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# Phylogenetic reconstruction and shell evolution of the Diplommatinidae (Gastropoda: Caenogastropoda)

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ABSTRACT

The fascinating and often unlikely shell shapes in the terrestrial micromollusc family Diplommatinidae (Gastropoda: Caenogastropoda) provide a particularly attractive set of multiple morphological traits to investigate evolutionary patterns of shape variation. Here, a molecular phylogenetic reconstruction, based on five genes and 2700 bp, was undertaken for this family, integrated with ancestral state reconstruction and phylogenetic PCA of discrete and quantitative traits, respectively. We found strong support for the Diplommatininae as a monophyletic group, separating the Cochlostomatidae into a separate family. Five main clades appear within the Diplommatininae, corresponding with both coiling direction and biogeographic patterns. A Belau clade (A) with highly diverse (but always sinistral) morphology comprised *Hungerfordia*, *Palaina*, and some *Diplommatina*. *Arinia* (dextral) and *Opisthostoma* (sinistroid) are sister groups in clade B. Clade C and D solely contain sinistral *Diplommatina* that are robust and little ornamented (clade C) or slender and sculptured (clade D). Clade E is dextral but biogeographically diverse with species from all sampled regions save the Caroline Islands. *Adelopoma*, *Diplommatina*, *Palaina*, and *Hungerfordia* require revision to allow taxonomy to reflect phylogeny, whereas *Opisthostoma* is clearly monophyletic. Ancestral state reconstruction suggests a sinistral origin for the Diplommatinidae, with three reversals to dextrality.

## 1. Introduction

Attempting to understand the origin of morphological diversity in a strongly radiated group of animals lies at the core of zoosystematics (Glaubrecht, 2010). While experimental studies and the exploration of phylogeographic patterns at the species level may provide insight into the evolutionary processes responsible (Rundell and Price, 2009), a reconstruction of the evolutionary trajectories followed to reach the present (and past) filling of morphospace requires a phylogenetic framework (Harvey et al., 1996). By mapping morphological characters onto a molecular phylogeny, we can test for variation in rates of diversification (Ricklefs, 2007), examine the homology of the characters of interest, and reveal morphological conservatism and homoplasy (Brown et al., 2000). Especially when applied to a group in which morphological diversity can be captured by a shared set of traits, and in which species-level studies exist that provide insight into the selective pressures at work, such an approach can be very valuable for a deeper understanding of evolutionary radiations and in linking micro-evolutionary processes with macro-evolutionary patterns. We here apply such an approach to the terrestrial microsnail family Diplommatinidae.

Like in all mollusks, the diplommatinid shell is basically a coiled tube, the shape of which is defined by a small set of parameters (Vermeij, 1993). However, unlike most other gastropod families, diplommatinids have additional morphological complexity in the presence of multiple coiling axes (Clements et al., 2008; Gittenberger, 1995; Vermeulen and Clements, 2008), and elaborate ornamentation (Fig. 1). One interesting characteristic of the Diplommatinidae is its chiral diversity. Not only is a large proportion of its species unusual among gastropods for being coiled counter-clockwise (sinistrally), but diplommatinids also have a third, unique chiral form, termed sinistroid, found in the genus *Opisthosoma*, where the coiling direction reverses during growth, forming a sinistral tuba on a dextral shell (Clements et al., 2008; Gittenberger, 1995; Vermeulen and Clements, 2008). Sinistrality itself is quite rare in gastropods, especially in non-pulmonates, as the vast majority of groups are exclusively dextral (Vermeij, 1975).

Diplommatininae are highly endemic, and many species are restricted to small areas, often only a single limestone hill, making species vulnerable to extinction (Vermeulen, 1993). A comparative

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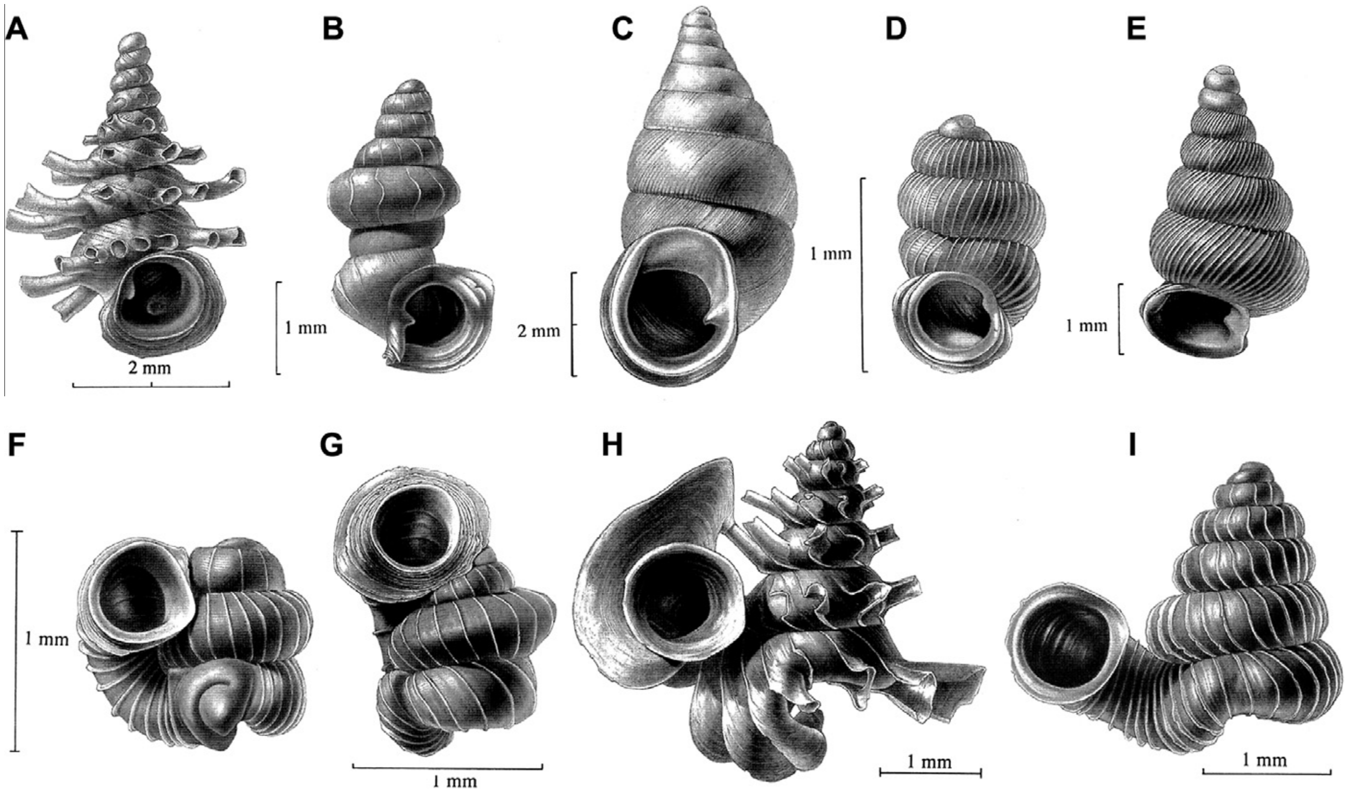

**Fig. 1.** Shell diversity in two genera of diplommatinids, *Diplommatina* (A–E) and *Opisthostoma* (F–I). A. *Diplommatina miraculumdei*. B. *Diplommatina everetti*. C. *Diplommatina rubicunda*. D. *Diplommatina whiteheadi*. E. *Diplommatina isseli*. F. *Opisthostoma sulcatum*. G. *Opisthostoma crassicolle*. H. *Opisthostoma mirabile*. I. *Opisthostoma obliquedentatum*. Figures adapted from Vermeulen (1993, 1994).

analysis of morphological traits mapped on a molecular phylogeny can also resolve standing taxonomic issues. The currently recognized subfamilies Diplommatininae and Cochlostomatinae consist of ten Central Indo-Pacific genera with hundreds of species, and a single European genus, respectively (Beesley et al., 1998; Bouchet et al., 2005; Vermeulen, 1993). The subfamilies have been considered separate families on occasion (Fehér, 2004), and the taxonomic structure within the Diplommatininae has been hampered by convergence in shell shape (Rundell, 2008; Solem, 1959; Tillier, 1981; Vermeulen, 1994). Rundell (2008) produced a molecular phylogeny for the three genera found on Belau, viz. *Diplommatina, Hungerfordia,* and *Palaina*. Her results showed that these are each non-monophyletic, and that the radiation does not follow a simple progression pattern relating to island age.

Studies aimed at understanding the shell diversity in this group have so far mostly focused on the morphologically exceptional genus *Opisthostoma*. Although Schilthuizen (2003) hypothesized that shell evolution in this group may be driven by sexual selection, there is better evidence that evolutionary arms races with predators play an important role. Schilthuizen et al. (2006) found correlations between the location of molluscivorous slug predation marks and the morphological variation between different *O. concinnum* populations, implying adaptive causes for the modifications in shell shape. Habitat has also been shown to affect shell morphology. Tillier (1981) found a correlation between height/width ratio and precipitation, with more elongate *Palaina* shells being found in drier areas. These studies suggest several distinct adaptive responses in multivariate shell morphospace for this group.

Here, we produce a molecular phylogenetic reconstruction of the Diplommatinidae and investigate evolutionary patterns in shell morphology. We demonstrate that the current genera require revision, that sinistrality is the ancestral state, and that the species can be clustered into clades based on their chirality, shell morphology, and biogeography.

## 2. Material and methods

### 2.1. Taxon sampling

DNA sequences from 71 specimens were used in this study (Table 1), including 54 Diplommatinidae from seven genera. A further 17 outgroup samples were used: three non-cyclophoroid caenogastropods and members of four other cyclophoroid families. Sequenced samples were collected between 1997–2010 and preserved in absolute ethanol, either by the authors or colleagues. Most species were sampled only once, as this study was aimed at family level diversity. Species that are represented more than once have been sampled from different populations (*Diplommatina hidagai, Diplommatina electa*, and *Diplommatina rubra*). Although in most cases, the entire snail was used for DNA extraction, other specimens from the same sample are available as vouchers.

### 2.2. DNA extraction and sequencing

The E.Z.N.A. Mollusc DNA kit (OMEGA bio-tek) was used to extract DNA from whole snails, shell included. For a few of the larger outgroup species, only a portion of the preserved animal was used.

Five genes were chosen for amplification, with varying expected levels of conservation, and are commonly used in gastropod family level phylogenies (Colgan et al., 2007; Jorgensen et al., 2008; Sengupta et al., 2009). By using genes from a variety of sources, we help ensure that we are not simply analyzing the evolution of a single gene, but of the species themselves. These are three ribosomal genes, 16S (mitochondrial), 18S (nuclear), and 28S (nuclear),

**Table 1**
Taxon Sampling. BB, Bram Breure; FMNH, The Field Museum and Rebecca Rundell; GB, Gary Barker; MS, Menno Schilthuizen; RMNH, Rijksmuseum van Natuurlijke Historie; PD, Pongrat Dumrongrojwattatna; TL, Thor-Seng Liew. Samples without voucher codes represent sequences that were obtained, rather than sequenced by the authors.

| Classification | Species | Voucher codes | | Locality | Source | Genbank accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | RMNH | FMNH | | | 16S | 18S | 28S | COI | H3 |
| Caenogastropoda | | | | | | | | | | |
| Ampullarioidea | | | | | | | | | | |
| Ampullariidae | *Pomacea insularum* (D'Orbigny, 1839) | | | | Genbank | FJ710229.1 | FJ710267.1 | | FJ946828.1 | FJ710375.1 |
| Conoidea | | | | | | | | | | |
| Conidae | *Conus miles* (L. 1758) | | | | Genbank | FJ868145.1 | DQ916538.1 | | AY588202.1 | AF033684.2 |
| | *Conus miliaris* (Hwass in Bruguière, 1792) | | | | Genbank | AF174181.1 | | | FJ411486.1 | |
| Cyclophoroidea | | | | | | | | | | |
| Cyclophoridae | *Alycaeus cf. kelantanensis* Sykes 1902 | MOL.119765 | | Malaysia, Perak | MS | HM753481 | HM753427 | HM753371 | | HM753273 |
| | *Alycaeus jagori* (von Martens, 1859) | MOL.119766 | | Malaysia, Kelantan | MS | HM753482 | HM753428 | HM753372 | HM753330 | |
| | *Alycaeus perakensis* (Crosse, 1879) | MOL.119767 | | Malaysia, Perak | MS | HM753483 | HM753429 | HM753373 | | HM753274 |
| | *Chamalycaeus everetti* (Godwin et Austin, 1889) | MOL.119764 | | Malaysia, Borneo, Sabah | MS | HM753480 | HM753426 | HM753370 | HM753329 | HM753272 |
| | *Cyclophorus latus* (Kuroda, 1941) | MOL.119768 | | Taiwan | GB | HM753484 | HM753430 | HM753374 | HM753331 | HM753275 |
| | *Cyclotus taivanus* (Adams, 1870) | MOL.119769 | | Taiwan | GB | HM753485 | HM753431 | HM753375 | | HM753276 |
| | *Japonia spec.* | MOL.119770 | | Malaysia, Borneo, Sabah | MS | HM753486 | HM753432 | HM753376 | HM753332 | HM753277 |
| | *Leptopoma pellucida* (Grateloup, 1840) | MOL.119771 | | Australia, QLD | GB | HM753487 | HM753433 | | | HM753278 |
| | *Opisthoporus birostris* (Pfeiffer, 1854) | MOL.119772 | | Malaysia, Borneo, Sabah | MS | HM753488 | HM753434 | HM753377 | HM753333 | HM753279 |
| Diplommatinidae | | | | | | | | | | |
| Cochlostomatinae | *Cochlostoma elegans* (Clessin, 1879) | MOL.119773 | | Croatia, Velebit | GB | HM753489 | HM753435 | HM753378 | HM753334 | HM753280 |
| | *Cochlostoma roseoli* (Wagner, 1901) | MOL.119774 | | Hungary, Mont | GB | HM753490 | HM753436 | HM753379 | | HM753281 |
| | *Cochlostoma septemspirale* (Wagner, 1897) | MOL.119825 | | Switzerland | MS | HM753497 | HM753423 | HM753367 | HM753326 | HM753269 |
| Diplommatininae | *Adelopoma tucma* (Döring, 1884) | MOL.119828 | | Argentina, Tucuman | BB | HM753534 | HM753450 | HM753393 | HM753341 | |
| | *Arinia paricostata* (Vermeulen, 1996) | MOL.119779 | | Malaysia, Borneo, Sabah | MS | HM753500 | HM753441 | HM753384 | | HM753284 |
| | *Diplommatina acme* (Laidlaw, 1949) | MOL.119781 | | Malaysia, Perlis | MS | | HM753443 | HM753386 | | HM753286 |
| | *Diplommatina calvula* (Vermeulen, 1993) | MOL.119784 | | Malaysia, Borneo, Sabah | MS | HM753502 | HM753444 | HM753387 | | HM753287 |
| | *Diplommatina canaliculata* (Möllendorff, 1886) | MOL.119783 | | Malaysia, Pahang | MS | HM753504 | HM753445 | HM753388 | HM753338 | HM753288 |
| | *Diplommatina centralis* Vermeulen, 1993 | MOL.119785 | | Malaysia, Borneo, Sabah | MS | HM753505 | HM753446 | HM753389 | HM753339 | HM753289 |
| | *Diplommatina cf. lenggongensis* (Tomlin, 1941) | MOL.119786 | | Malaysia, Perak | MS | HM753506 | HM753447 | HM753390 | | HM753290 |
| | *Diplommatina demorgani* (Laidlaw, 1949) | MOL.119787 | | Malaysia, Pahang | MS | HM753507 | HM753448 | HM753391 | HM753340 | HM753291 |
| | *Diplommatina diminuta* (Möllendorff, 1891) | MOL.119788 | | Malaysia, Perak | MS | HM753508 | HM753449 | HM753392 | | HM753292 |
| | *Diplommatina electa* A (Fulton, 1905) | MOL.119815 | | Malaysia, Borneo, Sabah | TL | HM753503 | HM753473 | HM753413 | HM753359 | HM753316 |
| | *Diplommatina electa* B (Fulton, 1905) | MOL.119816 | | Malaysia, Borneo, Sabah | TL | HM753517 | HM753474 | HM753414 | HM753360 | HM753317 |
| | *Diplommatina electa* D (Fulton, 1905) | MOL.119820 | | Malaysia, Borneo, Sabah | TL | HM753521 | HM753478 | HM753418 | | HM753321 |
| | *Diplommatina gomantongensis* (Smith, 1894) | MOL.119800 | | Malaysia, Borneo, Sabah | MS | HM753509 | HM753451 | HM753394 | HM753342 | HM753294 |
| | *Diplommatina hidagai* A (Panha, 1997) | MOL.119791 | | Thailand, Trang | PD | HM753498 | HM753424 | HM753368 | HM753327 | HM753270 |
| | *Diplommatina hidagai* B (Panha, 1997) | MOL.119826 | | Thailand, Trang | PD | HM753510 | HM753452 | HM753395 | HM753343 | HM753295 |
| | *Diplommatina isseli* (Godwin Austen, 1889) | MOL.119792 | | Malaysia, Borneo, Sabah | MS | HM753511 | HM753453 | HM753396 | | HM753296 |
| | *Diplommatina laidlawi* (Sykes, 1903) | MOL.119821 | | Malaysia, Kelantan | TL | HM753522 | | HM753419 | HM753364 | HM753322 |
| | *Diplommatina naiyanetri* (Panha, 1997) | MOL.119794 | | Thailand, Trang | PD | HM753512 | HM753454 | HM753397 | HM753344 | HM753297 |
| | *Diplommatina plecta* (Fulton, 1901) | MOL.119818 | | Malaysia, Borneo, Sabah | TL | HM753519 | HM753476 | HM753416 | HM753362 | HM753319 |
| | *Diplommatina prava* (Pilsbry and Hirase, 1905) | MOL.119796 | | Taiwan | GB | HM753513 | HM753455 | HM753398 | HM753345 | HM753298 |
| | *Diplommatina ringens* | | | | Genbank | EU742031 | | EU742072 | EU742112 | |
| | *Diplommatina rubicunda* (Von Martens, 1864) | MOL.119819 | | Malaysia, Borneo, Sabah | TL | HM753520 | HM753477 | HM753417 | HM753363 | HM753320 |
| | *Diplommatina rubra* A (Godwin Austen, 1889) | MOL.119797 | | Malaysia, Borneo, Sabah | MS | HM753514 | HM753456 | HM753399 | HM753346 | HM753299 |
| | *Diplommatina rubra* B (Godwin Austen, 1889) | MOL.119814 | | Malaysia, Borneo, Sabah | TL | HM753516 | HM753472 | HM753412 | HM753358 | HM753315 |
| | *Diplommatina sp. nov. AG* | MOL.119798 | 310833 | Belau, Peleliu | FMNH | HM753524 | HM753457 | HM753400 | HM753347 | HM753300 |
| | *Diplommatina sp. nov. AK* | MOL.119799 | 310775 | Belau, Airai | FMNH | HM753525 | HM753458 | HM753401 | HM753348 | HM753301 |
| | *Diplommatina sp. nov. K* | MOL.119817 | | Malaysia, Borneo, Sabah | TL | HM753518 | HM753475 | HM753415 | HM753361 | HM753318 |
| | *Diplommatina sp. nov. M* | MOL.119813 | | Malaysia, Borneo, Sabah | TL | HM753515 | HM753471 | HM753411 | HM753357 | HM753314 |
| | *Diplommatina sp. nov. V*$^{***}$ | MOL.119780 | | Malaysia, Borneo, Sabah | MS | HM753501 | HM753442 | HM753385 | HM753337 | HM753285 |
| | *Diplommatina sp. nov. Z* | MOL.119822 | | | TL | HM753523 | HM753479 | HM753420 | HM753365 | HM753323 |
| | *Diplommatina superba brevior* (Laidlaw, 1949) | MOL.119790 | | Malaysia, Perak | MS | | HM753459 | HM753402 | HM753349 | HM753302 |
| | *Diplommatina suratensis* (Panha and Burch, 1996) | MOL.119827 | | Thailand, Krabi | PD | HM753499 | HM753425 | HM753369 | HM753328 | HM753271 |

(*continued on next page*)

**Table 1** (*continued*)

| Classification | Species | Voucher codes | | Locality | Source | Genbank accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | RMNH | FMNH | | | 16S | 18S | 28S | COI | H3 |
| | *Diplommatina sykesi* (Fulton, 1901) | MOL.119801 | | Malaysia, Borneo, Sabah | MS | | HM753460 | HM753403 | HM753350 | HM753303 |
| | *Diplommatina ventriculus* (Möllendorff, 1891) | MOL.119823 | | Malaysia, Perak | MS | HM753495 | HM753421 | | HM753324 | HM753267 |
| | *Diplommatina whiteheadi* (Smith, 1898) | MOL.119802 | | Malaysia, Borneo, Sabah | MS | | HM753461 | | | HM753304 |
| | *Hunderfordia sp. nov. A* | MOL.119804 | 310854 | Belau | FMNH | HM753527 | HM753463 | HM753405 | HM753352 | HM753306 |
| | *Hungerfordia sp. nov. J* | MOL.119803 | 310918 | Belau | FMNH | HM753526 | HM753462 | HM753404 | HM753351 | HM753305 |
| | *Hungerfordia sp. nov. T* | | | | Genbank | EU742038 | | EU742079 | EU742119 | |
| | *Opisthostoma fraternum* (Smith, 1905) | | | Malaysia, Borneo, Sabah | Genbank | DQ235746 | DQ235709 | | | |
| | *Opisthostoma cf. sinyumensis* (Maasen, 2001) | | | Malaysia, Pahang | LT | | | | JF342448 | |
| | *Opisthostoma concinnum* (Fulton, 1901) | MOL.119805 | | Malaysia, Borneo, Sabah | MS | HM753528 | HM753465 | | | HM753308 |
| | *Opisthostoma fraternum* (Smith, 1905 | MOL.119824 | | Malaysia, Borneo, Sabah | MS | | HM753464 | | | HM753307 |
| | *Opisthostoma jucundum* (Smith, 1893) | MOL.119806 | | Malaysia, Borneo, Sabah | MS | HM753496 | HM753422 | HM753366 | HM753325 | HM753268 |
| | *Opisthostoma lissopleuron* (Vermeulen, 1994) | | | Malaysia, Borneo, Sabah | LT | | | | JF342449 | |
| | *Opisthostoma mirabile* (Smith, 1893) | MOL.119807 | | Malaysia, Borneo, Sabah | MS | HM753529 | HM753466 | HM753406 | HM753353 | HM753309 |
| | *Opisthostoma obliquedentatum* (Vermeulen, 1994) | MOL.119808 | | Malaysia, Borneo, Sabah | MS | HM753530 | HM753467 | HM753407 | | HM753310 |
| | *Opisthostoma simplex* (Fulton, 1901) | MOL.119809 | | Malaysia, Borneo, Sabah | MS | | HM753468 | HM753408 | | HM753311 |
| | *Palaina albata* (Beddome, 1889) | MOL.119810 | 310836 | Belau, Peleliu | FMNH | HM753531 | HM753469 | HM753409 | HM753354 | HM753312 |
| | *Palaina doliolum* (Möllendorff, 1897) | | | | Genbank | EU742006 | | EU747047 | EU742087 | |
| | *Palaina moussoni* (Crosse, 1866) | MOL.119811 | 310757 | Belau, Kayangel | FMNH | HM753532 | | | HM753355 | |
| | *Palaina striolata* (Crosse, 1866) | MOL.119812 | 310634 | Belau, Ngerekebesang | FMNH | HM753533 | HM753470 | HM753410 | HM753356 | HM753313 |
| Megalostomatidae | *Acroptychia bathiei* (Fischer-Piette and Bedoucha, 1965) | MOL.119775 | | Madagascar, Ambonarabe | GB | HM753491 | HM753437 | HM753380 | HM753335 | |
| | *Acroptychia milloti* (Fischer-Piette and Bedoucha, 1965) | MOL.119776 | | Madagascar, Kelifely | GB | HM753492 | HM753438 | HM753381 | HM753336 | |
| Neocyclotidae | *Aperostoma palmeri* (Bartsch and Morrison, 1942) | | | | Genbank | DQ093479.1 | DQ093435.1 | DQ279983.1 | DQ093523.1 | DQ093505.1 |
| Pupinidae | *Pupina hosei* (Godwin Austen, 1889) | MOL.119777 | | Malaysia, Borneo, Sabah | MS | HM753493 | HM753439 | HM753382 | | HM753282 |
| | *Pupinella swinhoei* (Adams, 1866) | MOL.119778 | | Taiwan | GB | HM753494 | HM753440 | HM753383 | | HM753283 |

as well as two protein coding genes, Cytochrome c oxidase I (COI; mitochondrial) and Histone 3 (H3; nuclear). We attempted to amplify all genes in all samples, and all samples included in the analysis had a minimum of two gene sequences, to minimize confounding factors of too much missing data (Table 1).

PCR mix and cycling profiles followed Groenenberg et al. (2009) with the following modifications: 1.5 μl of 2.5 mM $MgCl_2$ was added to 16S, 18S, and COI, and 5.5 μl of 5 × Q solution (Qiagen) was added to 16S, 18S, and 28S PCR mixtures. Primers and annealing temperatures used can be found in the Supplementary material (Supplementary Primer Data).

PCR products were sequenced in both directions by Macrogen Europe. Consensus sequences were generated using Sequencher 4.2 (Gene Codes Corporation). Regions with poor sequencing quality were removed. Histone 3 is a multicopy gene, and some copies have very slightly different sequences, here only 1–3 polymorphisms were detected per sequence. These single nucleotide polymorphisms were coded accordingly: e.g. M = A or C. Any questionable sequences were repeated, including the extraction when possible. All sequences produced by the authors were deposited in Genbank under accession numbers HM753267–HM753534 and JF342448–JF342449, and additional sequences were obtained from Genbank (Table 1).

### 2.3. Phylogenetic reconstruction

Sequences were initially aligned in ClustalX 1.82 (Larkin et al., 2007), then manually adjusted in MacClade 4 (Maddison and Maddison, 2005). Sequence portions that could not be aligned unambiguously were removed. Remaining gaps were treated as missing data in all analyses, as a fifth state for gaps is unavailable in Maximum Likelihood and Bayesian analyses.

Mr.Modeltest 2.3 (Nylander, 2004) was performed on each of the five genes, as well as each of the three codon positions for the protein coding genes. The most appropriate model was selected based on the AIC (Akaike Information Criterion) and shown in Table 2.

Saturation of genes and codon positions was assessed using the 'transitions and transversions vs. divergence' graphic function in Dambe (Xia, 2001). This was to ensure that the sequences had not changed so much that they obscure the pattern of evolution. Possible saturation effects were noted for 18S, as well as for COI codon position 3, and H3 codon positions 1 and 2. Bayesian analyses were used to assess the effect of this saturation. In all cases, trees including the possibly saturated positions showed (nearly) identical topologies, with on average higher support values than trees with those positions excluded. Thus all positions were included in all further analyses.

The optimal number of partitions was assessed by calculating Bayes factors using Tracer v1.4.1 (Rambaut and Drummond, 2007). Five possibilities were tested for all genes together, namely: (1) all genes combined; (2) each gene in a separate partition; (3) one for each gene, as well as each COI codon position; (4) one for each gene, as well as each H3 codon position; (5) nine partitions, one for each gene, as well as one for each codon position of both COI and H3. A $\log_{10}$ Bayes factor greater than 2 was considered a significant improvement (Kass and Raftery, 1995). Nine partitions, one for each gene as well as each COI and H3 codon position were used. The partitions were assessed to allow the model to compensate for different rates of evolution in each of the separate genes.

A Congruence Among Distance Matrices (CADM) test was performed to estimate the congruence in the data partitions using R (Campbell et al., 2011; R Core development team, 2011). There was no indication of incongruence ($p < 0.001$, nperm = 999) for both the global and *a posteriori* tests.

All three major phylogenetic analysis approaches were used to confirm a consensus topology that is well supported by multiple methodologies. Bayesian analyses were run on the CIPRES Portal v2.2 (Miller et al., 2010) using Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). In one analysis, three non-cyclophoroid taxa (*Conus miles*, *Conus miliaris*, and *Pomacea insularum*) were used to confirm monophyly of the Cyclophoroidea. As their inclusion reduced branch support values they were not used in further analyses. The analyses consisted of 40 million generations, sampling every 100 generations, with four simultaneous runs of four chains, and a temperature of 0.02. Convergence was confirmed by verifying that the standard deviations of split frequencies were below 0.01, and by examining the 'compare' and 'cumulative' plots using AWTY (Wilgenbusch et al., 2004). A burn-in value of 25% was determined by examining the 'compare' plots in AWTY.

A Maximum Likelihood analysis was run using RaxML7.2.6 (Stamatakis, 2006) as implemented on CIPRES portal v2.2 (Miller et al., 2010) on all the genes together, with 1000 rapid bootstraps using GTR + Γ. The data was divided into nine partitions, all analyzed with a GTR substitution model.

The Parsimony analyses were run using PAUP⁎ (Swofford, 1998). A bootstrapped heuristic search with 1000 bootstrap replicates, and 100 random addition sequence heuristic search replicates, with a rearrangement limit of 10 million rearrangements per replicate was carried out, with 50% as the minimum bootstrap support included.

### 2.4. Morphology

For each individual, we measured a range of shell traits commonly reported to differ at various taxonomic levels in the Diplommatinidae (Vermeulen, 1991, 1993, 1994, 1996a,b). The measurements are described in Fig. 2 and in the supplementary material (Supplementary Measurement Descriptions). One specimen per molecular sample was photographed using a Leica DCF480 microscope and Leica Application Suite 2.8.1 (Leica Microsystems) in montage mode, and measured from the photographs using ImageJ 1.43 (Rasband 1997–2011).

### 2.5. Ancestral state reconstruction of chirality

We used the combined Bayesian tree for ancestral state reconstruction. We carried out ancestral state reconstruction on a chronogram derived from the non-ultrametric Bayesian tree following Sanderson (2002), assuming a saturated model. In this manner, the time since divergence starting from a particular ancestral state becomes equal for pairs of descendant species. Thus we assume that the probability of chirality change is proportional to divergence time. Sinistroid shells were considered dextral. We used maximum likelihood estimation of models with either equal or different rates of transitions between the two chirality states along branches of the phylogeny (Pagel, 1994). AIC was used to compare the two models, the parameter estimates and their standard errors. The ancestral state reconstruction of chirality was done using the phylogenetics package APE (Paradis et al., 2004) for R (R Core development team, 2011), following the method described in Schluter et al. (1997). This method yields likelihoods of each chirality state at each ancestral node. The chirality state with the largest relative likelihood at an ancestral node is the maximum likelihood ancestral state. Per ancestral node, we plot the relative likelihoods of both states in a pie diagram, to allow an assessment of the strength of evidence for a particular ancestral state.

**Table 2**
Models used for each of the nine partitions. GTR, general time reversible model; SYM, symmetrical model; I, propotion of invariant sites; Γ, rate heterogeneity.

| Gene partition | Model |
|---|---|
| 16S | GTR + I + Γ |
| 18S | SYM + I + Γ |
| 28S | GTR + I + Γ |
| COI codon position 1 | GTR + I + Γ |
| COI codon position 2 | GTR + I + Γ |
| COI codon position 3 | GTR + I + Γ |
| H3 codon position 1 | GTR + I |
| H3 codon position 2 | SYM |
| H3 codon position 3 | GTR + I + Γ |

### 2.6. Phylogenetic principal component analysis

We applied a recently proposed method for analyzing morphological variation in a phylogenetic context, Phylogenetic Principal Component Analysis (pPCA; Jombart et al., 2010b). pPCA can be used to describe principal components of shape variation which either vary with phylogenetic distance, or which change mostly over small phylogenetic distances (Jombart et al., 2010a). Thus it is possible to distinguish between combinations of characters that change gradually with phylogenetic distance (global patterns), those that appear to change independently from a shared evolutionary history and character combinations that mostly vary between neighboring tips of the phylogeny (local patterns). This descriptive technique yields a clearer picture of the multivariate patterns and processes involved in the evolution of morphological characters. We used pPCA to investigate the changes in continuous morphological characters throughout the phylogeny. It was implemented using the R package 'adephylo' (Jombart et al., 2010a), as described by Jombart et al. (2010b). Three taxa and one shell measurement were removed from the analysis as they had more than 50% missing data. We analyzed the trait using two pPCAs. First, we carried out an analysis on all traits which we expected to correlate strongly with overall size, the size pPCA. These traits (indicated in Fig. 2) were measured in all species and shared the same unit of measurement. To calculate phylogenetic proximities, we used the modification of Abouheifs proximity, as proposed by Jombart et al. (2010b). For this metric, using the non-ultrametric Bayesian tree or the chronogram resulted in an identical matrix of phylogenetic proximities. We did not rescale trait variances in this analysis. A second pPCA was carried out on size-corrected trait values of the remaining traits, a shape pPCA. For size correction, we did a non-phylogenetic PCA on the first set of traits, calculated the score for the principal component with the largest eigenvalue, and used that score as a proxy for overall size (Berner, 2011; McCoy et al., 2006). We fitted a linear model with this proxy as explanatory variable to each trait to be used in the shape pPCA, and the residuals of these linear models were used as the size-corrected trait values in that second pPCA. Missing values in these traits were replaced by trait-specific median values. We rescaled all trait variances to unit variance in the shape pPCA.

In both pPCAs, we investigated whether global phylogenetic patterns occur in certain trait combinations. These are then represented by phylogenetic principal components (PCs) with a large positive eigenvalue, i.e. PCs with a large variance and a positive phylogenetic autocorrelation (Jombart et al., 2010b). Local phylogenetic patterns were also investigated, by inspecting phylogenetic PCs with a large variance and a negative phylogenetic autocorrelation. These weighted trait combinations then change most over short phylogenetic distances. There is no generally accepted procedure to determine the number of non-trivial axes in a (p)PCA (Dray, 2008; Jackson, 1993; Longman et al., 1989; Peres-Neto et al., 2005). We decided to support our choice of which PCs to consider as important in a phylogenetic context with simulations of a "null" model. Per pPCA, we made 1000 datasets where the trait vectors were randomized over the species such that traits per species became independent

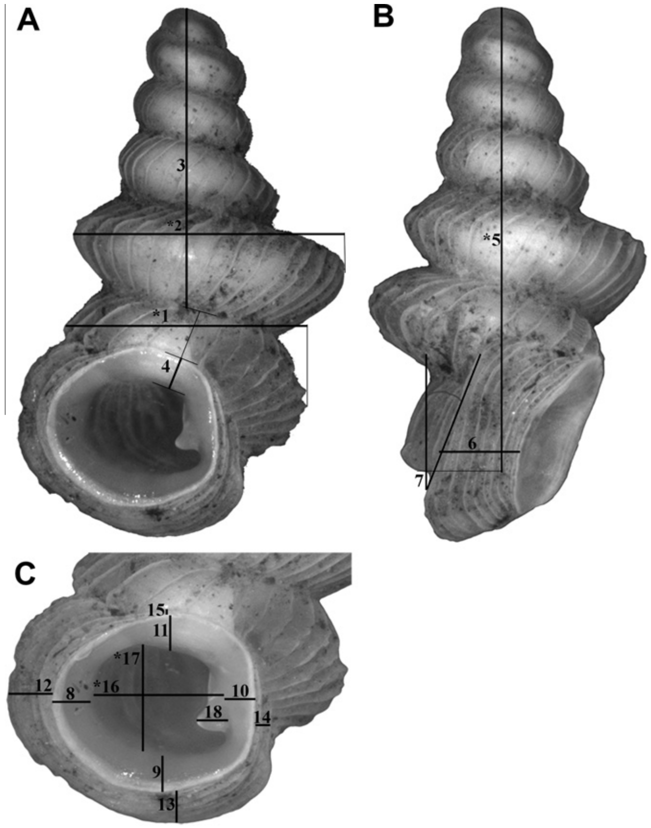

**Fig. 2.** Morphological measurements. 1. Width of last whorl. 2.Width. 3. Height to constriction. 4. Proportion of aperture that covers the penultimate whorl. 5. Height. 6. Inter outer Peristome distance. 7. Aperture angle. 8. Inner palatal. 9. Inner umbilical. 10. Inner columellar. 11. Inner apex. 12. Outer palatal. 13. Outer umbilical. 14. Outer columellar. 15. Outer apex. 16. Apertural width. 17. Apertural height 18. Columellaris. ∗ indicates measurements used in the size PCA.

of phylogenetic distance. From these simulated datasets, we obtained and inspected distributions of eigenvalues, variances, and autocorrelations of all principal components per analysis. We observed that for each simulated dataset, positive and negative eigenvalues occur, and that average autocorrelations and variances systematically decrease with PC eigenvalue, as in the actual data. We decided to inspect a limited number of successive PCs per actual analysis which had the largest (smallest) eigenvalues and which appeared to have eigenvalues outside of the distribution of the corresponding global (local) PC of the randomizations. We stress that this remains a relatively subjective selection procedure.

To determine which traits were associated with a certain PC, a clear break in the relative contributions was used, with nothing below 20% being examined. To assess effects of within-species variation on our results, we added additional amounts of extra error variation to all trait values and redid the analysis for a number of proportional amounts added to the entire dataset. To each trait value, we added Gaussian error contributions with zero mean and a standard deviation of 5%, 10%, 20%, 40% or 80%, 160% or 320% of the standard deviation of that trait. We inspected the effects on eigenvalues of the pPCA and the phylogenetic autocorrelations averaged across 20 replicate analyses.

## 3. Results

### 3.1. Sequence alignment and gene conservation

Sequences from 16S and 28S contained regions that were too variable for an accurate alignment and these regions were removed from the analysis. All other genes were unambiguously aligned, and all three ribosomal genes had remaining gaps (Table 3).

### 3.2. Phylogeny

The Maximum Parsimony and Maximum Likelihood analyses (Supplementary Phylogenies) produced very similar, but much less well supported topologies than the Bayesian phylogeny, which was used in all further analyses (Fig. 3).

The Cyclophoroidea were recovered as a monophyletic group with respect to *Pomacea* and *Conus*, and all cyclophoroid families, save Diplommatinidae, also appear monophyletic. Pupinidae, Cyclophoridae, and Megalostomatidae form well supported clades (PP [Posterior Probability] = 100%), where Neocyclotidae (*Aperostoma*) is sister to the remaining cyclophoroids, followed by Pupinidae, and then the rest of the clade (Fig. 3).

The two diplommatinid subfamilies, Diplommatininae and Cochlostomatinae, are both well supported clades (PP = 100%). However, Cochlostomatinae as the sister clade to the Diplommatininae is clearly refuted, making Diplommatinidae paraphyletic. Instead, Megalostomatidae and Cochlostomatinae are sister groups. Three families together ((Megalostomatidae + Cochlostomatinae) + Cyclophoridae) form the sister group to Diplommatininae. All of these nodes are well supported (PP > 97%).

The topology of the Diplommatininae suggests five well supported clades (labeled A–E in Fig. 3) and a few individual taxa. Within the Diplommatininae, *Opisthostoma* is the only genus sampled whose monophyly is clearly supported (PP = 96%). *Arinia* and *Adelopoma* were represented only by a single species, *Palaina* and *Hungerfordia* are paraphyletic, and *Diplommatina* is polyphyletic.

Clade A is sister to the remaining Diplommatininae, and is formed from *Palaina, Hungerfordia*, and a few *Diplommatina*. All species within clade A were collected from the Caroline Islands. *Palaina doliolum*, from Pohnpei, is separated phylogenetically from the remaining species that were collected on Belau. The remaining *Palaina* cluster together as a sister clade to *Hungerfordia* and the clade A *Diplommatina*, which do not group congenerically. Clade B consists of *Arinia*, and its sister group *Opisthostoma* (PP = 91%). There is very little structure recovered within *Opisthostoma*. Only that *O. mirabile* and *O. fraternum* are sister species is well supported (PP = 100%). The remaining *Diplommatina* samples form the rest of the tree, with the single *Adelopoma* specimen. There are four species here that do not clearly cluster into a clade. These are *Adelopoma tucma, D. laidlawi, D. isseli, and D. whiteheadi.* Clade C contains most, but not all, Bornean *Diplommatina* (PP = 100%). There is little well supported structure within clade C, and *D. isseli* is suggested as the sister species (PP = 65%). The several *D. electa* specimens do not clearly cluster together, and the two *D. rubra* specimens are clearly separate. Clade D consists solely of Thai and peninsular Malaysian species (PP = 100%). The topology of this clade is well supported with *D. superba brevior* as sister to the rest of the clade. This last clade, clade E, consists of a broad mix of species from different geographical regions: Borneo, Thailand, Peninsular Malaysia, and Taiwan (PP = 91%). *Diplommatina whiteheadi* is sister to Clade E (PP = 89%).

### 3.3. Reconstruction of chirality

The model with equal transition rates between chiral states (AIC: 38.0) had a slightly larger AIC value than the model with unequal rates (AIC: 38.1). However, the difference is minor and confidence intervals for the unequal parameters overlap. This leads us to prefer the equal rates model. Sinistrality is reconstructed as the plesiomorphic state for the Diplommatininae (Fig. 4), where the remaining cyclophoroids are ancestrally dextral. Furthermore, chirality is strongly conserved within clades; only one clade has both dextral and sinistral members; *D. calvula* is a dextral member of the sinistral clade C. Clades A and D are entirely sinistral. Clades B and E are entirely dextral, as well as all outgroup species. Overall, there appear to have been three reversals to dextrality within the Diplommatininae: one at the origin of clade B, one for *D. calvula*, and one at the origin of clade E.

### *3.4. Phylogenetic principal component analysis*

#### *3.4.1. Size and shape pPCA*

In our 'size' pPCA, on size-related variables, two global principal components were inspected, no local principal components were retained (Supplementary Phylogenetic PCA). The first global PC explained 97.0% of variation in the variables. The PC had a positive phylogenetic autocorrelation (Moran's $I = 0.63$; Jombart et al., 2010b). Most of these size related changes were associated with shell height (Fig. 5). The second global PC in this phylogenetic PCA explained 2.3% of trait variation. Inspecting the trait loadings reveals that this PC has a strong positive loading of shell height and strong negative loading of width of the last whorl (Fig. 5). It therefore represents variation between tall, narrow shells and short, wide shells. The phylogenetic autocorrelation for this PC is $I = 0.47$.

In the shape pPCA, on size-corrected data, again two global principal components and no local principal components were retained (Supplementary Phylogenetic PCA). Both PCs were global PCs, indicative of trait patterns gradually changing with phylogenetic distance. The first global PC represents 24.6% of total PC variation, it has a phylogenetic autocorrelation of $I = 0.72$. The loadings of this PC were complex, without an easily determined general pattern (Fig. 5). The second shape PC represents 8.5% of total variation and had an autocorrelation of $I = 0.57$, with a complex pattern of character loadings (Fig. 5). For the set of phylogenetic proximities given by our tree, the minimum and maximum possible values of Moran's I were −0.53 and 1.00, respectively (de Jong et al., 1984).

Mapping these PCs on the phylogeny demonstrates some morphological consistency within the clades (Fig. 6). Size GPC1 was most positive in clade C, as well as '*Hungerfordia*' in clade A. Size GPC2 was most positive in some members of clades C–E, and most positive in *D. cf. lenggongensis* and *D. naiyanetri*. This suggests long, narrow shells in these species. It was generally most negative in members of clade B, suggesting short, wider shells. Shape GPC1 clearly separated the Diplommatininae into a largely positive grade including clades A and B, and a more derived largely negative clade, including the remaining snails. Shape GPC2 was strongly positive in *Hungerfordia* and the *Diplommatina* of clade A, and strongly negative in most of clade C, as well as *D. electa D.*

#### *3.4.2. Variance error*

The analysis of the extra error variances of our trait values showed the following pattern (Supplementary Phylogenetic PCA). Within-species variation decreased the proportion of variation explained by the first PC in both pPCAs, and decreased the phylogenetic autocorrelations. The first size-related PC explained less than 90% of total variation when more than 40% error variation was added. In the size-corrected (shape) pPCA, the effects on most eigenvalues were very small. Phylogenetic autocorrelations overall decreased with the amount of variation added, but the decrease was gradual for most PC and only conspicuous when 40% or more error is added. The analysis suggests that small to moderate amounts of within-species variation in our data were unlikely to lead to the selection of a different number of PCs to interpret. Among selected PCs, mostly on the size-related traits, it might lead to underestimated phylogenetic autocorrelations.

## 4. Discussion

### *4.1. Phylogeny*

The combined Bayesian tree produces a clear picture of the relationships within the Diplommatinidae as well as with the outgroups. Although not all nodes are resolved or have strong support, the major clades are well supported.

This is the first study showing the phylogenetic relationships between the various cyclophoroid families. The sampling of the various families was quite small, with only a single specimen for Neocyclotidae, two species each for Pupinidae and Megalostomatidae, and nine species for the Cyclophoridae. Clearly, further sampling will be required to resolve these relationships, but a framework on which further studies can be based has been produced. Lee et al. (2008) completed a molecular phylogenetic reconstruction of the Cyclophoridae, the only previous phylogeny of members of the outgroup. The cyclophorid relationships recovered here do not conflict with those results, although neither study strongly supports a certain topology. The fact that the *Japonia* sample. clusters with *Leptopoma* in this analysis, and has longitudinal red-brown stripes on the shell, suggests that it belongs, in fact, to *Pilosphaera*, the new genus erected by Lee et al. (2008) to rectify the polyphyly of *Japonia*.

Cochlostomatinae as the sister group to Megalostomatidae is well supported in this molecular phylogeny. As a result, the Cochlostomatinae is here raised to the level of family and referred to as Cochlostomatidae, leaving the Diplommatininae as the sole subfamily of the Diplommatinidae. The Diplommatininae (now Diplommatinidae) is a clearly monophyletic group, however, many of the included genera require some reassessment.

#### *4.1.1. Clade A*

This clade (all from the Caroline Islands) is the most diverse group, both morphologically and taxonomically, with representatives assigned to three different genera. The taxonomic results have been previously reported by Rundell (2008), on the basis of a larger sampling of the diplommatinids from this region. The current generic assignments within this clade are questionable. The morphological analyses show high levels of morphological variation within clade A. Interestingly, the two *Hungerfordia* specimens are morphologically very similar despite not forming a monophyletic group,

**Table 3**
Character numbers and characteristics for each gene alignment.

| Gene | Total bases | Unambiguously aligned sites (bp) | Variable sites (bp) | Variable sites (%) | Parsimony informative sites (bp) |
|---|---|---|---|---|---|
| 16S | 550 | 398 | 237 | 60 | 192 |
| 18S | 374 | 374 | 33 | 9 | 19 |
| 28S | 874 | 711 | 171 | 24 | 117 |
| COI | 658 | 658 | 342 | 52 | 304 |
| H3 | 267 | 267 | 111 | 42 | 99 |
| Total | 2723 | 2408 | 894 | 37 | 731 |

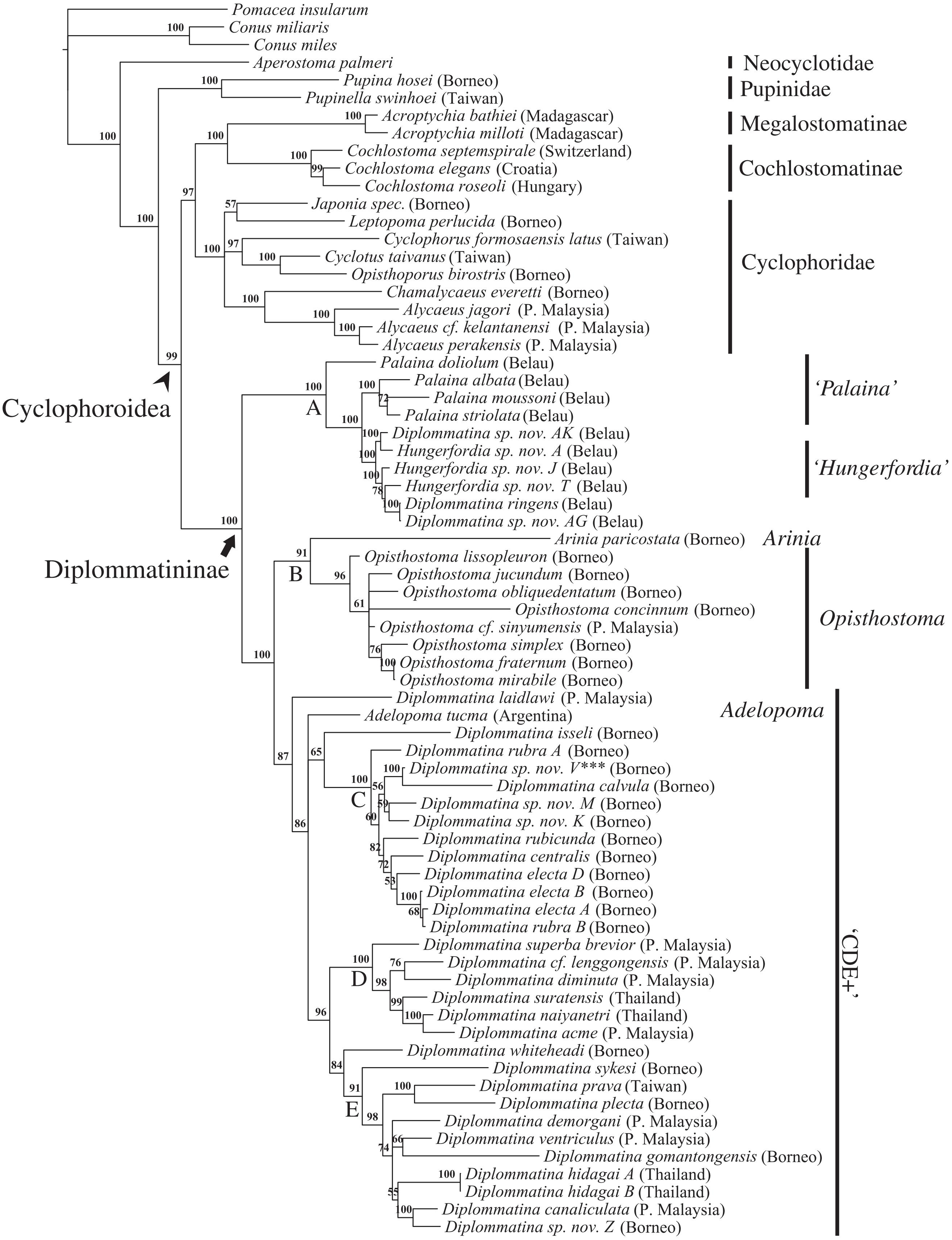

**Fig. 3.** Phylogeny inferred from the combined data set. Bayesian posterior probabilities are shown above the node. Clades are lettered as in text.

implying convergent evolution. In contrast, the short branch lengths seen throughout Clade A suggest little molecular divergence, which may mean that this group has been under strong evolutionary pressure to produce such morphological disparity within a short time period.

### 4.1.2. Clade B

Morphologically, shells of members of clade B are distinctly shorter and wider than other Diplommatinidae, and are very similar morphologically according to the shape pPCA. These features are more prominent in *Opisthostoma* than *Arinia.*

While its monophyly is strongly supported, the relationships within *Opisthostoma* are essentially unresolved. The pPCA does not indicate a strong morphological difference between the two genera of clade B, despite the evolution of sinistroidy in *Opisthostoma*. This may have been complicated by the fact that *Opisthostoma* specific measurements were excluded from the pPCAs as they were missing for all other taxa. *Opisthostoma cf. sinyumensis* is not sinistroid, so we would expect it to be separate from the rest of the genus, but this is not the case. The analysis is not resolved enough to strongly support this result, but, if true, it does have interesting implications for the evolution of this group and its unique coiling pattern. A study focusing on this genus with much greater species sampling and less conserved markers is currently ongoing (Liew, unpubl.).

*Arinia*, the sister group to *Opisthostoma,* was represented by a single species. This might explain the long branch seen for *Arinia paricostata*, which would almost certainly be attenuated by congeneric specimens. This could break up the variation in the genus, and help place it with better support.

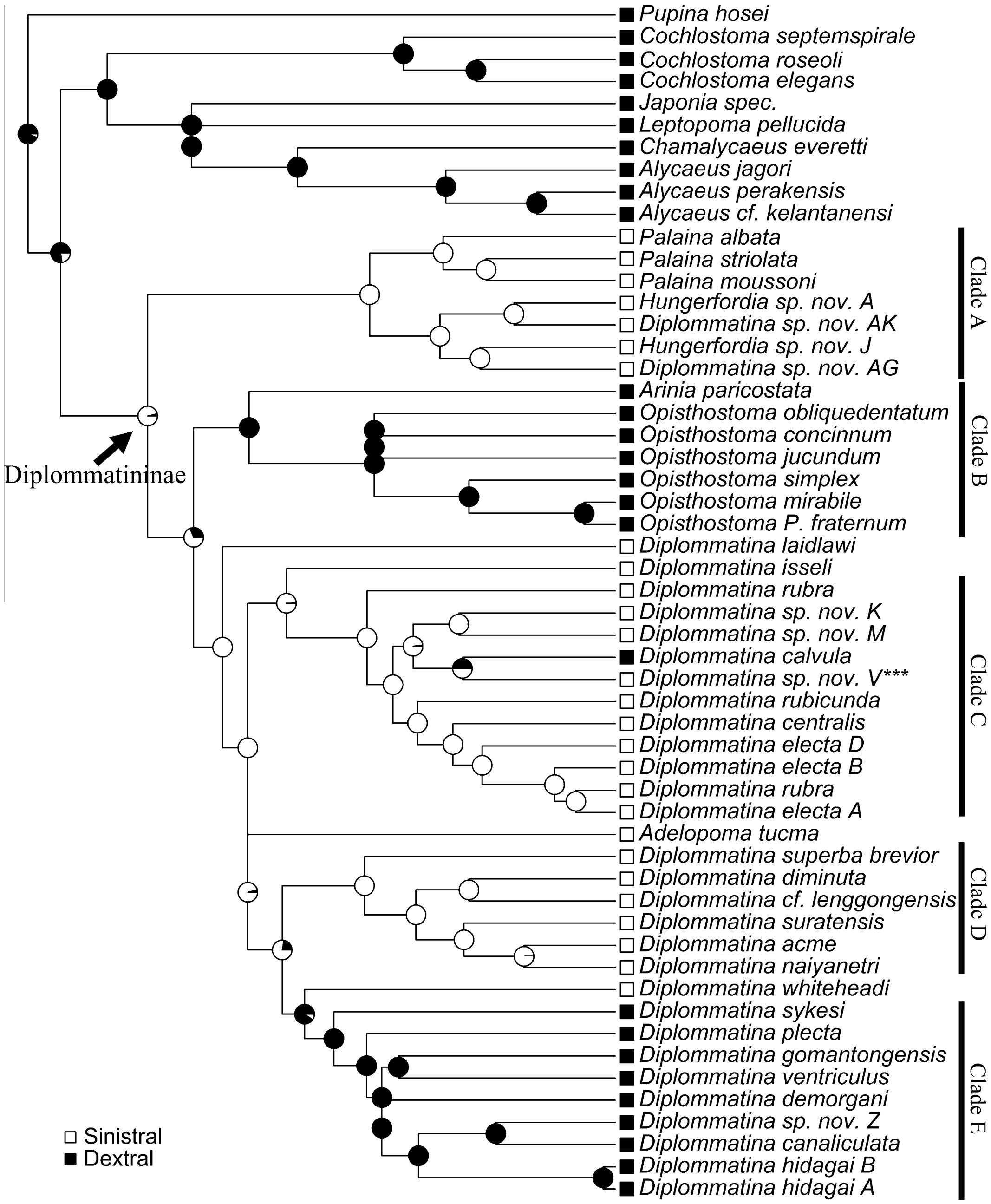

**Fig. 4.** Reconstruction of chirality. Squares indicate actual species chirality. Circles indicate ancestral state reconstruction, where the proportion of the circle that is white represents the relative likelihood that the ancestor at this node was sinistral.

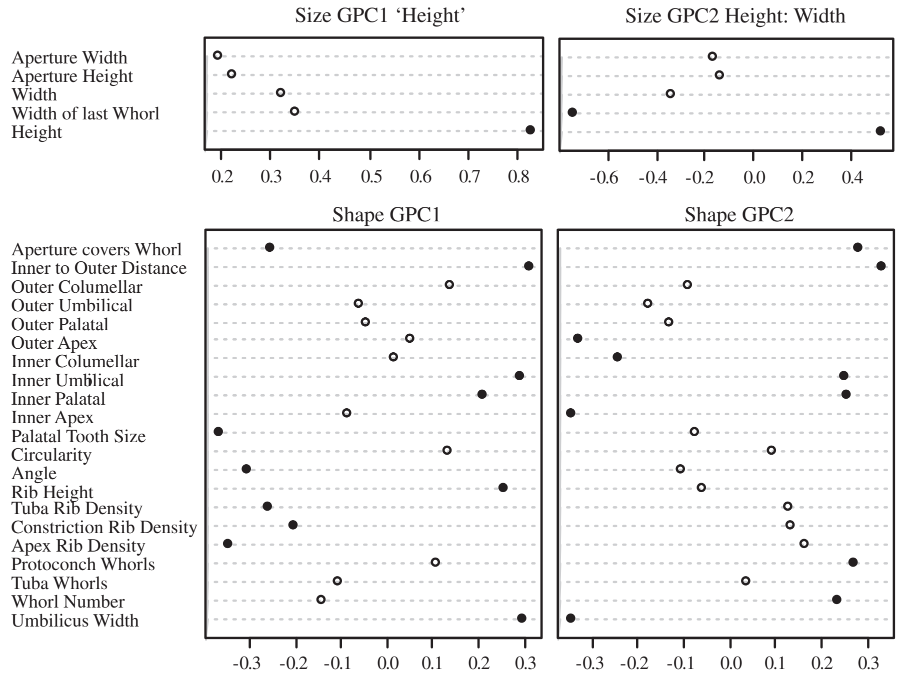

**Fig. 5.** Contributions to PCs examined, showing direction and weight for each character. Black dots represent characters that contribute strongly to each PC.

#### 4.1.3. CDE+

Those species falling outside of clades A and B appear to form a derived clade, 'CDE+', with several clear morphological differences, supported mainly by the results of shape GPC1. Despite being composed nearly entirely of *Diplommatina*, 'CDE+' includes *Adelopoma*, and therefore cannot correspond to a monophyletic *Diplommatina*.

The placement of the neotropical *Adelopoma* within 'CDE+' is surprising, considering that the rest of 'CDE+' are from South East Asia. Due to the geographic distance of this genus from the rest of Diplommatinidae, a more isolated placement would be expected, with high levels of both morphological and molecular divergence. This is clearly not the case. This has implications for the evolution of *Adelopoma*, and its dispersal to South America. Migration from Australia, through Antarctica to South America was possible only until about 40 Ma (Briggs, 1995, 2003). Clade A is a maximum of 37.7 Ma old, the maximum geologic age of Palau, and probably arose less than 10 Ma (Kobayashi, 2004; Rundell, 2008). If the branch lengths can be assumed to represent relative time, then *Adelopoma* will have arisen after this time, and the Antarctic route would have been closed. Thus it seems most likely that *Adelopoma* dispersed to South America across the Pacific Ocean. Although dispersing across the Pacific seems improbable, long-distance dispersal is not unprecedented in land snails (Gittenberger et al., 2006; Greve et al., 2010; Miura et al., 2011), especially in small-bodied species (Peake, 1981). Clade E also supports the high dispersability of diplommatinids, as it contains species from all over the region in a single clade.

#### 4.1.4. Clade C

Clade C is a well supported clade, being composed of primarily sinistral species, and contains all species that used to be part of *Gastroptychia*. All are from Borneo, have a generally darker shell (most are orange), all lack striations, and none have a distinct angle to the protoconch (supplementary material). Despite these similarities, the pPCAs suggest a high level of morphological variation in this clade. Except for *D. calvula*, all have distinctly large values of size GPC1, suggesting that clade C is larger than other Diplommatinidae, and that this is phylogenetically relevant. There is one dextral species in clade C, *D. calvula*, with a long branch, and it is also morphologically unusual for this group, with a complete lack of ribs, and having a very narrow last whorl and long tuba.

#### 4.1.5. Clades D and E

Clades D and E can mainly be distinguished by their opposing chiralities. Both clades contain all species once assigned to the *Sinica* subgenus. Clade D contains only sinistral species from the South East Asian mainland (Peninsular Malaysia and Thailand) and the shell is tall and narrow. Clade E is the most geographically diverse clade, containing dextral species from all sampled regions save the Caroline Islands.

The phylogeny produced here provides a new context for previous studies on this group. Peake (1973), for example, showed some very interesting morphological results for sympatric, similarly sized dextral and sinistral species. He suggested that in sympatry, sinistral shells would be longer and thinner than dextral shells, and designated this sinistral morphology as morphological type 3. This type 3 morphology is very similar to what is seen in the sinistral clade D of our study (Fig. 7). Peake (1973) assigned dextral shells a type 1 or 2 morphology, which generally compare to the morphologies seen in clade C (sinistral) and clade E (dextral). If Peake's 'sympatric sinistral species' belonged to clade D, while the sympatric dextral species belonged to clade E, it is thus possible that the morphological trends he uncovered are in fact due to phylogenetic conservatism rather than natural selection. Peake made little effort to identify the species he examined, thus their interrelationships are unknown. Alternatively, his results may suggest that there is a fundamental difference or niche partitioning between clades D and E, allowing them to exist in sympatry where others cannot.

### 4.2. Morphology

#### 4.2.1. Intraspecific variation

Many diplommatinid species have been described as being morphologically variable (Vermeulen, 1991, 1993, 1994, 1996a,b). It was impossible to take this variation into account for a family level analysis. First, the intention of this study was to examine the whole family, and focusing on intraspecific variation would take away from the larger scale of this analysis. Second, the soft tissue

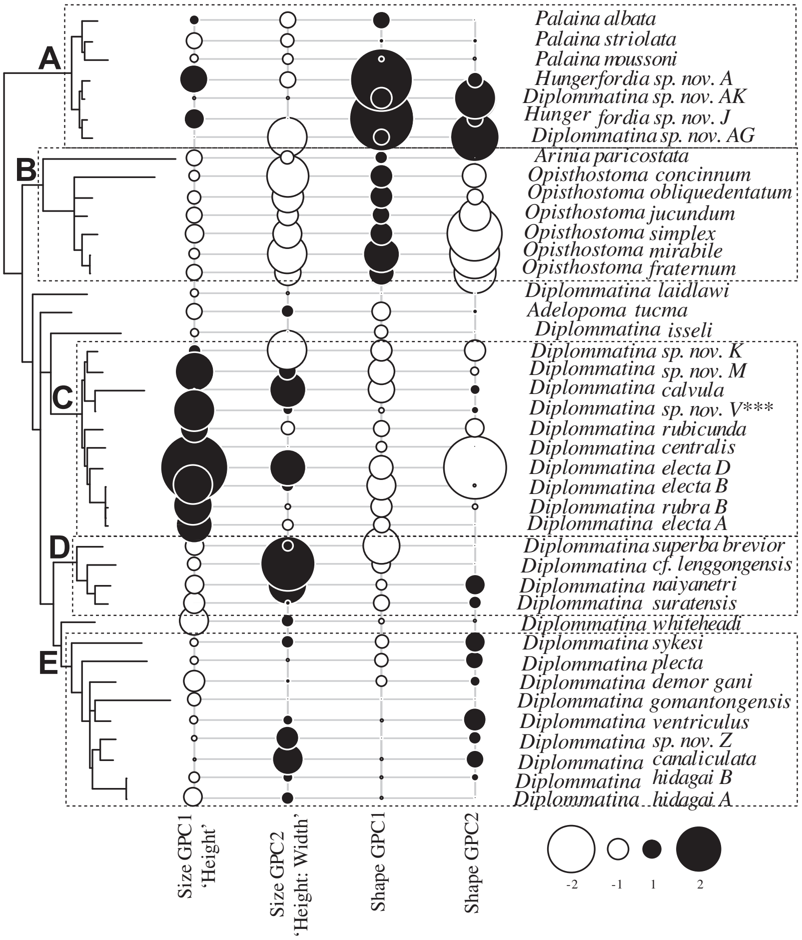

**Fig. 6.** Scores for each taxon for the four PCs. Black circles are positive scores, white circles are negative scores. Size of the circles indicates magnitude of the score. Clades and PCs labeled as in text.

material for most species is very limited, and it would have been impossible to assess intraspecific variability while maintaining a direct link to the molecular phylogeny.

### 4.2.2. Chirality

Snail chirality is directed by a single, maternally expressed nuclear locus (Schilthuizen and Davison, 2005). Transitioning from dextral (the most common state) to sinistral is probably rare due to frequency dependent selection, as inter-chiral mating is difficult to impossible due to physical limitations of mismatching anatomy, at least in pulmonate land snails (Gittenberger, 1988; Schilthuizen and Davison, 2005). However, developmental constraints might also affect the appearance of species with chirality reversals (Schilthuizen and Haase, 2010; Utsuno et al., 2011).

The current phylogeny suggests a sinistral origin for the Diplommatinidae, which is confirmed by the ancestral state reconstruction. A sinistral origin requires three changes to dextrality, whereas a dextral origin requires four changes. This result may be very sensitive to sampling bias and depends highly on basal sinistrality for the Diplommatinidae. No specific morphological changes appear to be associated with the chirality reversals, as relatively narrow and wide shells are present in both chiral states in this family. Whichever the ancestral chirality, the phylogenetic pattern indicates that reversals are rare, but do occur. The family thus allows a comparative analysis of selective factors associated with the reversals.

### 4.2.3. Morphological evolution

pPCA is a new methodology that allows PCA to be used in a phylogenetic context in order to summarize variation in many quantitative traits in a reduced number of dimensions. It allows separating trait combinations which vary globally and gradually with phylogenetic distance, and trait combinations with local phylogenetic variation. Examining the phylogenetic pattern of shell morphologies shows similar conserved patterns to those highlighted by the pPCA (Fig. 7). This layout shows the large morphological differences within Clade A. It demonstrates the conserved shell morphology within the other clades. Clade D seems to have the most recognizable form comprising narrow shells, with angular whorls, and round peristomes. Overall, this method seems to have captured the general morphological trends of the Diplommatinidae, and would be useful for further studies, or for similar work in other taxa.

Here we have produced a molecular phylogeny and mapped morphological variation onto it. We have separated the diplommatinid diversity into five general clades, and determined associated shell morphologies. We have highlighted groups in need of further revision to resolve paraphyly, specifically *Adelopoma* and Clade A. We have separated the Cochlostomatidae into a separate family to properly reflect evolutionary relationships. We have also demonstrated that Diplommatinidae is likely one of few snail families with a sinistral origin. Further work is required, both to confirm the broader applicability of the evolutionary relationships determined here and to explain the pattern of morphological diversity of this clade. We have nonetheless provided a strong reference for all further work on the family Diplommatinidae, and placed them in context with other cyclophoroids. We have further more illustrated the useful combination of a molecular phylogenetic framework with morphological analyses for other evolutionary studies.

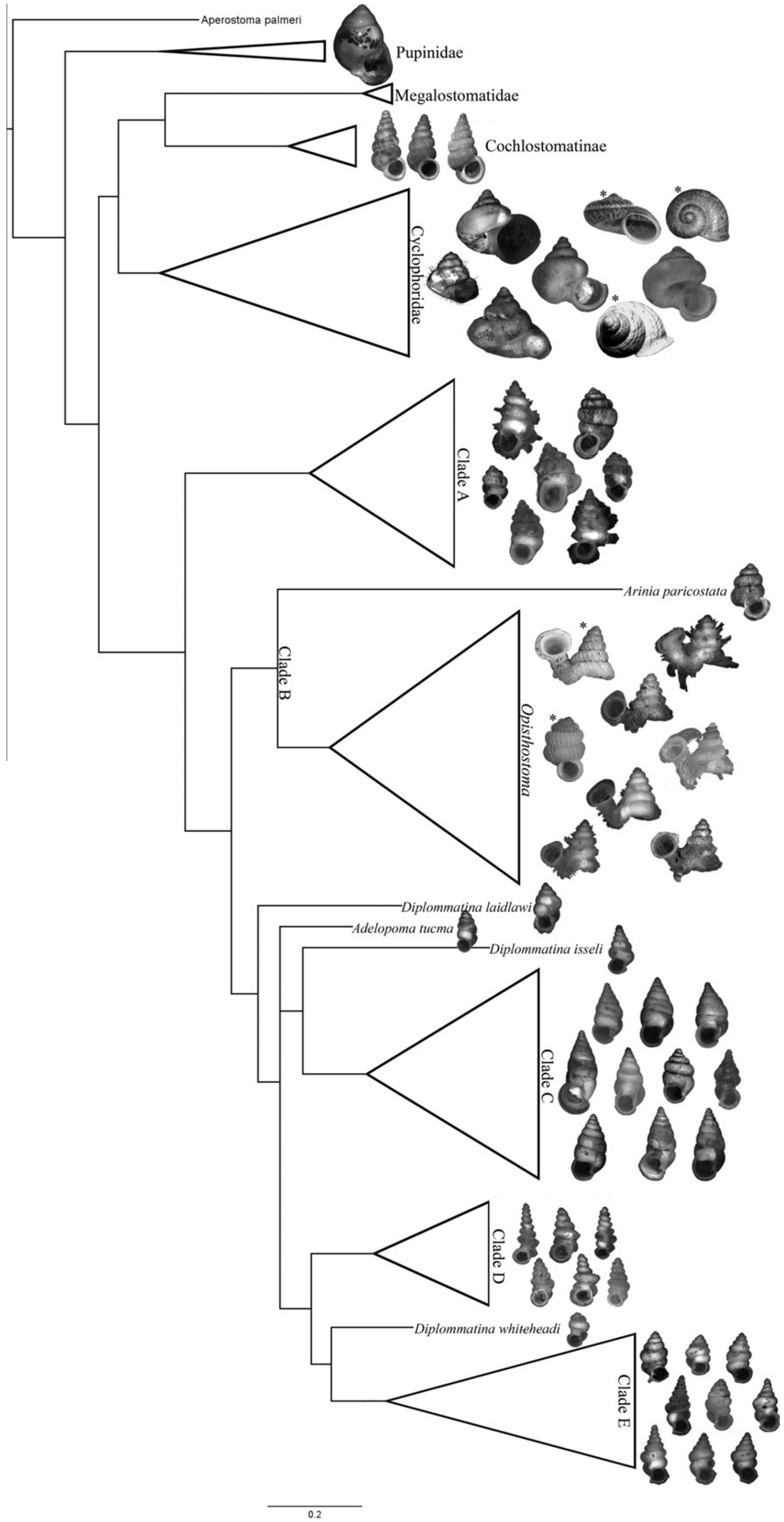

**Fig. 7.** Shells placed on a collapsed phylogeny to demonstrate overall morphological variation within and between the major groups in the phylogeny. $*$ indicates taxa not included in the morphological analysis.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2012.02.004.

## References

Beesley, P.L., Ross, G.J.B., Wells, A. (Eds.), 1998. Mollusca: The Southern Synthesis. Fauna of Australia, Part B, vol. 5. CSIRO Publishing, Melbourne.

Berner, D., 2011. Size correction in biology: how reliable are approaches based on (common) principal component analysis? Oecologia 166, 961–971.

Bouchet, P., Rocroi, J.P., Fryda, J., Hausdorf, B., Ponder, W., Valdés, A., Warén, A., 2005. Classification and nomenclator of gastropod families. Malacologia 47, 1–368.

Briggs, J.C., 1995. Global Biogeography. Elsevier, Amsterdam.

Briggs, J.C., 2003. Fishes and birds: Gondwana life rafts revisited. Syst. Biol. 52, 548–553.

Brown, B., Emberson, R.M., Paterson, A.M., 2000. Morphological character evolution in hepialid moths (Lepidoptera: Hepialidae) from New Zealand. Biol. J. Linn. Soc. 69, 383–397.

Campbell, V., Legendre, P., Lapointe, F.-J., 2011. The performance of the Congruence Among Distance Matrices (CADM) test in phylogenetic analysis. BMC Evol. Biol. 11, 64.

Clements, R., Liew, T.S., Vermeulen, J.J., Schilthuizen, M., 2008. Further twists in gastropod shell evolution. Biol. Lett. 4, 179–182.

Colgan, D.J., Ponder, W.F., Beacham, E., Macaranas, J., 2007. Molecular phylogenetics of Caenogastropoda (Gastropoda: Mollusca). Mol. Phylogenet. Evol. 42, 717–737.

de Jong, P., Sprenger, C., van Veen, F., 1984. On extreme values of Moran's I and Geary's c. Geogr. Anal. 16, 17–24.

Dray, S., 2008. On the number of principal components: a test of dimensionality based on measurements of similarity between matrices. Comput. Stat. Data Anal. 52, 2228–2237.

Fehér, Z., 2004. A revision of the genus *Cochlostoma*, subgenus *Titanopoma* (Gastropoda, Caenogastropoda, Cochlostomatidae), in particular the forms occurring in Albania. Basteria 68, 25–44.

Gittenberger, E., 1988. Sympatric speciation in snails: a largely neglected model. Evolution 42, 826–828.

Gittenberger, E., 1995. On the one hand. Nature 373, 19.

Gittenberger, E., Groenenberg, D.S.J., Kokshoorn, B., Preece, R.C., 2006. Molecular trails from hitch-hiking snails. Nature 439, 409.

Glaubrecht, M., 2010. Evolution in Action. Case Studies in Adaptive Radiation, Speciation, and the Origin of Biodiversity. Springer.

Greve, C., Hutterer, R., Groh, K., Haase, M., Misof, B., 2010. Evolutionary diversification of the genus *Theba* (Gastropoda: Helicidae) in space and time: a land snail conquering islands and continents. Mol. Phylogenet. Evol. 57, 572–584.

Groenenberg, D.S.J., Goud, J., De Heij, A., Gittenberger, E., 2009. Molecular phylogeny of North Sea Sepiolinae (Cephalopoda: Sepiolidae) reveals an overlooked species. J. Moll. Stud. 75, 361–369.

Harvey, P.H., Brown, A.J.L., Smith, J.M. (Eds.), 1996. New Uses for New Phylogenies. Oxford University Press, Oxford.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Jackson, D.A., 1993. Stopping rules in principal components analysis: a comparison of heuristical and statistical approaches. Ecology 74, 2204–2214.

Jombart, T., Balloux, F., Dray, S., 2010a. Adephylo: new tools for investigating the phylogenetic signal in biological traits. Bioinformatics 26, 1907–1909.

Jombart, T., Pavoine, S., Devillard, S.B., Pontier, D., 2010b. Putting phylogeny into the analysis of biological traits: a methodological approach. J. Theor. Biol. 264, 693–701.

Jorgensen, A., Kristensen, T.K., Madsen, H., 2008. A molecular phylogeny of apple snails (Gastropoda, Caenogastropoda, Ampullariidae) with an emphasis on African species. Zool. Scr. 37, 245–252.

Kass, R.E., Raftery, A.E., 1995. Bayes factors. J. Am. Stat. Assoc. 90, 773–795.

Kobayashi, K., 2004. Origin of the Palau and Yap trench-arc systems. Geophys. J. Int. 157, 1303–1315.

Larkin, M.A., Blackschields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.F., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, . Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lee, Y.C., Lue, K.Y., Wu, W.L., 2008. Molecular evidence for a polyphyletic genus *Japonia* (Architaenioglossa: Cyclophoridae) and with the description of a new genus and two new species. Zootaxa 1792, 22–38.

Longman, R.S., Cota, A.A., Holden, R.R., Fekken, G.C., 1989. A regression equation for the parallel analysis criterion in principal components analysis: mean and 95th percentile Eigen values. Multivar. Behav. Res. 24, 59–69.

Maddison, D., Maddison, W., 2005. MacClade 4.08. University of Arizona.

McCoy, M.W., Bolker, B.M., Osenburg, C.W., Miner, B.G., Vonesh, J.R., 2006. Size correction: comparing morphological traits among populations and environments. Oecologia 148, 547–554.

Miller, M., Holder, M., Vos, R., Midford, P., Liebowitz, T., Chan, L., Hoover, P., Warnow, T., 2010. The CIPRES Portals. CIPRES v2.2. <http://www.phylo.org/sub_sections/portal> (accessed 18.09.10).

Miura, O., Torchin, M.E., Bermingham, E., Jacobs, D.K., Hechinger, R.F., 2011. Flying shells: historical dispersal of marine snails across Central America. Proc. R. Soc. Biol. Sci. Ser. B. <http://dx.doi.org/10.1098/rspb.2011.1599> (accessed 04.10.11).

Nylander, J.A.A., 2004. Mr. Modeltest v2 2.3. Evolutionary Biology Centre, Uppsala University.

Pagel, M., 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. Proc. R. Soc. Lond. B: Biol. Sci. 255, 37–45.

Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290.

Peake, J.F., 1973. Species isolation in sympatric populations of the genus *Diplommatina* (Gastropoda, Prosobranchia, Cyclophoridae, Diplommatininae). Malacologia 14, 303–312.

Peake, J.F., 1981. The land snails of islands – a dispersalist's viewpoint. In: Foery, P.L. (Eds.), The Evolving Biosphere: Change, Chance and Challenge. University Press, Cambridge (Chapter 19).

Peres-Neto, P.R., Jackson, D.A., Somers, K.M., 2005. How many principal components? Stopping rules for determining the number of non-trivial axes revisited. Comput. Statist. Data Anal. 49, 974–997.

R Core development team, 2011. R: A Language and Environment for Statistical Computing. 2.12.2. R Foundation for Statistical Computing.

Rambaut, A., Drummond, A., 2007. Tracer v1.4. <http://beast.bio.ed.ac.uk/Tracer>.

Rasband, W.S., 1997–2011. ImageJ 1.43u. US National Institutes of Health, Bethesda, Maryland, USA. <http://rsbweb.nih.gov/ij/>.

Ricklefs, R.E., 2007. Estimating diversification rates from phylogenetic information. Trends Ecol. Evol. 22, 601–610.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rundell, R.J., 2008. Cryptic diversity, molecular phylogeny and biogeography of the rock- and leaf litter-dwelling land snails of Belau (Republic of Palau, Oceania). Philos. Trans. R. Soc. Lond. B: Biol. Sci. 363, 3401–3412.

Rundell, R.J., Price, T.D., 2009. Adaptive radiation, nonadaptive radiation, ecological speciation and nonecological speciation. Trends Ecol. Evol. 24, 394–399.

Sanderson, M.J., 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19, 101–109.

Schilthuizen, M., 2003. Sexual selection on land snail shell ornamentation: a hypothesis that may explain shell diversity. BMC Evol. Biol. 3, 13.

Schilthuizen, M., Davison, A., 2005. The convoluted evolution of snail chirality. Naturwissenschaften 92, 504–515.

Schilthuizen, M., Haase, M., 2010. Disentangling true shape differences and experimenter bias: are dextral and sinistral snail shells exact mirror images? J. Zool. 282, 191–200.

Schilthuizen, M., van Til, A., Salverda, M., Liew, T.-S., James, S.S., bin Elahan, B., Vermeulen, J.J., 2006. Microgeographic evolution of snail shell shape and predator behaviour. Evolution 60, 1851–1858.

Schluter, D., Price, T., Mooers, A.O., Ludwig, D., 1997. Likelihood of ancestor states in adaptive radiation. Evolution 51, 1699–1711.

Sengupta, M.E., Kristensen, T.K., Madsen, H., Jorgensen, A., 2009. Molecular phylogenetic investigations of the Viviparidae (Gastropoda: Caenogastropoda) in the lakes of the Rift Valley area of Africa. Mol. Phylogenet. Evol. 52, 797–805.

Solem, A., 1959. Systematics and zoography of the land and fresh-water mollusca of the New Hebrides. Fieldiana: Zoology 43, 339.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods) 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Tillier, S., 1981. Clines, convergence and character displacement in New Caledonian diplommatinids (land prosobranchs). Malacologia 21, 177–208.

Utsuno, H., Asami, T., Van Dooren, T.J.M., Gittenberger, E., 2011. Internal selection against the evolution of left-right reversal. Evolution 65, 2399–2411.

Vermeij, G.J., 1975. Evolution and distribution of left-handed and planispiral coiling in snails. Nature 254, 419–420.

Vermeij, G.J., 1993. A Natural History of Shells. Princeton University Press, New Jersey.

Vermeulen, J.J., 1991. Notes on the non-marine molluscs of the island of Borneo 3. The genus *Platycochlium* (Gastropoda Pulmonata: Streptaxidae). Basteria 55, 165–171.

Vermeulen, J.J., 1993. Notes on the non-marine molluscs of the island of Borneo 5. The genus *Diplommatina* (Gastropoda Prosobranchia: Diplommatinidae). Basteria 57, 3–69.

Vermeulen, J.J., 1994. Notes on the non-marine molluscs of the island of Borneo. 6. The genus *Opisthostoma* (Gastropoda Prosobranchia: Diplommatinidae), part 2. Basteria 58, 75–191.

Vermeulen, J.J., 1996a. Notes on the non-marine molluscs of the island of Borneo 7. The genus *Niahia* gen. nov. (Gastropoda Prosobranchia: Diplommatinidae). Basteria 60, 67–69.

Vermeulen, J.J., 1996b. Notes on the non-marine molluscs of the island of Borneo 8. The genus *Arinia*; additions to the genera *Diplommatina* and *Opisthostoma* (Gastropoda Prosobranchia: Diplommatinidae). Basteria 60, 87–138.

Vermeulen, J.J., Clements, R., 2008. Another twist in the tale: a new species of *Opisthostoma* (Gastropoda, Diplommatinidae) from Peninsular Malaysia. Basteria 72, 263–266.

Wilgenbusch, J.C., Warren, D.L., Swofford, D.L., 2004. AWTY: A System for Graphical Exploration of MCMC Convergence in Bayesian Phylogenetic Inference. <http://ceb.csit.fsu.edu/awty>.

Xia, X., 2001. DAMBE: Data Analysis in Molecular Biology and Evolution 5.2.5. Kluwer Academic Publishers.