	AY 330806
		ZMB 200.268-1	Thailand	AY 330807
		ZMB 200.268-2	Thailand	AY 330811
	B. binodosa (Blanford, 1903)	ZMB 200.192	Thailand	AY 330815
		ZMB 200.202	Thailand	AY 330819
		ZMB 200.267	Thailand	AY 330818
		ZMB 200.269	Thailand	AY 330820
		ZMB 200.328	Thailand	AY 330816
	B. citrina (Brot, 1868)	ZMB 200.207	Thailand	AY 330798
		ZMB 200.212	Thailand	AY 330799
	B. costula herculea	ZMB 200.206	Thailand	AY 330787
		ZMB 200.209	Thailand	AY 330789
		ZMB 200.219	Thailand	AY 330790
		ZMB 200.220	Thailand	AY 242971
		ZMB 200.253	Thailand	AY 330788
	B. dautzenbergiana (Morlet, 1884)	ZMB 200.213	Thailand	AY 533176
		ZMB 200.226	Thailand	AY 330802
		ZMB 200.229	Thailand	AY 330800
	B. episcopalis (H. Lea and I. Lea, 1850)	ZMB 200.116	Sumatra	AY 330784
		ZMB 200.119	Sumatra	AY 330785
		ZMB 200.120	Sumatra	AY 3307876
	B. hainanensis (Brot, 1872)	ZMB 200.301	Hong Kong	AY 330778
	B. henriettae (Gray, 1824)	ZMB 200.210	Thailand	AY 330793
		ZMB 200.221	Thailand	AY 330794

APPENDIX Continued.

Genus	Species	Inventory no.	Origin	16S
	B. microsculpta Brandt, 1968	ZMB 200.191	Thailand	AY 330805
		ZMB 200.200	Thailand	AY 330804
		ZMB 200.266	Thailand	AY 330803
	B. pageli (Thiele, 1908)	ZMB 200.092	Malaysia, Sabah	AY 242952
		ZMB 200.053	Indoensia, Borneo	AH 012869
	B. pagodula (Gould, 1847)	ZMB 200.205	Thailand	AY 330795
		ZMB 200.208	Thailand	AY 172443
	B. peninsularis (Brandt, 1974)	ZMB 200.242	Thailand	AY 330791
		ZMB 200.046	Thailand	AY 330792
	B. pseudosulcospira (Brandt, 1968)	ZMB 200.196	Thailand	AY 330797
	B. solemiana (Brandt, 1968)	ZMB 200.174	Thailand	AY 330814
	B. testudinaria (von dem Busch, 1842)	ZMB 190.415	Java	AY 330777
		ZMB 190.416	Java	AY 330776
		ZMB 200.099	Java	AY 330775
		ZMB 200.100	Java	AY 242949
	B. solemiana (Brandt, 1968)	ZMB 200.174	Thailand	AY 330812
		ZMB 200.203		AY 330813
	B. torquata (von dem Busch, 1842)	ZMB 200.117	Sumatra	AY 330781
		ZMB 200.121	Sumatra	AY 330782
		ZMB 200.122	Sumatra	AY 330783
	B. verbecki (Brot, 1886)	ZMB 200.118-1	Sumatra	AY 330779
	B. wykoffi (Brandt, 1974)	ZMB 200.232	Thailand	AY 330796
Jagora	*J. asperata* (Lamarck, 1822)	ZMB 200.111	Philippines, Luzon	AY 172439
		ZMB 200.212	Philippines, Luzon	AY 172440
		ZMB 200.213	Philippines, Luzon	AY 172441
		ZMB 200.215	Philippines, Luzon	AY 172442
	J. dactylus (H. Lea and I. Lea, 1850)	ZMB 200.109	Philippines, Cebu	AY 172438
Paracrostoma	*P. huegeli* (Philippi, 1853)	ZMB 200.311	India, Karnataka	AY 330771
		ZMB 200.315	India, Karnataka	AY 330772
	P. sp.	ZMB 200.322	India, Karnataka	AY 330773
	P. sp.	ZMB 200.318	India, Karnataka	AY 330770
Pseudopotamis	*P. semoni* (von Martens, 1894)	ZMB 190.364	Torres Straits Islands	AY 242968
	P. supralirata (E.A. Smith, 1887)	ZMB 190.363	Torres Straits Islands	AY 242970
Tylomelania	*T. centaurus* (P. and F. Sarasin, 1898)	ZMB 190.012	Sulawesi	AY 311830
		ZMB 190.022	Sulawesi	AY 311832
	T. gemmifera (P and F. Sarasin, 1897)	ZMB 190.051	Sulawesi	AY 242954
	T. insulaesacrae (P. and F. Sarasin, 1897)	ZMB 190.116	Sulawesi	AY 311924
	T. kruimeli (Rintelen and Glaubrecht, 2003)	ZMB 190.155	Sulawesi	AY 311852
	T. kuli (P. and F. Sarasin, 1898)	ZMB 190.011	Sulawesi	AY 311852
	T. patriarchalis (P. and F. Sarasin, 1897)	ZMB 190.078	Sulawesi	AY 311867
	T. perconica (P. and F. Sarasin, 1898)	ZMB 190.083	Sulawesi	AY 311884
	T. perfecta (Mousson, 1849)	ZMB 190.008	Sulawesi	AY 311892
		ZMB 190.030	Sulawesi	AY 311895
		ZMB 190.079	Sulawesi	AY 311889
		ZMB 190.080	Sulawesi	AY 311890
		ZMB 190.187	Sulawesi	AY 311885
		ZMB 190.206	Sulawesi	AY 311887
	T. sarasinorum (Kruimel, 1913)	ZMB 190.212	Sulawesi	AY 311906
	T. towutensis (P. and F. Sarasin, 1897)	ZMB 190.113	Sulawesi	AY 311914
	T. towutica (Kruimel, 1913)	ZMB 190.214	Sulawesi	AY 311918
		ZMB 190.116	Sulawesi	AY 311924
	T. wallacei (Reeve, 1860)	ZMB 190.135	Sulawesi	AY 311926
		ZMB 190.211	Sulawesi	AY 311903
	T. zeamais (P. and F. Sarasin, 1897)	ZMB 190.052	Sulawesi	AY 311939
		ZMB 190.064	Sulawesi	AY 311932