The Shell evolution of the hydrocenidae of Malaysian Borneo

Bin Khalik, M.Z.

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Chapter 5

Shell evolution of the Bornean *Georissa* in a phylogenetic context

Mohd Zacaery Khalik[1,3,4], Martin Haase[2] and Menno Schilthuizen[1,3,5]

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1 Naturalis Biodiversity Center, Darwinweg 2, 2333CR Leiden, The Netherlands.
2 Zoological Institute and Museum, Greifswald University, 17489 Greifswald, Germany.
3 Institute of Biology Leiden, Faculty of Science, Leiden University, 2333 BE Leiden, The Netherlands.
4 Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
5 Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.

Abstract

Gastropod shells have experienced divergent, convergent, and parallel evolution at various taxonomical levels. In this paper, we attempt to elucidate the patterns of conchological character evolution in the minute *Georissa* land snails from Borneo. We performed phylogenetic analyses, incorporating taxa from Malaysian Borneo and other regions, inferred by mitochondrial (*COI* and *16S*) and nuclear (*18S* and *28S*) DNA sequences. Within this phylogenetic context we then investigated the evolutionary patterns of selected morphological features using a maximum likelihood ancestral state reconstruction and phylogenetic signal analysis. We found that the Bornean *Georissa* show significant parallelism in shell characters (i.e., shell scales, shell size, and general shape) as well as habitat. In addition, the non-monophyly of Bornean *Georissa* might indicate multiple invasions of ancestral species to Borneo and a history of long-distance dispersal.

Introduction

Gastropods are primarily single-shelled organisms which (except those who have secondarily lost their shell - i.e., true slugs) display great morphological diversity in shell shape. Generally, qualitative and quantitative approaches are applied in evaluating the evolution of gastropod shells. Although many taxa possess high intraspecific morphological variation, which may be due to either different selection pressures between different populations (Kemp and Bertness, 1984; Schilthuizen et al., 2005; Teshima et al., 2003) or phenotypic plasticity (Clewing et al., 2015; Verhaegen et al., 2018), the presence of unique morphological traits among closely related species could provide taxonomically informative characters for species identification and these characters could be used to explore evolutionary diversification of a particular taxon.

However, although detailed assessment of shell characters can provide substantial information for gastropod classification and identification, due to their long evolutionary history and diversification, gastropods also often possess similar phenotypic traits at various taxonomical levels as a result of morphological convergence (e.g., Dowle et al., 2015; Emberton, 1995; Goodacre and Wade, 2001; Köhler and Criscione, 2015; Pfenninger and

Magnin, 2001; Vermeij, 2017; Wagner and Erwin, 2006; West and Cohen, 1996) and parallelism (e.g. Butlin et al., 2014; Gittenberger and Schilthuizen, 1996; van Moorsel et al., 2000).

Recent taxonomic treatments of the Bornean *Georissa* have revealed that this genus of minute land snails is more diverse than previously anticipated. Our recent study of the Bornean species (**Chapters 2 and 3**) revealed a total of 29 species, many of which are narrowly endemic, and which vary in a number of trait systems, most conspicuously in the type and degree of expression of the sculpture of their shells. These varying conchological features make this group of minute snails a suitable and interesting model to study character evolution which may provide a basis to understand the adaptive function of these shell characters.

Study system

Georissa is the only genus from the family Hydrocenidae that is widespread in the limestone areas of Borneo. In general, the shell of *Georissa* consists of a moderate number of whorls with distinct morphological surface features. The internal shell walls are dissolved (**Chapters 2 and 3**; Haase and Schilthuizen, 2007; Thompson and Dance, 1983; Vermeulen and Junau, 2007; Vermeulen et al., 2015). Species diversity is particularly displayed in the presence or absence of protruding sculpture on the shell whorls and varying morphological features of the protoconch and teleoconch (see **Chapters 2 and 3**); for example, the number of whorls, ribbing patterns, shell shape, and shell size. The projected scale features on the whorls are superficially similar to those seen in the minute land snails of the genera *Opisthostoma*, *Plectostoma*, and *Diplommatina* (Diplommatinidae) of the same region (Webster, van Dooren and Schilthuizen, 2012). Studies on morphological evolution of those features suggest a function in anti-predation defense (see Liew and Schilthuizen, 2014; Schilthuizen et al., 2006), and this might be one of many evolutionary responses displayed by minute terrestrial snails in this region.

In Bornean *Georissa*, phylogenetically closely related species often share unique conchological characters relating to shell shape, size, and color (**Chapters 2 and 3**). Their adult height can range between 0.7 and 4.0 mm (see **Chapters 2 and 3**; Thompson and Dance, 1983; Vermeulen et al., 2015), their shape ranges from taller than wide to wider than tall, and they vary in shell color from unpigmented (white) over moderately pigmented (yellow to orange) to highly pigmented (red to brown).

The Bornean *Georissa* are usually found foraging at limestone outcrops and nearby vegetation, while some species (e.g., *G. filiasaulae*, *G. silaburensis*, *Georissa* "sp.") have diverged from the aboveground environment by occupying a cave environment. The first recorded hypogean species, *G. filiasaulae* from the interior province of Sepulut, Sabah (see Schilthuizen et al., 2005; Haase and Schilthuizen, 2007) showed a marked niche shift accompanied by morphological changes. Similarly, a microgeographic study of *Georissa* from Mount Silabur, Sarawak (see **Chapter 4**), revealed morphological parallelism between two sympatric, but reproductively isolated, species (*G. silaburensis* and *Georissa* "sp. Silabur"), both occupying a hypogean environment.

Here, we explore the evolution of shell shape in the Bornean *Georissa*. We conducted ancestral character state reconstruction by employing the ultrametric tree presented in **Chapter 1**. Additionally, we performed phylogenetic signal analysis to determine whether genetically closely related species are likely to share similar morphological traits. We aim to obtain answers to the question to what extent are character state changes conserved within the phylogeny of *Georissa*?

Materials and Methods

Taxon sampling for molecular phylogenetic analysis

We sampled 23 species of Bornean *Georissa*, including the paraphyletic species *G. kobelti*, *G. saulae*, *G. xesta*, and *G. pyrrhoderma* (**Chapters 2 and 3**; Schilthuizen et al., 2012), each represented by one individual. Six species could not be investigated, because we failed to obtain genetic sequences (namely, *G. borneensis*, *G. leucococca*, *G. trusmadi*, *G. williamsi*, *G. corrugata*, and *G. scalinella*). As the Bornean species are not likely to form a monophyletic group, we also incorporated two species from Vietnam (*Georissa* sp. Vietnam 1 and *Georissa* sp. Vietnam 2), one from Australia (*Georissa multilirata*) and one from Japan (*Georissa shikokuensis*). For the outgroup, we obtained the DNA sequences of *Bathynerita naticoidea*, *Theodoxus baeticus*, *T. meridionalis*, *Nerita patula*, *N. maxima*, and *N. olivaria* from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Details of specimens used in this study and their accession numbers are listed in **Table 5.1**.

Shell characters and morphological assessment

The morphological characters were compiled for the species used in the molecular phylogeny based on shells examined from newly collected specimens, museum material, as well as literature data. The resulting character matrix contained one continuous and nine discrete shell characters as listed in **Table 5.2**. We categorized the continuous shell character into three discrete traits, which we defined in **Table 5.2**. This set of characters is considered as taxonomically informative for the Bornean *Georissa* (**Chapters 2 and 3**; Haase and Schilthuizen, 2007; Thompson and Dance, 1983; Vermeulen and Junau, 2007; Vermeulen et al., 2015). We used the previously taken shell measurements from **Chapters 2, 3 and 4** for shell height, shell width, aperture height, aperture width, and angles. The measurements were taken as shown in **Figure 5.1**. We measured 4 to 20 adult shells of each species (based on the available material). For shell color, we referred only to freshly collected specimens. Details of all scored characters are presented with reference to representative shells in **Figures 5.1 and 5.2**.

Table 5.1 List of specimens used in molecular analyses with their voucher, locality and GenBank accession numbers. Sources for the sequences are encoded as follows: [a]Aktipis and Giribet (2010); [b]Bunje and Lindberg (2007); [c]Frey and Vermeij (2008); [d]Khalik et al. (2018); [e]Khalik et al. (2019a); [f]Khalik et al. (2019b); [g]Current study.

Species	Voucher	Locality	GenBank accession numbers			
			COI	16S	28S	18S
Bathynerita naticoidea	MCZ DNA 102209	-	FJ977768[a]	FJ977721[a]	-	-
Theodoxus baeticus	-	Quart, Spain	AY771277[b]	AY771234[b]	-	-
Theodoxus meridionalis	-	Sicily, Italy	AY771291[b]	AY771252[b]	-	-
Nerita patula	-	Philippines	EU732286[c]	EU732123[c]	-	-
Nerita maxima	-	Papua New Guinea	EU732274[c]	EU73211 [c]	-	-
Nerita olivaria	-	Indonesia	EU732281[c]	EU732118[c]	-	-
Georissa hungerfordi	MZU/MOL 16.12	Sarawak, Borneo	MK505435[e]	MK411780 [e]	Current study	Current study
Georissa sepulutensis	BOR/MOL 12278	Sabah, Borneo	MH033955[d]	MG982360[d]	MK775833[f]	Current study
Georissa kinabatanganensis	MZU/MOL 17.26	Sabah, Borneo	MH033959[d]	MG982349[d]	Current study	Current study
Georissa silaburensis	MZU/MOL 17.07	Sarawak, Borneo	MH033946[d]	MG982316[d]	MK775941[f]	Current study
Georissa bauensis	MZU/MOL 16.01	Sarawak, Borneo	MH033936[d]	MG982309[d]	Current study	Current study

Georissa pyrrhoderma	MZU/MOL 17.21	Sarawak, Borneo	MK811486[d]	MK775808[d]	MK775903[f]	Current study
Georissa "sp. Silabur"	MZU/MOL 17.05	Sarawak, Borneo	MK811455[f]	MK775775[f]	MK775892[f]	Current study
Georissa hosei	MZU/MOL 16.04	Sarawak, Borneo	MH033905[d]	MG982338[d]	MK775914[f]	Current study
Georissa anyiensis	MZU/MOL 17.51	Sarawak, Borneo	MH033927[d]	MG982278[d]	Current study	Current study
Georissa muluensis	MZU/MOL 17.31	Sarawak, Borneo	MH033893[d]	MG982288[d]	Current study	Current study
Georissa hadra	MZU/MOL 17.32	Sarawak, Borneo	MH033897[d]	MG982282[d]	Current study	Current study
Georissa kobelti	MZU/MOL 17.38	Sarawak, Borneo	MH033882[d]	MG982290[d]	Current study	Current study
Georissa niahensis	MZU/MOL 17.25	Sarawak, Borneo	MH033954[d]	MG982298[d]	Current study	Current study
Georissa gomantonensis	BOR/MOL 7389	Sabah, Borneo	MH033876[d]	MG982259[d]	MK775829[f]	Current study
Georissa saulae	BOR/MOL 12770	Sabah, Borneo	Current study	MG982266[d]	Current study	Current study
Georissa filiasaulae	BOR/MOL 12768	Sabah, Borneo	MK505425[e]	MK411785[e]	Current study	Current study
Georissa insulae	MZU/MOL 18.02	Sabah, Borneo	Current study	MK411801[e]	Current study	Current study
Georissa pachysoma	MZU/MOL 17.63	Sarawak, Borneo	MK505443[e]	MK411789[e]	Current study	Current study
Georissa similis	MZU/MOL 16.14	Sabah, Borneo	MK505446[e]	MK411792[e]	Current study	Current study
Georissa flavescens	BOR/MOL 7416	Sabah, Borneo	MH254769[e]	MK403003[e]	Current study	Current study
Georissa bangueyensis	RMNH/MOL 5005057	Sabah, Borneo	MH254416[e]	MK402997[e]	Current study	Current study

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Georissa xesta	BOR/MOL 7258	Sabah, Borneo	MH254698[e]	MK403000[e]	Current study	Current study
Georissa nephrostoma	MZU/MOL 17.29	Sabah, Borneo	Current study	MK411798[e]	Current study	Current study
Georissa “sp. 1 Vietnam”	-	Vietnam	Current study	Current study	Current study	Current study
Georissa “sp. 2 Vietnam”	-	Vietnam	Current study	Current study	Current study	Current study
Georissa multilirata	-	Australia	Current study	Current study	Current study	Current study
Georissa shikokuensis	-	Japan	Current study	Current study	Current study	Current study

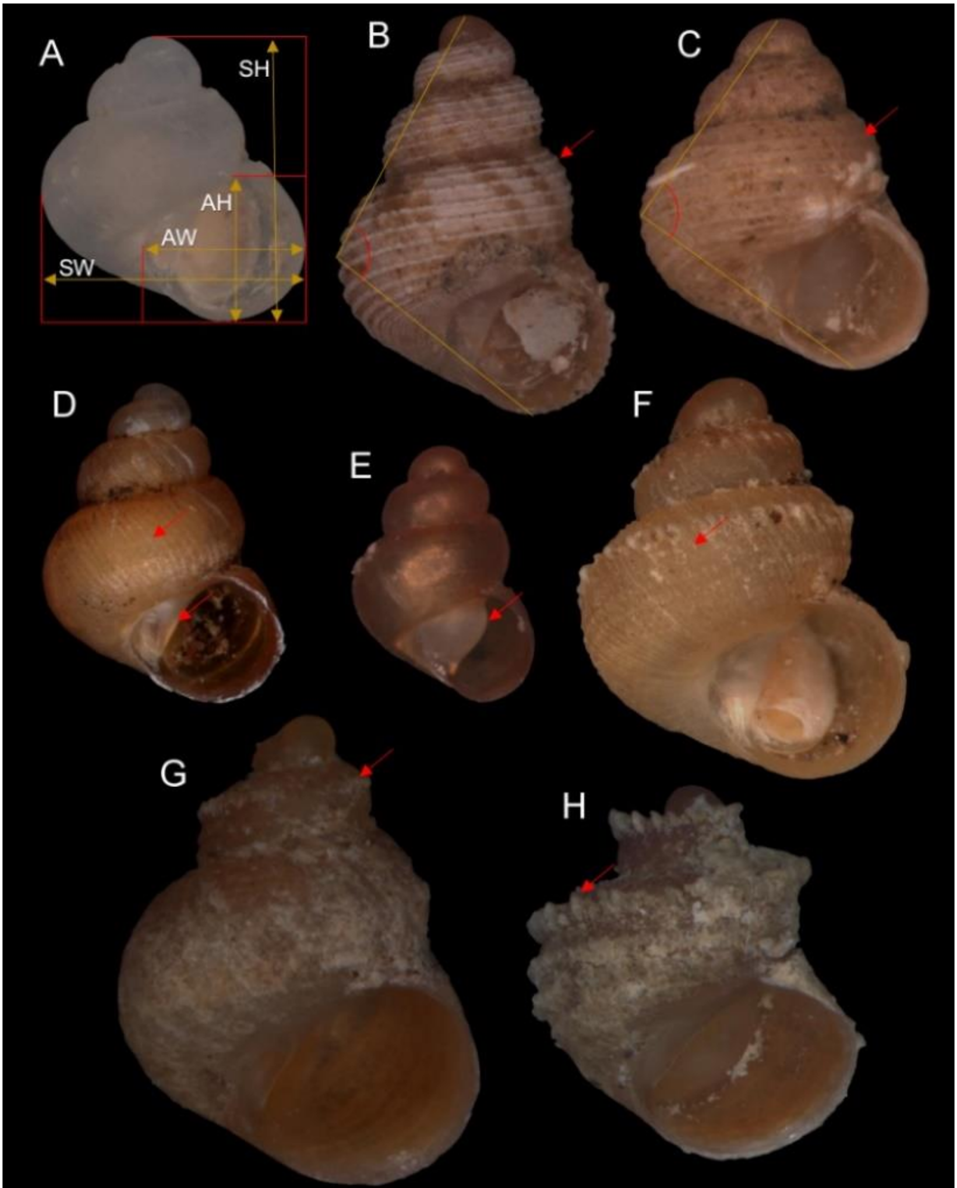

Figure 5.1 Morphological characters of the Bornean *Georissa* shown by representative species. The examined characters include shell height (SH), shell width (SW), aperture height (AH), aperture width (AW), spiral and radial ribbings, columella, shell shape, color and size (see Table 5.2). Measurements were taken as shown in **A** (*G. filiasaulae*). Other characters are indicated by red arrows on representative species (detailed in Table 5.2). **B** and **C**: spiral ribs and shell shape-angle (< 90° = broad, > 90° = slender) (*G. insulae* and *G. hungerfordi*, respectively), **D**: radial ribs and columellar wall (*G. similis*), **E**: columellar wall (*G. nephrostoma*), **F**: radial ribs (*G. saulae*), **G** and **H**: scales (*G. niahensis* and *G. anyiensis*, respectively). Scale bar equals 500 µm.

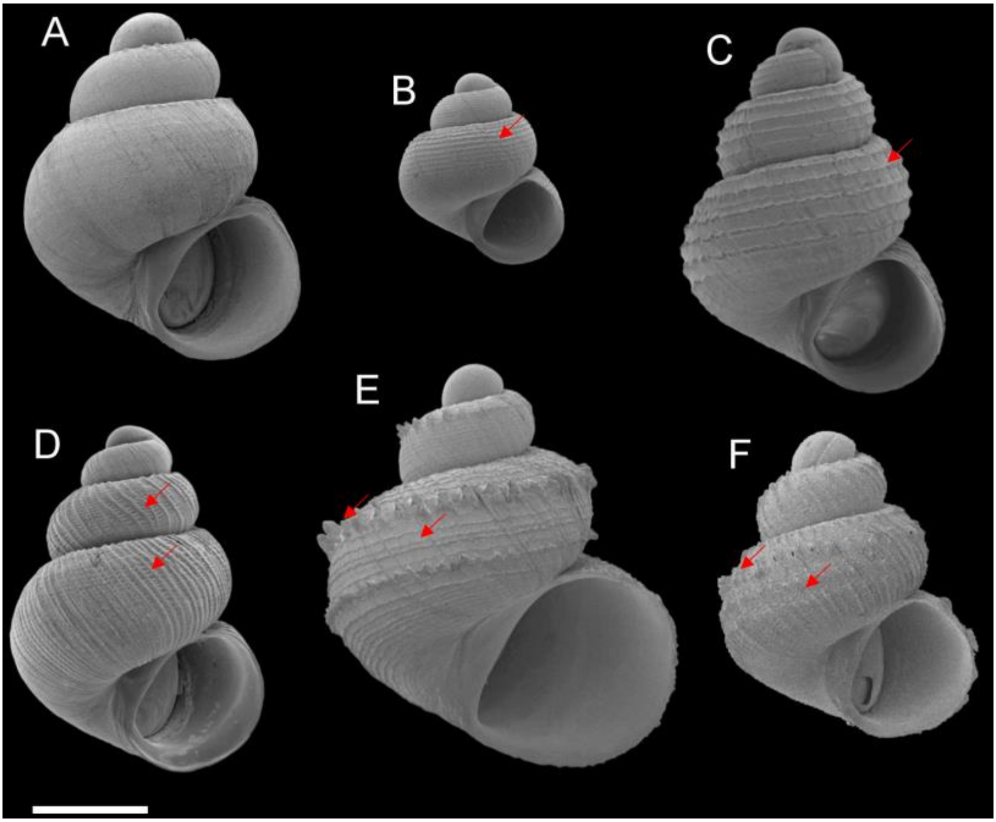

Figure 5.2 Morphological details shown by SEM. Red arrows indicate characters on representative shells (detailed in Table 5.2). **A**: smooth shell (*G. filiasaulae*), **B** and **C**: spiral ribs (*G. flavescens* and *G. hungerfordi*, respectively), **D**: radial ribs (*G. similis*), **E**: scales and spiral ribs (*G. muluensis*), **F**: scales and radial ribs (*G. saulae*). Scale bar equals 500 µm.

Table 5.2 List of characters and scoring of seven morphological traits, with references to examples illustrated in Figs 5.1 and 5.2. Selected models for ancestral state reconstruction (ASR) were determined as explained further in the methodology section.

Characters	States	ASR model	Examples
Shell surface	(0) smooth; (1) ribbed; (2) scaly	ER	Smooth: Figs 5.1A, and 5.2A Ribbed: Figs 5.1B-D and F, 5.2B-F Scaly: Figs 5.1F-H, 2E-F
Primary scale pattern	(0) none; (1) low; (2) acute	SYM	Low: Figs 5.1F-G, and 2F Acute: Figs 5.1H, 2 E
Spiral rib pattern	(0) none; (1) straight; (2) wavy/distorted	SYM	None: Figs 5.1A, 5.2A Straight: Figs 5.1B-C, 5.2D-E Wavy: Fig 5.2B Distorted: Fig 5.2D
Radial rib pattern	(0) none; (1) straight; (2) distorted	SYM	Straight: Fig 5.2D Distorted: Fig 5.2F
Shell shape	(0) slender; (1) broad	ARD	Slender: Fig 5.1B Broad: Fig 5.1C The apical angle for a slender shell > 90°, for a broad shell < 90°.
Shell size	(0) small; (1) moderate; (2) large	ER	Small: shell height < 1.5 mm Moderate: 1.5 mm < shell height < 2.5 mm Large: shell height > 2.5 mm
Shell color	(0) unpigmented; (1) moderately pigmented; (2) highly pigmented	SYM	Unpigmented: Fig 5.1A (white) Moderately pigmented: Figs 5.1C, 5.1F (from yellow to orange) Highly pigmented: Figs 5.2D-E (from red to brown)

DNA extraction, amplification, and sequencing

We extracted genomic DNA of the specimens with the Qiagen DNeasy Blood and Tissue kit using the manufacturer's protocol. The whole organism was used in the extraction procedure, with the shell (partially) included. We amplified three partial ribosomal genes, namely, *16S* (mitochondrial), *18S* (nuclear) and *28S* (nuclear), and also the partial *COI* (mitochondrial) protein coding gene. Polymerase chain reactions (PCR) were performed in 25 µL reaction volumes containing 1.0 µL undiluted DNA template following the master-mix and temperature profiling as in **Chapters 2 and 3** for *16S* and *COI* genes, Park and O'Foighil (2000) for *28S*, and Webster et al. (2012) for *18S*. Details on each primer set and temperature profiling are listed in Supplementary material 1 (**Table S1**).

The amplified fragments were Sanger-sequenced at BaseClear B.V. (Leiden, The Netherlands). The genes for which we failed to obtain sequences were treated as missing data. Accession numbers of newly obtained sequences and sequences retrieved from GenBank are given in **Table 5.1**.

Sequence alignment and phylogenetic analyses

The sequenced products were assembled in Geneious 10.2.3 assembler, manually checked and edited, and primer sequences were trimmed. The sequences were aligned using default parameters of MUSCLE (Edgar, 2004). The alignments were checked and edited including removal of the ambiguous ends. For *COI* sequences, the codon positions were identified in Geneious 10.2.3. We inferred the best fitting substitution models for each gene (partial *16S*, *18S*, and *28S* genes) and all three codon positions of *COI* in MrModelTest 2.4 (Nylander, 2004) based on the Akaike Information Criterion (AIC). The sequence data were concatenated and partitioned for further analysis. We performed phylogenetic analyses with Bayesian inference (BI) and maximum likelihood (ML) methods.

Bayesian inference was performed with MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) with the following MCMC parameters: number of generation (ngen) = 20 × 10^6; number of runs (nruns) = 2; number of chains (nchains) = 4; temperature profile (temp) = 0.2, tree sampling frequency (samplefreq) = 1,000. The log data of each independent run and both combined were inspected in Tracer 1.7.1 (Rambaut et al., 2018) to check for convergence of parameter estimates with effective sample sizes > 200. We discarded the initial 20% of the generated trees as burn-in and used the remaining trees to generate the consensus tree. We performed a maximum likelihood (ML) analysis using the same dataset with 10,000 ultrafast bootstrap replicates (Hoang et al., 2017) using IQ-TREE 1.6.3 (Nguyen et al., 2015) using a command line *-s -spp -m MF -bb 10000 -AIC* (Kalyaanamoorthy et al., 2017). The best suited nucleotide substitution models were determined using ModelFinder based on Akaike Information Criterion (AIC). We then evaluated the posterior probability (PP) for Bayesian inference and bootstrap support (BS) for maximum likelihood analyses.

Ancestral state reconstruction

The ancestral state reconstructions of the selected characters listed in **Table 5.2** were conducted in a maximum likelihood framework (Bouckaert et al., 2014) using the *ace* function in the package *phytools* (Revell, 2012), implemented in RStudio based on the maximum clade credibility tree from the BEAST 2 analysis. The best-suiting models were determined by comparing the *p*-values of log likelihoods between equal rates (ER), symmetrical (SYM), and all rates different (ARD). For each character, we did stochastic mapping on the tree with 1,000 replicates using the function *make.simmap* and *describe.simmap* (Bollback, 2006; Revell, 2012) to calculate the transition and proportion of time spent of each character state on the branches. For the ASR, we used the ultrametric tree we presented in **Chapter 1**.

Phylogenetic signal

The phylogenetic signal of each trait was estimated again in a ML framework based on the same ultrametric tree. We used two different models, *Blomberg's K* (Blomberg et al., 2003) and *Lambda* (Pagel, 1999) applying the *phylosig* function in the *phytool* package implemented in RStudio. In addition, we applied 1,000 simulation tests on all character state estimations for both models for an estimate of robustness.

Results

Sequence alignment and phylogenetic analyses

Our alignment of concatenated sequences of *COI*, *16S*, *28S*, and *18S* gene fragments had a total length of 3,009 base pairs, including gaps. The concatenated sequences were partitioned based on their codon positions and gene fragments. The model selection analysis showed that the best fitting nucleotide substitution model was GTR+I+G for all data partitions except for *18S*, where the K80+I substitution model gave a better fit. We applied these substitution models in our BI analysis in MrBayes.

Both ML and BI produced nearly identical topologies, except for Clade B, where *G. bauensis* + *G. silaburensis* are sister to all the scaly species in the BI analysis, while in the ML analysis they are not (see Supplementary material 2, **Figures S1**). *Georissa* splits into two likewise well supported clades A and B. Both clades consist of species of Sabah and Sarawak (Malaysian Borneo), and other regions (i.e., Australia, Japan, and Vietnam). Thus, the Bornean species are not monophyletic (**Figure 5.5**). Our ML and BI analyses show multiple well-supported nodes, with PP >0.95 and BS >75% values.

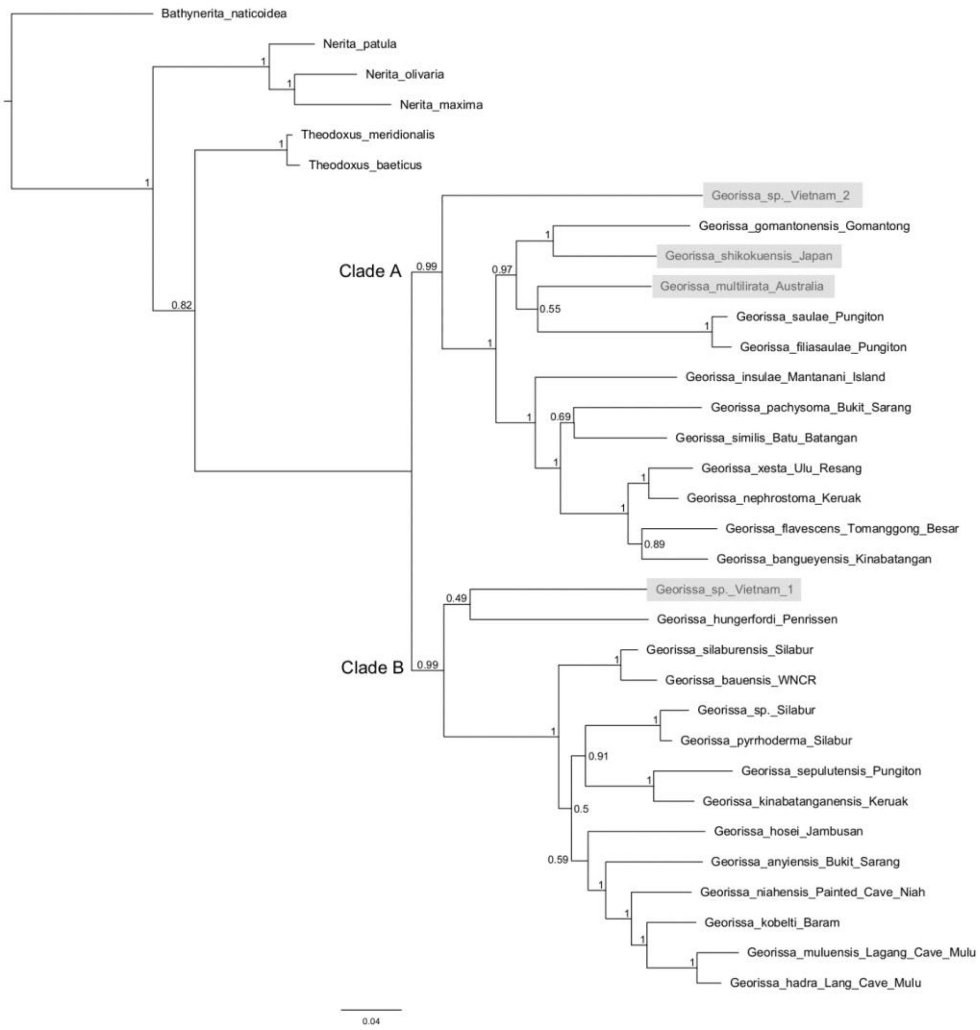

Figure 5.5 Phylogenetic tree from Bayesian inference. Non-Bornean species shaded in grey. Node support given as posterior probabilities. Scale in substitutions per site.

Ancestral state reconstruction

The results for the ancestral state reconstructions are shown in **Figures 5.6-5.9**. The absence of scale-like projections on the shell whorls of *Georissa* is likely to be ancestral. Hence, it is inferred that the character state change in the evolution of scales occurred at least twice in the phylogeny (**Figure 5.6**). For the “scaliness” of the primary scale (the first scale series on the whorls), we found that the species of clade B tend to possess a more acute scale projection (i.e., *G. anyiensis*, *G. hosei*, *G. kinabatanganensis*, and *G. muluensis*). With respect to other sculptural patterns (i.e., the radial and spiral ribbings on the shell whorls), we found that among the Bornean species, only *G. filiasaulae* has non-conspicuous sculpture and a smooth shell, and two species evolved conspicuous spiral and radial sculptures, *G. saulae* and *G. similis* (see **Figure 5.7**). For ribbing patterns, our ASR shows absence of radial ribbing as the ancestral character state of *Georissa*, while for spiral ribbing the ancestral state cannot be reconstructed unambiguously as both states "straight" and "distorted" are similarly common and scattered across the taxa suggesting multiple events of convergence.

Regarding shell shape (apical angle) and color (**Figures 5.8 and 5.9**) we found similar character state distributions in the two lineages of the Bornean *Georissa*. Broad-shelled species are generally poorly pigmented or unpigmented, with the exception of the more strongly pigmented *G. bauensis*, *G. saulae*, and *G. sepulutensis*. For shell size, medium size (between 1.5 to 2.5 mm) was reconstructed as ancestral in the Bornean species. Large-shelled species from Borneo evolved only in northern Sarawak (e.g., *G. hadra* and *G. niahensis*). As for the habitat, at least three Bornean species (e.g., *Georissa* "sp. Silabur", *G. silaburensis*, and *G. filiasaulae*) are known to occupy a hypogean habitat, while all others occupy epigean environments. However, only *G. filiasaulae* has lost the epidermal pigment in the cave environment.

Figure 5.6 Ancestral state reconstructions for shell surface (ER) and primary scale patterns (SYM). Characters mapped on the ultrametric tree resulting from BEAST 2 analysis.

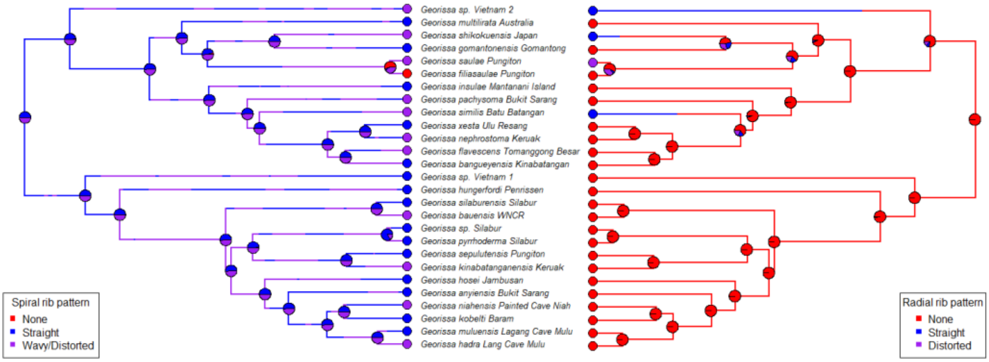

Figure 5.7 Ancestral state reconstructions for spiral rib (SYM) and radial rib (ARD) patterns. Characters mapped on the ultrametric tree resulting from BEAST 2 analysis.

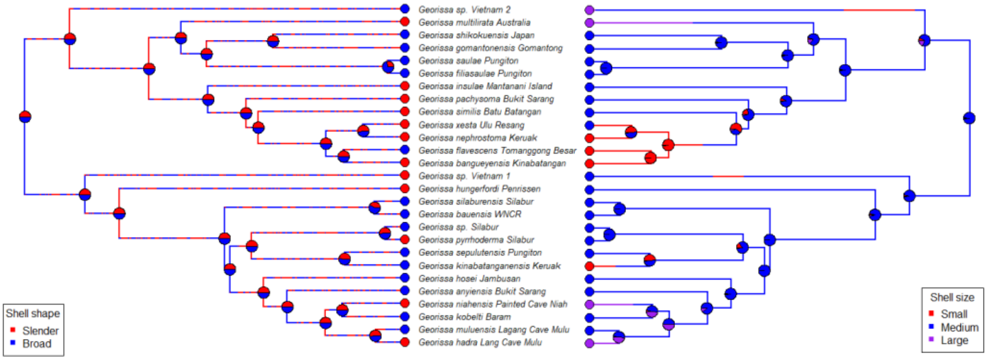

Figure 5.8 Ancestral state reconstructions for shell shape (ARD) and shell size (ER). Characters mapped on the ultrametric tree resulting from BEAST 2 analysis.

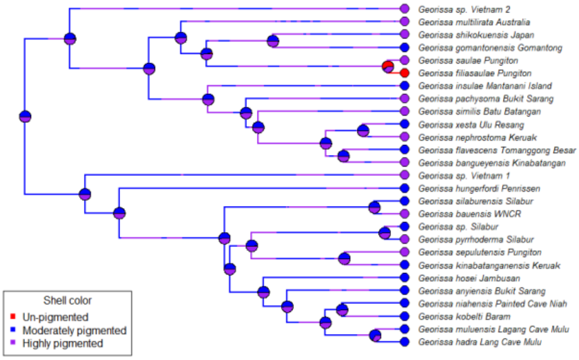

Figure 5.9 Ancestral state reconstruction for shell color (SYM). Character mapped on the ultrametric tree resulted from BEAST 2 analysis.

Phylogenetic signal

For morphological characters, our phylogenetic signal analysis based on both *lambda* (λ) and *Blomberg's K* show significant ($p < 0.05$) signal only for primary scale pattern. For shell surface, λ is significant ($p = < 0.001$) but *Blomberg's K* is not ($p = 0.533$). In addition, both methods are concordant for the remaining morphological characters, which appeared to evolve uncorrelated with the phylogeny, namely, spiral rib pattern, radial ribbing pattern, shell shape, size, and color.

Table 5.3 Phylogenetic signal and maximum likelihood model for each character in the ancestral state reconstruction analysis.

	Blomberg *K* method		Lambda method			
	K	*p*	Lambda	logL	logL0	*p*
Shell surface	0.261	0.533	0.463	-15.699	14.542	< 0.001
Primary scale pattern	0.723	0.005	0.672	-20.837	17.781	< 0.001
Spiral rib pattern	0.132	0.998	< 0.001	-22.382	-0.001	1
Radial rib pattern	0.154	0.893	0.144	-17.886	0.572	0.450
Shell shape	0.301	0.299	0.253	-19.111	0.636	0.425
Shell size	0.447	0.073	0.477	-21.287	1.205	0.272
Shell color	0.144	0.981	< 0.001	-22.738	< -0.001	1

Discussion

Phylogenetic relationships of the Bornean Georissa

Recent taxonomic treatment of the Bornean species (**Chapters 2 and 3**) delimited the species based on the combination of their geographical distribution, detailed conchology, and molecular phylogenetics. These studies have shown that the Bornean species have high allopatric and sympatric diversity. At least four species are known to be paraphyletic (*G. kobelti*, *G. saulae*, *G. xesta*, and *G. pyrrhoderma*). Our phylogenetic analyses inferred by both mitochondrial and nuclear genes, in which we incorporated the paraphyletic taxa, recovered similar relationships (see Supplementary material 2, **Figure S2**). Our results for this study are concordant with **Chapters 2 and**

3, where we also found that the “scaly” species grouped together in Clade B, with the exception of *G. saulae*.

Similarly, phylogenetic analyses showed that the Bornean *Georissa* are composed of two distantly related ancestral lineages, each of which also includes non-Bornean species, resulting in non-monophyly for the Bornean *Georissa*. Presumably, multiple invasions of several ancestral species took place in the region, similar to the situation with other minute land snails (e.g., *Diplommatina* and *Opisthostoma*; see Webster et al., 2012; Rundell, 2008).

Evolution of characters related to shell sculpture

The protruding microsculpture (i.e., ‘scales’) on the shell whorls of *Georissa* are one of the phenotypically informative traits for species identification in the genus. Our results (**Figure 5.6**) suggest a “non-scaly” origin of the Bornean *Georissa*. Based on the phylogeny and our ancestral state reconstruction, scales were obtained at least twice by the Bornean species. Although the precise function of the scales remains unclear, environmental selection in subterranean conditions could possibly cause the loss (e.g., *G. filiasaulae*) or reduction (e.g., *G. silaburensis* and *Georissa* “sp. Silabur”) of the scales on the shell whorls (see **Chapter 4;** Schilthuizen et al., 2005; Haase and Schilthuizen, 2007). As in other minute land snails from Borneo (i.e., Diplommatinidae), a stronger expression of protruding sculpture may relate to the evolution of mechanisms that defend against predators (Liew and Schilthuizen, 2014; Schilthuizen et al., 2006). *Georissa* could possibly experience similar ecological forces that lead toward similar morphological features.

Our phylogenetic analysis of the Bornean *Georissa* shows that most of the “scaly” species are grouped together in Clade B. Only the scaly *G. saulae* is grouped with Clade A, otherwise entirely consisting of “non-scaly” species.

Regarding acuteness of the scales, we found that only medium to small shelled *Georissa* possess acute projections of the scales. Our ancestral state reconstruction shows that scale acuteness diverged at least four times, and reversed at least twice in the phylogeny (Clade B). The development of acute scales could be a structural modification in response to predation. Large shelled species (e.g., *G. hadra* and *G. niahensis*) tend to possess low and minute scales on the whorls, which may indicate that the size and robustness of their shell is sufficient to resist predation.

Other phenotypically informative features of the Bornean *Georissa* are the spiral and radial ribs on the shell whorls. Despite species specificity, we found that these traits contain little phylogenetic signal (only one significant signal for Blomberg *K* and *lambda*, shell surface), and therefore probably evolve independently and repeatedly. Furthermore, there are only two not closely related species of the Bornean *Georissa* (e.g., *G. similis* and *G. saulae*) with conspicuous radial ribbing, one species with a smooth shell (e.g., *G. filiasaulae*) and one species with inflated columellar aperture wall (e.g., *G. nephrostoma*). These species are strongly diverged from their sister species, by independently having evolved novel morphological traits.

Shell size

Given the strong correlation, genus-wide, of shell height and width, we may use shell height to represent the shell size of each species. Our results based on the ancestral state reconstruction and phylogenetic signal analyses show that shell height of *Georissa* is phylogenetically conserved. We found that the Bornean *Georissa* possess a medium shell size, despite some recently diverged species having attained a larger shell (i.e., *G. niahensis* and *G. hadra*).

In addition, *Georissa* from the lower Kinabatangan valley, Sabah, possess a small shell compared to species from other geographical areas, while *Georissa* from Northern Miri, Sarawak, tend to possess relatively large shells. Thus, phylogenetically closely related species tend to evolve independently and show concerted shifts in shell size. In more specific cases (see **Chapter 4** and Haase and Schilthuizen, 2007), below-ground (cave) species often appear to have a relatively large and broad shell compared to closely related above-ground species. In general, strong shell size divergence among closely related land snail species is well known (e.g., see Chiba, 2004; Fiorentino et al., 2008; Hoso and Hori, 2008; Teshima et al., 2003), similar to what we found for *Georissa*.

Shell color

The analysis of shell color was somewhat restricted by the type of materials available for examination. Old dry specimens show decolorisation of the shell. We found no correlation between shell color and sculpture based on our ancestral state reconstruction. The hypogean species (e.g., *G. filiasaulae*, *G. silaburensis* and *Georissa* "sp. Silabur") show less pigmentation than closely related above-ground species, which indicates that shell color divergence may be driven by different habitat types (e.g., Baur, 1988; Cameron and Cook, 1989; Chiba, 2004; Chiu et al., 2002; Goodfriend, 1986).

Conclusion

Our current study revealed that the Bornean species of *Georissa* are divided into two major lineages (clades A and B). Clade A comprises species with no protruding sculptures on the shell surface, with the exception of *G. saulae*, and clade B comprises subclades of the "non-scaly" (*G.* "sp. Vietnam 1" + *G. hungerfordi*) and the "scaly" groups (all "scaly" species, except *G. saulae*). Our ancestral character state reconstructions show that many character states have evolved repeatedly and retain little phylogenetic value, with the exception of those relating to surface sculpture. Future studies which focus on morphological evolutionary patterns in *Georissa* might need to assess the ecological pressures, behavior, and diets of the individual species that possibly play important roles to the morphological divergence of the genus *Georissa*.

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Supplementary material

1. Supplementary material 1. Table S1. Primers and their respective sequences used in DNA extraction.
2. Supplementary material 2. Maximum likelihood phylogenies.

Link to supplementary material:
https://drive.google.com/drive/folders/1YBeLAomLIv8n88xGOUC9ALnwYOA23jjc