The Shell evolution of the hydrocenidae of Malaysian Borneo
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Chapter 1

A general perspective on animal diversification and the introduction to the model organism

A general perspective on diversification

Evolutionary biology is the field of study that aims to understand the evolutionary history of organisms through a certain period of time. One of its objectives is to understand how organisms respond genetically to environmental changes and the concomitant selection, and to determine which forces maintain, change, or deplete genetic variability (Houle, 1991). These responses may lead not only to anagenesis (evolution within a single lineage) but also to cladogenesis (speciation), which results in a tree-like multifurcation. Post-hoc, the relationship among two or more different species may be determined by reconstructing the phylogeny, based on molecular data and morphological characters.

Many morphological radiations in organisms occur due to new ecological opportunities in their surroundings, and this forces the organism to adapt, resulting in a continued persistence and branching out in the ecosystem (Mousseau and Roff, 1987). Accumulation of the adaptative results of selection could lead to multiple subsequent appearances or disappearances of genetic traits which, taken together, result in major phenotypic changes and the arising of new species (Chazot et al., 2016; Danowitz et al., 2015; Price et al., 2003) (**Figure 1.1**). In brief, this means that changes in the ecological system could induce changes in a species' morphological characters (Price et al., 2003). In many studies related to morphological character evolution and divergence, the researchers aim to link the morphological adaptation of their model organism with environmental change (e.g., Lovern et al., 2004; Hendenstrom and Moller, 1992; Freeman, 1981).

As niche characteristics may be geographically variable, species usually will display a spatially restricted range since it cannot establish itself beyond this range (Bridle and Vines, 2007). When such species do disperse beyond this range, they will suffer deleterious effects, leading to a negative population growth rate (Bridle and Vines, 2007). Only by evolving the ability to occupy a novel habitat and the accompanying niche, range expansion will be possible. However, this process generally requires long periods of time, from hundreds to millions of years (Pearman et al., 2008).
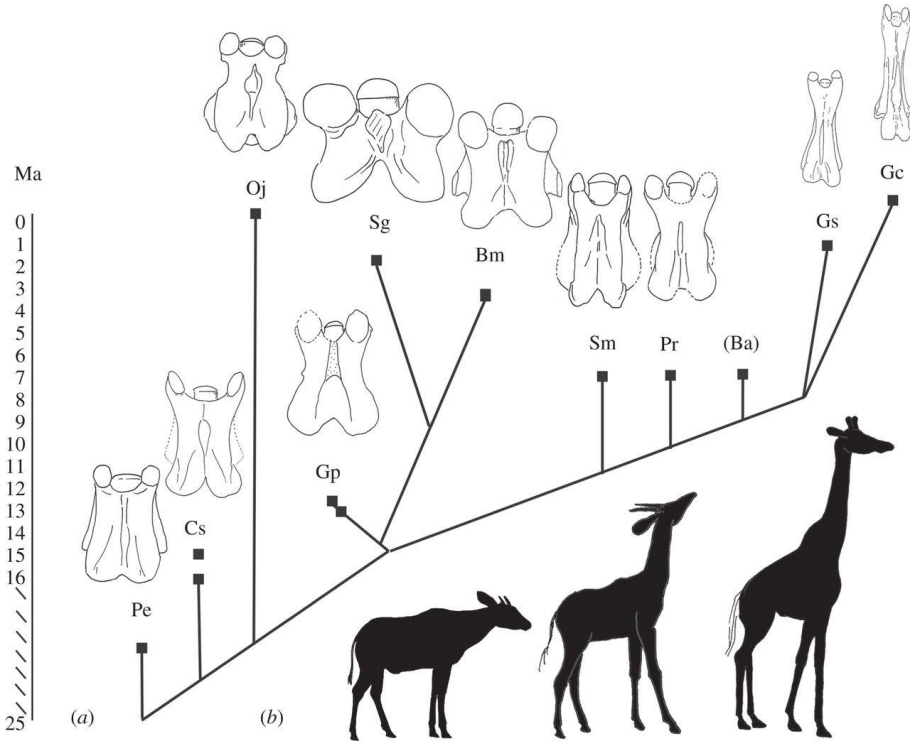
Figure 1.1 A cladogram with geological age of evaluated taxa and the dorsal view of their C3 vertebrae. Pe, *Prodremotherium elongatum*; Cs, *Canthumeryx sirtensis*; Oj, *Okapia johnstoni*; Gp, *Giraffokeryx punjabiensis*; Sg, *Sivatherium giganteum*; Bm, *Bramatherium megacephalum*; Sm, *Samotherium major*; Pr, *Palaeotragus rouenii*; Ba, *Bohlinia attica*; Gs, *Giraffa sivalensis*; Gc, *Giraffa camelopardalis*. The clades are terminated at the square points and each point indicates the age of the taxon in millions of years. The isometrically scaled V3 vertebrae are made to the equal length and they show shape/size changes through time (adapted from Danowitz et al., 2015).

In addition, we could observe that resource availability also plays an important role in the adaptive pattern of species in a complex niche area (Kohn, 1971). Competition for specific food resources in the same geographical region could induce selection to outcompete other individuals. Experimental physiology is one good approach to observe and determine the effects of different types of diets on organisms in the framework of adaptive radiation (e.g., Alföldi et al., 2011). This kind of study could explain how different types of diet (e.g., food item size) would affect the real adaptation process in the wild.

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Determining the modes of adaptation and speciation based on a particular set of niche parameters is one key point in evolutionary biology. This assists the evolutionary biologist to infer the main force(s) that drive, channel, or impede the evolutionary trajectory of a particular species. The complete set of information, data, and various kinds of analyses could be used to reconstruct the evolutionary relationships among the selected species, finding the answers of the coexistence of species, and theoretically discuss the factors that drive the species to undergo phenotypic and genotypic variation.

Evolutionary radiations in gastropods

The class Gastropoda (snails and slugs) is the largest class within the phylum of the Mollusca and estimated to contribute approximately 80% of species to all molluscan taxa (Bieler, 1992). They are widely variable with distinct characters, which differentiate them from other classes within the Mollusca. Gastropods possess shells which are best described as coiled tubes made of calcium carbonate and proteins, an extra-cellularly deposited 'organ' common to all gastropods (except those who have secondarily lost it – i.e., true slugs). Recent studies of gastropod shell evolution focus on characters such as color, size, shape, growth, and presence or absence of an umbilicus (Barker, 2005; Fiorentino et al., 2010; Hausdorf, 2006; Kuris and Brody, 1976; Liew et al., 2014; Schilthuizen et al., 2005; Teshima et al., 2003).

Studies on the evolution of land snail shell shape and size (e.g., Fiorentino et al., 2010; Liew and Schilthuizen, 2016; Teshima et al., 2003) have shown that gastropods may display very distinct morphometric characters, even at the subgeneric level, and even among phylogenetically very closely related species. Major morphological evolution in gastropod shell shape may even occur within the same species, if different populations experience different selection pressures (Kemp and Bertness, 1984; Schilthuizen et al., 2005; Teshima et al., 2003). In addition to response to directional selection, shell evolution may also be steered by 'constraints', which may take the shape of genetic, selective, developmental, and functional constraints (**Figure 1.2**), that affect the evolutionary processes and history of a species (Arnold, 1992).
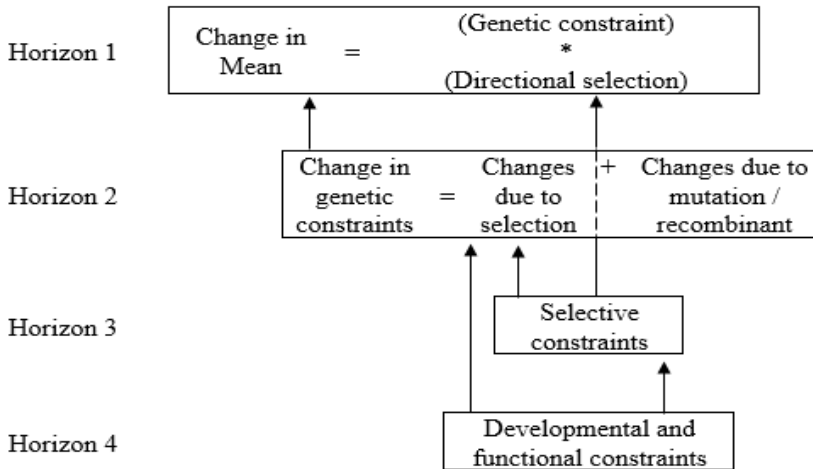
Figure 1.2 A framework for the main theoretical connection between genetic, selective, developmental, and functional constraint. Immediate consequences are shown by arrows. Adapted from Arnold (1992).

Interaction between shell and niche evolution of gastropods

The functional aspects of shell morphology are of great interest in the study of shell evolution, due to the fact that distinct characters or shell morphometrics could help unravel the evolutionary history of a population. (DeWitt et al., 2000; Hausdorf, 2006; Schilthuizen et al., 2006; Teshima et al., 2003). Teshima et al. (2003) explained adaptive shell morphology based on the patterns in a hybrid zone in *Ainohelix editha* in Japan. Using transects, they found that the angularity and globularity of the shells changed at two independent hybrid zones as the result of selective forces by the surrounding environment, with flat, keeled shells apparently adapted to either vertical or crevice-like substrates (Teshima et al., 2003). This is one example of how a new ecological niche will allow a species to change morphologically as well as to change or adjust its distribution range (Pearman et al., 2008).

In the perspective of predator-prey interaction, shelled organisms diverge in shell morphology in response to predation (Schilthuizen et al., 2006; Liew and Schilthuizen, 2014). As an example, *Plectostoma concinnum* evolved anti-predation traits (protruding radial ribs and distorted coiling of the shell), which appear to allow them to avoid predation by *Atopos* slugs. This study revealed

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how this defense mechanism works for this species, while the same characters do not seem to be the effect of adaptation to other (abiotic) ecological parameters.

As shown above, snails may evolutionarily adjust their shell morphology to suit biotic and abiotic ecological aspects of their environment, and may even experience speciation, either in sympatry or in allopatry, due to these processes. However, it is also possible that the evolution of the ecological niche is not reflected in the shell shape and that different cryptic species may be present, each with its own niche, which have solved the demands of these differing niches purely by behavioral and physiological, or soft-body morphological adaptation. Such cryptic species may only be detected and studied using genetic or anatomical means.

The model organism

Gastropoda from the clade Neritimorpha comprise four superfamilies: Helicenoidea, Hydrocenoidea, Neritoidea, and Neritopsoidea (Bouchet et al., 2005), which are thought to have diverged between the Paleozoic and early Mesozoic eras (Uribe et al., 2016). Hydrocenidae as the sole family in Hydrocenoidea consists of two widely accepted genera: *Hydrocena* and *Georissa* (Uribe et al, 2016). The genus *Georissa* is wide-spread in the Indo-West Pacific regions, and has approximately 70 described species. It is mainly characterised by dextral and calcareous shell whorls, with the presence of a rounded to ovate operculum, where a straight to curved peg emerges toward the inner part of the shell (**Chapters 2 and 3**; Thompson and Dance, 1983; Vermeulen et al., 2015).

As for the Bornean *Georissa*, Godwin-Austen (1889) conducted the first systematic study with the description of four conchologically distinct species: *G. hosei*, *G. niahensis*, *G. williamsi*, and *G. hungerfordi*. The work by Godwin-Austen (1889) was then followed by Smith (1893, 1895), Gredler (1902), and van Benthem-Jutting (1966), resulting in the description of an additional nine Bornean species. With the increasing amount of materials collected from Borneo, Thompson and Dance (1983) made the first overview on the systematics and biogeography of Bornean *Georissa*, and described three new species. Several additional Bornean *Georissa* have been described

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only recently by Haase and Schilthuizen (2007), Vermeulen and Junau (2007), and Vermeulen et al. (2015).

Habitat and distribution of the Bornean *Georissa*

Taking into account the work described in this thesis, there are now at least 29 known species of *Georissa* endemic to Borneo (see **Chapters 2 and 3**). They are particularly restricted to environments rich in calcium carbonate ($CaCO_3$) and can be found in variable abundances on wet and shaded limestone walls or rocks. *Georissa saulae* and *G. gomantongensis* on the other hand, are occasionally found on sandstone rocks and in vegetation, respectively, which are not associated with a rocky substrate (**Chapters 2 and 3**; Haase and Schilthuizen, 2007). Of all Bornean *Georissa*, at least three species are hypogean (cave species), while the rest are epigean.

The evolutionary dynamics of the Bornean *Georissa*

The overview presented by Thompson and Dance (1983) was, until now, the only revision for the Bornean *Georissa*. Furthermore, the evaluation of species validity has traditionally depended mostly on their morphological characters. Therefore, often conchologically similar species may have been misinterpreted as intraspecific variation of a species. For this reason, sympatric and/or cryptic diversity of the Bornean *Georissa* was not well understood.

Haase and Schilthuizen (2007) described the troglobitic *Georissa filiasaulae* from Sanaron cave in Sabah, Malaysian Borneo. *G. filiasaulae* has descended from the above-ground *G. saulae*, which lives on the surface of the limestone outcrop that holds Sanaron cave, and gene-flow between the two species is still occurring at cave entrances (Schilthuizen et al., 2012). *G. filiasaulae* has diverged strongly from *G. saulae* in shell sculpture, color, genitalia, and the digestive system, as well as in *16S* mtDNA sequences (Schilthuizen et al., 2005; Schilthuizen et al., 2012). These findings have led to a type of population and evolutionary studies of *Georissa* with systematic sampling design to understand the evolutionary pattern of this interesting minute terrestrial snail at a small geographical scale (see also Hendriks et al., 2019); this type of study has also been adopted in this thesis (see **Chapters 4 and 5**).

The aim of the study

In this PhD project, I am focusing on the evolutionary patterns of the *Georissa* species of Malaysian Borneo to reveal and interpret the divergence in two-character systems (a-b). To be able to correlate (a) shell shape changes in phylogeny with shifts in the environment, I will also undertake the study of (b) habitat niche evolution. Thus, the study aims to answer several questions related to physical and biological characters that occur within the genus *Georissa* which are:

- How do shell traits evolve in this genus?
- How do niche traits evolve in this genus?
- What correlations exist among the evolutionary pathways of these two sets of traits?

A first introduction to quantification and phylogenetic background of the shell characters in Bornean *Georissa*

Below, I present some preliminary attempts to quantify shell shape differences in Borneo *Georissa* and to generate divergence times for the species. I have decided to present these results here in the Introduction, rather than in any of the analytical chapters, because (a) the absence of regularly spaced landmarks on the outside of the shell, and the dissolution of the inner walls make it difficult to generate an ontogenetic axis, and (b) the absence of reliably dated calibration points renders the timetree quite imprecise. Nonetheless, I think these preliminary studies could provide a starting point for further work, which is why I have chosen to include them in my thesis, though not in any of the chapters that are intended for publication in peer-reviewed journals.

Micro-computed tomography for shell shape

The quantification of morphological divergence in gastropod shells has been discussed extensively (e.g., Chiba, 2009; Goodfriend, 1986; Haase and Misof, 2009; Liew and Schilthuizen, 2016; Schilthuizen et al., 2005). The diversity of shell morphologies may require different approaches of quantification. For example, Liew and Schilthuizen (2016) quantified the shell growth pattern of *Plectostoma* by using the ontogeny axis and the protruding apertural structures on the shell whorls. This was done by micro-computed tomography (μCT), to

obtain the complete 3-dimensional (3D) structure of the shell. The quantification of the shell may provide an insight into how the shell shape of an individual species is generated. In *Georissa*, however, the dissolved inner wall and the absence of radial ribs make it hard to define the ontogeny axis. We did µCT-scanning of the shell of the *Georissa* to obtain their 3D structure in order to determine shell shape patterns, but we applied different ontogeny and measurement approaches than Liew and Schilthuizen (2016).

The measurements were carried out in Avizo 9.4 (FEI Visualisation Sciences Group, https://www.fei.com/software/avizo/materials-science/) on the 3D reconstructed images of the shell which were segmented using the same software. Each species was represented by an individual adult shell. We measured the distances from the tip of the protoconch (a reference point) to the ontogeny axis, which we assigned to the line of points on the whorls which had the largest distance to the vertical axis of the shell (**Figure 1.3**). The ontogeny axis was also determined by following the spiral ribbing on the shell whorls (if present). The distances from the reference point along the ontogeny axis were measured based on the applied Python script (Supplementary material 1, **File 1**; see also below) as implemented in Avizo 9.4. The measurement data of each shell were extracted and plotted with RStudio (RStudio Team, 2016).
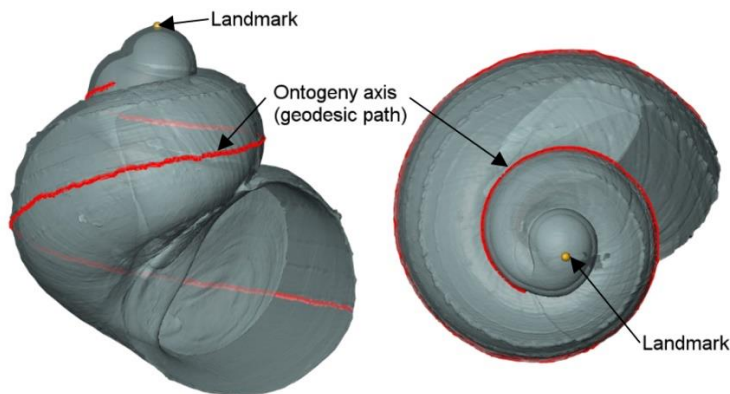

Figure 1.3 A landmark on the tip of the protoconch as a reference point for measurement purposes with an ontogeny axis, which we defined as the widest points on the shell whorl. The measurement is taken at each point on the shell whorl (pre-defined as in the Python script – one point at every one-pixel unit).

The measurement of the shape of each species of Bornean *Georissa* was conducted based on distance travelled from the tip of the protoconch along the ontogeny axis. The results of measurements on each shell (Supplementary material 2, **Table S1**) define the general shape of each species. In general, the Bornean species have a similar shell shape with linear shell growth as shown in **Figure 1.4**. The distance travelled from the landmark along the ontogeny axis serves as a reference to reflect shape of each species (i.e., greater slope indicates a more slender shell and vice versa), while the number of points indicate the length of the shell whorl. The number of points on the shell whorl were determined by the geodesic path on the shell as implemented in the Python script; one point at each 1-pixel unit or 1 μm.
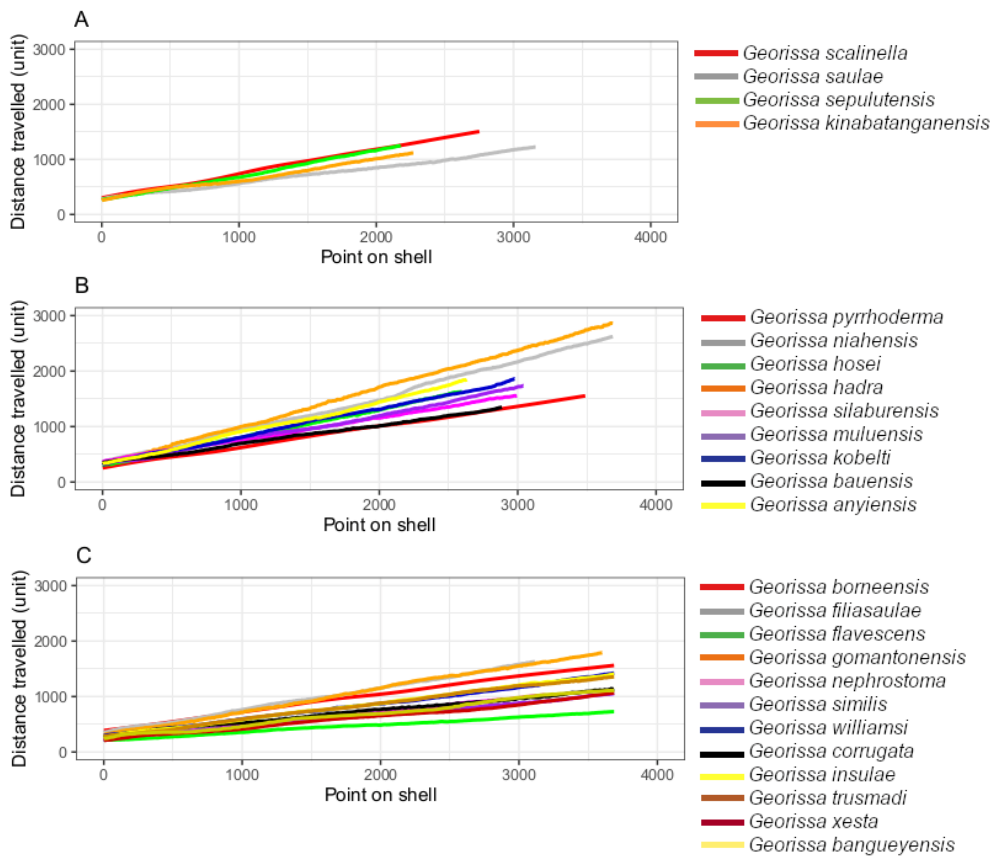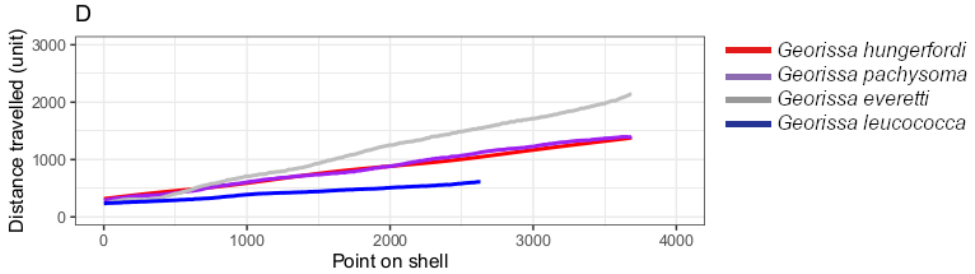
Figure 1.4 Plots of shell measurement based on the distance travelled from the tip of protoconch along the ontogeny axis (1000 units ~ 1 mm). **A** Measurement plot of the scaly *Georissa* of Sabah. **B** Measurement plot of the scaly *Georissa* of Sarawak. **C** Measurement plot of the non-scaly *Georissa* of Sabah. **D** Measurement plot of the non-scaly *Georissa* of Sarawak with two species (i.e., *G. everetti and G. leucococca*) ocurring in both Sabah and Sarawak.

All hydrocenid species have a simple coiled shell and show nearly linear shell tube expansion, resulting in an approximately regular cone (**Figure 1.4**). Slight deviations from a linear graph are discernible, which represent species-specific shell shape characteristics. Unlike the situation in *Plectostoma* (see Liew and Schilthuizen, 2016), our measurement plots provide no information regarding the shell growth rate, since there is no specific morphological feature(s) to determine increments with specific time intervals or different stages of shell growth. The plots form a visual summary of shell shape differences among species, independent of size, which are further elaborated upon in a qualitative manner in the taxonomic chapters (**Chapters 2 and 3**).

Estimation of divergence time of *Georissa*

We employed BEAST 2 (Bouckaert et al., 2014) to estimate the divergence times between the hydrocenid species and to obtain an ultrametric tree for the ancestral state reconstruction in Chapter 5. The parameters for the BEAST 2 analysis were set in BEAUTi 2 where the genes were unlinked for substitution model and linked for clock model as well as tree topology. We used bModelTest 1.1.2 (Bouckaert and Drummond, 2017) in the estimation of the Bayesian site models for each partition, selecting the transition + transversion split, since the rates of these substitutions are rarely equal. We applied an uncorrelated relaxed log-normal clock. The best fitted clock type was not tested. For the tree prior, we selected the Yule model of speciation. We ran the

analysis with 50×10^6 generations and set the sampling frequency at every 1,000 generations. The tree was calibrated at the most recent ancestor of the genus *Nerita* assuming minimum and maximum ages of 49 and 56 my, respectively, following Frey and Vermeij (2008) and Uribe et al. (2016). The MCMC results were again inspected in Tracer 1.7.1 (Rambaut et al., 2018). The maximum clade credibility tree with mean node heights was generated after discarding a burn-in of 20% using TreeAnnotater 2.5.2 (a utility program distributed together with BEAST 2).
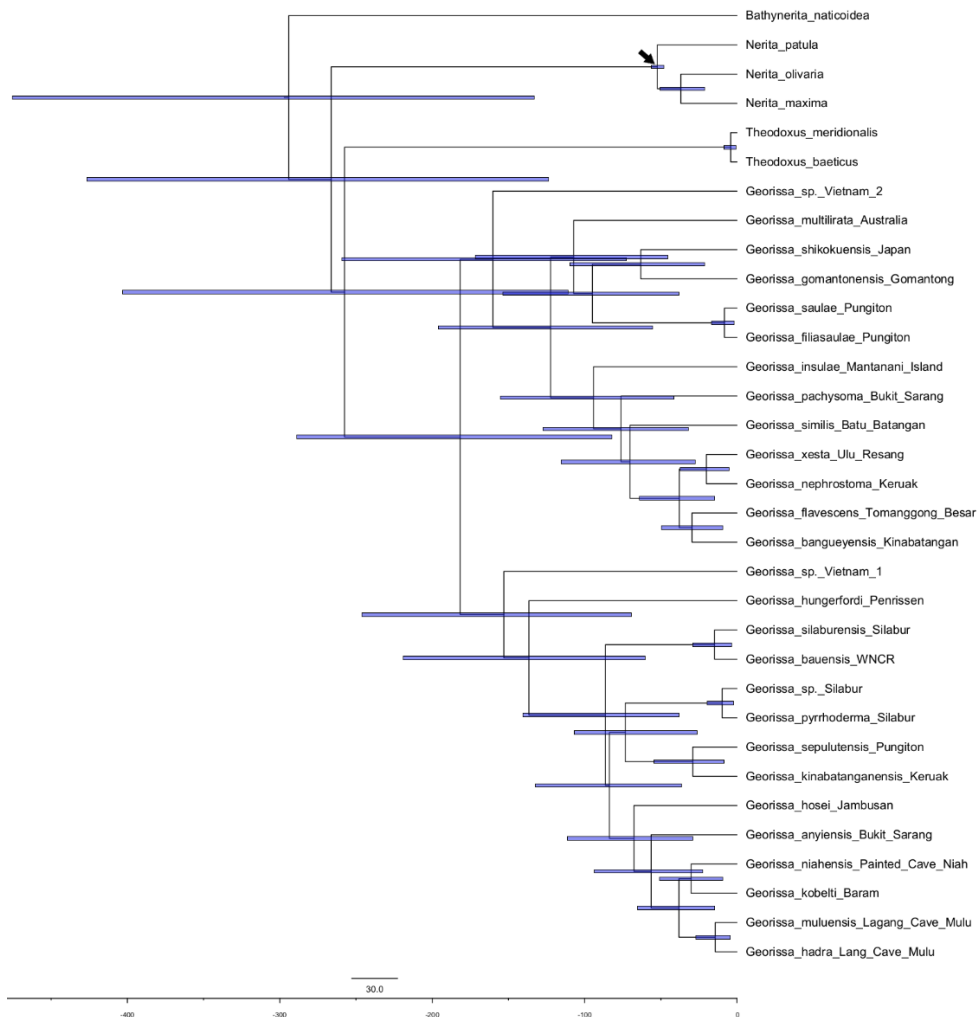

Figure 1.5 Chronogram of Hydrocenidae inferred by partial COI, 16S, 18S and 28S genes, calibrated at the node of genus *Nerita* (denoted by arrow). The horizontal bars represent the 95% highest posterior density intervals for time estimation in million years.

Our divergence time analysis based on BEAST 2 suggested that the divergence of the two major clades of Bornean *Georissa* took place between the Permian and Cretaceous (**Figure 1.5**). However, since any other calibrated time points are unavailable, these dates are to be considered tentative at best. The structure (though not the timing) of the ultrametric tree constructed in this analysis is used in our ancestral state reconstruction and phylogenetic signal analysis (**Chapter 5**).

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Supplementary material

1. File 1. Python script objects.
2. Table S1. Shell measurement.

Link to supplementary material
https://drive.google.com/drive/folders/1_iXsjYH1P2yfphCkNZN7hyJFUxgPyCo3