LAND SNAILS OF *LEPTOPOMA* PFEIFFER, 1847 IN SABAH, NORTHERN BORNEO (CAENOGASTROPODA: CYCLOPHORIDAE): AN ANALYSIS OF MOLECULAR PHYLOGENY AND GEOGRAPHICAL VARIATIONS IN SHELL FORM ~~DUE TO GEOGRAPHY~~

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Abstract. ~~The~~ *Leptopoma* is a species rich genus with approximately 100 species documented ~~according to shell morphology and animal anatomy. Many~~. The identification of ~~the~~ *Leptopoma* species ~~are described in terms of~~was mainly dependent on shell morphology (e.g. shell size, shape, ~~sculpture~~ and colour patterns) and animal anatomy of ~~a~~ small number of examined materials. However, the implications of the inter- and intra-species variations in shell form to the taxonomy of *Leptopoma* species and the congruency of its current shell based taxonomy with its molecular phylogeny are still unclear. ~~Over the last decade, more than 900 collection lots consisting of more than 4000 *Leptopoma* specimens have been obtained~~There are four *Leptopoma* species found in Sabah, Borneo, and ~~deposited in *BORNEENSIS* at Universiti Malaysia Sabah. Access~~their taxonomyic status remains uncertain due to ~~this collection gave us the opportunity to examine the geographical variations~~substantial variation in shell form~~s and the~~ . ~~In t~~This study~~,~~ focuses on the phylogenetic relationships and geographical variation in shell form of three *Leptopoma* species from Sabah ~~were focused in the study of phylogenetic relationship of *Leptopoma* species~~and ~~geographical variation in shell form based on more than 4000 *Leptopoma* specimens collected between 2000 and 2016 in Sabah~~. The phylogenetic relationship of ~~three *Leptopoma*~~these species was first estimated by performing ~~maximum likelihood~~Maximum Likelihood and Bayesian analysis based on mitochondrial genes (16S and COI) and nuclear gene (ITS-1). ~~After this~~Then, a total of six quantitative shell characters (i.e. shell height, shell width, aperture height, aperture width, shell spire height, and ratio of shell height ~~and~~to width) and three qualitative shell characters (i.e. shell colour patterns, spiral ridges, and dark ~~ring~~apertural band ~~in aperture~~) of the specimens were mapped across the phylogenetic tree and tested for phylogenetic signals. Data on shell characters of *Leptopoma sericatum* and *Leptopoma pellucidum* from two different locations (i.e. Balambangan Island and Kinabatangan) where both species occurred sympatrically were then obtained to examine the geographical variations in shell form. The molecular phylogenetic analyses suggested that each of the three *Leptopoma* species was monophyletic and indicated congruence with only one of the shell characters (i.e. shell spiral ridges) in the current morphological-based classification. ~~Other qualitative and quantitative shell characters were incongruent with the *Leptopoma* species phylogeny.~~ Although the geographical variation analyses suggested some of the shell characters indicating inter-species differences between the two *Leptopoma* species, these also pointed to intra-species differences between populations from different locations. ~~This study provides an initiation to resolve the taxonomy conundrum for the remaining 100 little known *Leptopoma* species from other regions~~This ~~is the first~~ study on *Leptopoma* species is based on small sample sizes and the findings appear only applicable

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Commented [R1]: It is not quite clear whether you describe the past situation or your current study. How about something like "Species-level identification in this group has been based on shell morphology and colouratio as well as some anatomical features based on small samp sizes."

to *Leptopoma* species in Sabah. Nevertheless, we anticipate this study to be a starting point for more detaileds investigations, especially on to include the other still rest little known (*ca*. 100) *Leptopoma* species and highlights a need to assess variations in shell characters before they could be used in species classification.

Keywords: Borneo, Cyclophoridae, integrative taxonomy, Malaysia, phylogenetic signals, Sabah, shell morphology variations.

INTRODUCTION

The terrestrial snail genus *Leptopoma* is one of 35 genera in the family Cyclophoridae family (Kobelt, 1902) with), which has a wide global distribution range that covers, extending across much of the Oriental and Australasia zoogeographical regions. An early global overview of worldwidespecies-level diversity in *Leptopoma* species classified the genus *Leptopoma* into four subgenera with a total of 105 species (Kobelt, 1902). Several subsequent regional taxonomic reviews of *Leptopoma* were conducted infor the Philippines (Zilch, 1956), Ceylon and BurmaSouth Asia (Gude, 1921), and most recently infor Borneo (Vermeulen, 1999). To date, taxonomicaltaxonomic works on *Leptopoma* (Kobelt, 1902; Gude, 1921; Zilch, 1954; Vermeulen, 1999) have been based mainly based on shell morphology. (i.e shell size, shape, colour pattern and sculpture). Besides shell morphology, other anatomical characteristicscharacters of the soft body such as radula, operculum, and genital duct have been used limitedlyfrom time to time in the species delimitation (Sarasin & Sarasin, 1899; Jonges, 1980). The phylogenetic relationship of *Leptopoma* species *per se* is not known althoughAlthough several species werehave been included in phylogenetic studies offocussing on other taxa as outgroup (Colgan *et al*., 2000, 2003, 2007; Lee *et al*. 2008a, 2008b; Nantarat *et al*., 2014a).), little is known about the relationship within the genus *Leptopom*a itself.

The genus *Leptopoma* is abundantwidespread in the Philippines and the adjacent Malaysian state of Sabah, which is located atin the northern part of Borneo Island (Godwin-Austen, 1891; Laidlaw, 1937; Vermeulen, 1999; Schilthuizen & Rutjes, 2001; Uchidal *et al*., 2013). Currently, four *Leptopoma* species could be identified from the specimens collected in Sabah with. Of these, *Leptopoma undatum* (Metcalfe, 1851) distinguished by its uniformed shell colour (translucent when young and white when old) and shell shape (less convex whorl and sharp keep at the last whorl). The other three species —*Leptopoma atricapillum* (Sowerby, 1843),) can be found in the Borneo and the Philippines (Vermeulen, 1999). *Leptopoma sericatum* (Pfeiffer, 1851) is anddistributed in the Borneo (Vermeulen, 1999). For *Leptopoma pellucidum* (Grateloup, 1840) are very similar in terms of shell shape with all showing and sharing colour pattern polymorphism. The *Leptopoma sericatum* (Pfeiffer, 1851) differs from *Leptopoma pellucidum* (Grateloup, 1840)), this species is widely spread in Sabah but the actual global range is unknown due to the presence of stronger spiral thread-like ridges (Vermeulen, 1999). *Leptopoma atricapillum* (Sowerby, 1843) – not included in Vermeulen (1999) – taxonomy uncertainty (Vermeulen, 1999). Its putative synonym, *L. vitreum* (Lesson, 1830), has a more pronounced spiral ridges and are hence more easily identified.wide range encompassing Taiwan, South Asia, and Papua New Guinea (Vermeulen, 1999).

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Leptopoma undatum is readily distinguished from the others by its uniform white shell (translucent when young and opaque when old) and distinctive shell shape (i.e. relatively less convex whorls and sharply keeled at the last whorl). The other three species, *Leptopoma atricapillum, L.~~eptopoma~~ sericatum,* and *L.~~eptopoma~~ pellucidum,* are very similar in terms of shell shape with all showing and sharing colour pattern polymorphism. *L. atricapillum* and *L. sericatum*, however have strongly-defined spiral ridges on the shell surface, with more pronounced spiral ridges in the former, whereas the spiral ridges of *L. pellucidum* are only weakly defined.

The Cyclophoridae represents the most diverse Caenogastropoda family~~ yet less~~, but is remains poorly resolved taxonomically~~ resolved~~. ~~Specific d~~Delimitation among subgenera and species in Cyclophoridae has long been a conundrum for taxonomists due to exceptionally diverse variation in morphology (e.g. subgenera in genus *Cyclophorus* and *Alycaeus* (Kobelt, 1902; Gude, 1921)). Vermeulen (1999) identified two major challenges ~~when~~with using shell characters ~~as diagnostic indicators for the Bornean~~to discriminate between six species of Borne~~o~~an *Leptopoma*~~ species. Firstly~~. First, the majority of species ~~were~~are similar in shell ~~form thus limiting~~morphology and this limits the number of shell characters that ~~could~~can be used as diagnostic ~~indicators~~characters at species level. ~~This problem was noted in Vermeulen's examination of six Bornean species. There is no doubt that this problem would become even more pronounced when examining the other *ca.* 100 species. Secondly~~Second, there ~~are intermediate shell forms between *Leptopoma* ~~appears to be a continuum of variation in some characters (i.e. size, shape, colour patterns), particularly between *L. pellucidum* and *L. sericatum* and this contributes to uncertainty when delimiting species~~ which could cause uncertainties in species delimitation.~~. Thus it is clear that to date, the implications of the intra- and inter-~~species variations in shell form, in terms of shape, size and colour patterns in the taxonomy of *Leptopoma* species~~specific variation in shell morphology have not been studied systematically and comprehensively in the context of the taxonomy of this genus.

~~Hence this~~This study ~~was conducted specifically to~~ had three aims. (1) To estimate the molecular ~~phylogeny~~phylogenetic relationship of three similar yet polymorphic *Leptopoma* species in Sabah ~~in order to~~to investigate the monophyly of *L. sericatum*, *L. pellucidum*, and *L. atricapillum* based on two mitochondrial genes (16S RNA and COI) and ~~a~~ nuclear gene (ITS-1~~),~~), (2) To test the phylogenetic signal of ~~the shell morphological characters in terms of~~ three qualitative ~~shell characters~~ and six quantitative shell ~~measurements across the phylogenetic trees in~~ characters ~~in order~~ to evaluate their reliability as diagnostic characters~~, and~~. (3) To compare the differences in shell characters of two *Leptopoma* species~~ namely,~~ *L. sericatum* and *L. pellucidum*, at two locations, where they ~~were~~co-occur and are abundant ~~and found sympatrically in order~~ to understand the geographical variations in shell ~~form~~characters under consideration and further assess their reliability as diagnostic characters. ~~The results of this study supported the monophyly of the three *Leptopoma* species in line with the current classifications by Vermeulen (1999) although only the qualitative shell character such as spiral ridges was reliable for species delimitation.~~

MATERIALS AND METHODS

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All the *Leptopoma* specimens included in this study were obtained from the *BORNEENSIS* ~~molluscaMollusca~~ collection at the Institute of Tropical Biology and Conservation in Universiti Malaysia Sabah. The collection ~~housed~~houses more than 4000 specimens of *Leptopoma* spp. collected since 2000 from various locations in Sabah (~~Fig.~~Figure 1). From this comprehensive collection, 77 ~~wet~~alcohol-preserved specimens of four species (*L. sericatum*, *L. pellucidum*, *L. atricapillum*, *L. undatum*) were selected for molecular analysis. 249 empty shells of adult snails of *L. sericatum* (114) and *L. pellucidum* (135) from Balambangan Island and the Kinabatangan region, where both species ~~existed~~exist sympatrically were selected for morphological analysis (~~Supplemetary~~Supplementary File 1). These *Leptopoma* specimens were identified ~~into~~as either *L. pellucidum* or *L. sericatum* ~~based~~ on the basis of the presence/absence of distinct spiral ridges on the shell (Vermeulen, 1999).

Data Collection

Genetic Data

Genomic DNA was extracted from a total of 77 ~~selected specimens stored~~individuals preserved in 70% ethanol ~~was isolated~~, but sequence data for at least two genes could only generated for 17 of these (Table S1 in Supplementary File 2). DNA was extracted from foot tissue by using DNeasy extraction kit (Qiagen Inc., Hilden, Germany) according to manufacturer instructions. We used two mitochondrial genes, the protein coding COI (cytochrome oxidase 1) and the non-coding 16S rRNA, and one nuclear gene ITS-1 (internal transcribed spacer 1). Universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') were used to amplify and sequence ~~mitochondrial cytochrome c oxidase subunit 1 (~~COI~~)~~ (Folmer *et al*., 1994). The 16s rRNA ~~mitochondrial gene~~region was amplified using primers 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (Kessing *et al*., 1989). ITS-1 region was PCR-amplified using the primers 5.8c (5'-GTGCGTTCGAAATGTCGATGTTCAA-3') and 18d (5'-CACACCGCCCGTCGCTACTACCGATTG-3') (Hillis & Dixon, 1991). Thermal cycling was performed with pre-denaturation at 90°C for 2 minutes, denaturation at 94°C for 45 seconds, one minute of annealing at 55°C, 60°C, and 54°C for COI, 16s, and ITS-1 respectively, extension ~~step~~ at 72°C for one minute followed by final extension at 72°C for 5 minutes. ~~Denaturation~~The denaturation, annealing and extension steps were repeated for 35 cycles. ~~Positive PCR results were obtained from 17 out of 77 DNA extracts for at least two genes (Table S1 in Supplementary File 2).~~ The PCR products were sequenced at Macrogen, Inc. (Korea). All sequences were subsequently uploaded and stored in Barcoding of Life Database (BOLD, http://www.boldsystems.org, Ratnasingham & Hebert, 2007), under the project title "*Leptopoma* in Sabah" (Code: LEPT).

Shell Morphological Characters Data

~~Quantitative~~Shell form in this study included both quantitative (i.e size and shape) and qualitative (i.e colour patterns, spiral ridges and presence/absence dark apertural band) shell characters. These morphological characters were ~~obtained~~evaluated from the ~~shell~~

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~~aperture~~apertural view of ~~all~~the 264 shells examined in total (i.e. the 249 ~~*Leptopoma*~~dry specimens and 14 adult specimens included in the phylogenetic analysis~~.~~). First, high quality photographs were taken of the aperture of each shell with the aid of a Leica Stereo Microscope M205. Five quantitative linear measurements, namely shell height (SH), shell width (SW), aperture height (AH), aperture width (AW), and shell spire height (SpH) were then ~~taken~~measured directly from the photographs ~~by~~ using Leica Application Suite software (Fig. 2A). ~~The~~A sixth quantitative shell character – the ratio ~~between~~of shell height ~~and~~to width – was computed ~~accordingly~~. Next, the states for ~~the~~ two qualitative shell characters (i.e. ~~the~~ eight ~~types of~~distinct shell colour patterns and presence/absence of the dark ~~ring~~apertural band ~~in aperture~~) were recorded for each of the shells (Figs. 2C, 2D; see Table S2 in Supplementary File 2 for the descriptions of the eight shell colour patterns).

Data Analysis

Molecular Phylogenetic Analysis

In addition to the sequences collected from 17 specimens in this study, 16S and COI sequences of *L. tigris*, *L. vitreum* and an outgroup species - *Cyclophorus formosensis* (Nevill, 1882) from Lee *et al.* (2008a) and Nantarat *et al.* (2014a) - were obtained from GenBank (Supplementary File S1, Page 1: Table S1 for informations of specimens). All the DNA sequences were aligned and checked manually using Bioedit v7.1.9 (Hall, 1999). In order to find the best-fit model of substitution, jModelTest2 (Darriba *et al.*, 2012) as implemented in CIPRES portal (Miller *et al.*, 2010) was performed based on corrected Akaike Information Criterion (AICc) for ITS-1 sequences, 16S sequences and each of the codon positions of COI sequences. Phylogenetic trees were estimated ~~by~~ using Maximum likelihood (ML) and Bayesian Inference methods (BI) as implemented in CIPRES portal (Miller *et al.*, 2010). ~~Maximum likelihood~~ML analysis was conducted using Raxml-HPC2 (Stamatakis, 2014) with ~~100~~1000 rapid bootstraps. ~~Bayesian Inference~~BI analysis was performed using MrBayes v3.2.3 (Huelsen & Ronquist, 2001~~) which~~). This consisted of running four simultaneous chains for 100,000 generations and 10 sampling frequency. The first 250 trees were discarded as burn-in, while the rest were used to obtain the final consensus tree.

Phylogenetic Signal Analysis

Phylogenetic signal analysis was ~~applied~~used to investigate the ~~congruence~~relationship between phylogeny and morphology, with all the analyses done in the R statistical environment, version 3.1.3 (R Core Team, 2015). The tips ~~of~~in the tree corresponding to juvenile specimens and outgroup taxa ~~in the phylogenetic tree~~ were excluded by using package 'ape' (Paradis *et al.*, 2004). The final tree for phylogenetic signal analysis consisted of 14 adults of the three *Leptopoma* species, *L. atricapillum*, *L. pellucidum*, and *L. sericatum*. The six quantitative and three qualitative shell characters were mapped onto the tree by utilising package 'phytools'. Phylogenetic signals for each of these nine shell characters were examined using maximum likelihood (λ) (Pagel, 1999) and K (Blomberg *et al.*, 2003). The consensus tree was transformed into an ultrametric tree after which a lambda analysis was performed using the 'chronopl' function from the 'ape' package (Paradis *et al.*, 2004). As a result, a chronogram was generated

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using penalised likelihood with an arbitrary lambda value of 0.1, the alternative model. A null model, the *Leptopoma* phylogenetic tree with $\lambda = 0$ (no phylogenetic signal), was generated using the 'rescale' function from the 'geiger' package (Harmon *et al*., 2008). The λ value of each shell character was estimated for both models using the 'fitDiscrete' function for three qualitative shell characters and 'fitContinuous' function for six quantitative shell characters in the 'geiger' package (Harmon *et al*., 2008). Likelihood scores for the alternative and null models were compared by performing a likelihood ratio test in order to examine the phylogenetic signal in each shell character, wherein Blomberg's K was calculated using the 'physig' function from the 'phytool' package (Revell, 2012; R script in Supplementary File 3).

Geographical Variation in Shell Morphology Analysis

Two-way ANOVA tests were performed to determine if there were differences in the six quantitative shell characters between: (i) the two *Leptopoma* species (*L. pellucidum* and *L. sericatum*), and (ii) the two locations (Balambangan Island and Kinabatangan). In addition, the interaction effects of both factors (species and location) were tested. A Shapiro-Wilk test for normality (Shapiro & Wilk, 1965), and a Levene's test (Brown & Forsythe, 1974) for homogeneity of variance, revealed that some datasets were not normally distributed and showed non-homogeneity of variances (Table S3 & ~~4~~S4 in Supplementary File 2). Nevertheless, two-way ANOVA tests were still conducted since the deviations of these datasets from the ANOVA assumption were considered not too serious (see boxplots of ~~Fig.~~Figure 5), and the ANOVA was considered a robust test against the normality assumption (Zar, 1999).

Chi-square two-way contingency table tests were performed to determine whether the ~~types of~~ shell colour patterns and the presence/absence of a dark ~~ring bands in the aperture~~apertural band were associated with species identity and location respectively. Prior to the analyses, four two-way contingency tables were produced ~~by summarising the frequency of the categories of~~. These summarised frequencies as follows: (1) shell colour patterns *vs.* species, (2) shell colour patterns *vs.* location, (3) dark ~~ring bands in aperture~~apertural band *vs.* species, and (4) dark ~~ring bands in aperture~~apertural band *vs.* location. Each of the tables was analysed by using Pearson's Chi-squared test. When the expected frequency in the contingency table was less than 5, a Fisher exact test was performed instead of the Pearson's Chi-squared test (Bower, 2003). All the statistical analyses were performed in the R statistical environment version 3.1.3 (R Core Team, 2015) with the significant p-values set at 0.05. (R script in Supplementary File 3).

RESULTS

The molecular phylogeny of the *Leptopoma* species in Sabah

A total of 660 nucleotide sites were aligned for the COI gene, 558 nucleotide sites for the 16S gene and 627 nucleotide sites for ITS-1 (Supplementary File 4). Gaps were treated as missing data and were retained in all phylogenetic analyses. The aligned COI dataset consisted of 36.9% GC content, 207 (31.4%) parsimony informative, and 253 (38.3%) variable sites. ~~Aligned~~The aligned 16s gene had 33.3% GC content with 276 (49.8%) parsimony informative and 406 (73.3%) variable sites. On the other hand, aligned ITS-1 gene had 48.6% GC content, 158

(25.2%) parsimony informative, and 274 (43.7%) variable sites. Phylogenetic analyses were run for four datasets: three single-gene datasets (ITS-1, COI, 16S) and a concatenated dataset of ~~ITS-1, COI and 16S whereby gaps were treated as missing data. Outgroup~~the three genes. The tree was rooted on the outgroup *Cyclophorus* ~~*formensis* was used to root the tree.~~ *formosensis*.

~~The best~~Best-fitted models were selected based on the corrected Akaike Information Criterion (AICc~~)~~); the models were TPM3uf+G for ITS-1, TIM3+G for 16S, TIM3ef+G ~~for COI first codon~~, TPM3uf+I ~~for second codon~~, and TPM3uf+G ~~for~~ respectively for first, second and third ~~codon in~~codons of COI. These models were ~~applied~~used in both ML and BI analyses. Phylogenetic trees ~~produced~~ from ~~both~~the ML and BI ~~based on~~ analyses of the concatenated dataset showed no conflict in tree ~~topologies. Therefore, only the BI tree based on concatenated dataset was shown (Figure 3). The resultant phylogenetic tree supported the~~ topology. The monophyly of the three *Leptopoma* species (*L. sericatum*, *L. pellucidum* and *L. atricapillum*) in Sabah~~. Each major clade formed by each species~~ was consistently strongly supported ~~by significant supporting values ((~~posterior probability of 100~~ PP~~%~~ and ML~~ bootstrap ~~larger~~support greater than 75~~%).~~).

Phylogenetic signals ~~relating to~~in shell characters ~~for~~of the *Leptopoma* species

Figure 4 shows the ~~inter-relation~~correlation between phylogeny and the quantitative and qualitative shell characters for *L. sericatum*, *L. atricapillum* and *L. pellucidum*. A ~~Phylogenetic~~phylogenetic signal test based on Pagel's λ and Blomberg's K showed that spiral ridges and the presence/absence of dark ~~ring~~apertural band ~~in aperture~~ represented a strong signal with λ = 1 and K > 1 (K= 4.536 for spiral ridges and K= 1.114 for dark ~~ring~~apertural band) (~~see~~ Table 1). However, shell ~~patterns~~colour pattern, a character that is often used as a diagnostic character in traditional classification ~~indicated~~, showed a weak phylogenetic signal (λ=0.997, K= 0.234). Among the quantitative shell characters, shell height exhibited a strong signal according to Pagel's λ although Blomberg's K ~~indicated~~gave a weak phylogenetic signal. The ratio of shell height to width (SH/SW) exhibited the weakest phylogenetic signal among all the shell characters (λ= 0, K= 0.054).

Geographical Variation in Shell ~~Morphology~~Forms

Two-way ANOVA showed that all shell quantitative characters (except aperture height) differed between the two locations (Table 2). In addition, all shell quantitative characters except shell width and aperture height also differed between the two species. There was interactive effect of species and location on the aperture height, shell spire height and ratio ~~between~~of shell height ~~and~~to width.

Chi-square analyses indicated a significant association between the frequencies of shell colour patterns and both ~~the factors of~~ species identity (Fisher's exact test: p =0.0000) and location (Fisher's exact test: p=0.0000). ~~Similarly~~On the other hand, there was a fairly

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significant association between the ~~frequencies~~frequency of ~~the~~ presence/absence of ~~the~~ dark ~~ring~~apertural band ~~in the aperture and both the factors of~~and species identity (Pearson's Chi-Squared with Yates' continuity correction: ~~X^2~~X^2 (1, N=249~~) =)~~=4.~~88~~019, p=0.~~0271) and~~0449) but not with location (Pearson's Chi-Squared with Yates' continuity correction: ~~X^2~~X^2 (1, N=249~~) = 12.910~~)=1.5505, p=0.~~0003~~2131). Both contingency tables are available in Table S5 & S6 in Supplementary File 2. Overall, the shell characters considered in this study did not show consistent differences between *L. pellucidum* and *L. sericatum* since the differences in shell ~~form~~forms were coupled with geographical variations and interaction effects between geography and species.

DISCUSSION

~~Although Cyclophoridae represents the most diverse family, it is also one of the less taxonomically resolved Caenogastropoda families. The current classification of many Cyclophorids was based solely on morphology characteristics where its reliability remains doubtful as this taxa possesses exceptionally diverse variations in morphology. Past research has shown that molecular phylogenetic analyses could provide insights into the taxonomy of morphologically ill land snails in this region (Nantarat *et al*., 2014a, 2014b; Liew *et al*., 2009; Liew *et al*., 2014).~~ This study presents the first molecular phylogeny investigation on genus *Leptopoma* in Sabah, one of the many understudied ~~taxa~~genera within the Cyclophoridae, and examines the concordance between morphology and phylogeny as well as geographical ~~variations~~variation in shell form.

~~All the phylogenetic trees based on different genes were congruent and provided~~We found consistently significant support for the monophyly of the three morphologically similar Sabah *Leptopoma* species ~~in Sabah namely~~, *L. pellucidum*, *L. sericatum* and *L. atricapillum*. ~~The phylogenetic placement of genus *Leptopoma* in Sabah was~~ This finding is in concordance with ~~its~~the existing traditional morphology-based classification. For example, take the placement of *Leptopoma pellucidum* 6014 (Fig. 3) in our phylogeny, this ~~study,~~ population was previously assumed ~~as~~to be *L. vitreum* (Lesson, 1830) due to its uniformly white ~~colour~~ shell ~~which differed from other *L. pellucidum*, was revealed as~~, but we have shown that it falls within the *L. pellucidum* clade ~~which supported~~, and this provides support to Vermeulen (1999)'s decision to assign *L. vitreum* ~~as synonymous~~from Sabah to *L. pellucidum*. In the case of *L. pellucidum* and *L. sericatum*, ~~Vermeulen separated them into two species provisionally due to the existence~~the presence of intermediate forms between the two species led Vermeulen to recognise the ~~two species~~ m as being distinct on a purely provisional basis. In this study, results suggested that the two species could be unequivocally regarded as separate. In short, the findings of this study are in line with past ~~research~~research~~es~~ in this region, which proposed that a combination of morphology and molecular approaches could improve taxonomy of land snails (Nantarat *et al*., 2014a, 2014b; Liew *et al*., 2009; Liew *et al*., 2014). ~~.~~However, we aware that this findings still need to be verified with more specimens of both species from their entire distribution range outside of Sabah.~~could be preliminary due to limited number of molecular specimens from small geographical coverage involved in this study~~

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A morphological character is assumed to have strong phylogenetic signal when the same character clusters together within closely-related species (Blomberg *et al*., 2003). This could be a useful diagnostic indicator for species delimitation. The phylogenetic signal tests showed that spiral ridges had a significant phylogenetic signal (λ=1, K>1). Distinct spiral ridges were present in *L. sericatum* and *L. atricapillum*, while *L. pellucidum* had weak spiral ridges. This indicated that weak spiral ridges might be an automorphy character for *L. pellucidum* which could be useful in discriminating *L. pellucidum* from *L. sericatum* and *L. atricapillum*. This result was in agreement with Vermeulen's (1999) work where spiral ridges were also used as a key to ~~delimitate between~~delimit *L. pellucidum* and *L. sericatum*.

The presence of a dark ~~ring~~apertural band ~~in the aperture of land snails~~ has not been observed in other ~~Cyclophorids~~cyclophorids and was not mentioned in other revisionary works of *Leptopoma* species. ~~A phylogenetic signal test~~Results showed that the presence/absence of a dark ~~ring~~apertural band exhibited a significant phylogenetic signal~~. However, this character was found to be strongly affected by geographical variations when two~~ and does not associate~~d~~ with geography for two of the species ~~from two different locations were compared. All shells with a~~. Nevertheless, association of dark ~~ring~~apertural band ~~located in the shell aperture were collected from a single location in Kinabatangan, i.e.~~ with species identity was fairly significant. Our results showed that dark apertural band only present in some *L. sericatum*, with this character being observed at just two sites (i.e. Kinabatangan and the Tabin Wildlife Reserve area~~. The dark ring band was presented in both species with *L.* ~~). However, when observed~~ing all the specimens in the *BORNEENSIS* collection, we found that such character is actually ~~present~~presents~~s~~ in both *L. pellucidum* ~~showing more instances than~~and *L. sericatum*. The ~~underlying causes of the presence~~intensity of ~~this shell character remain yet unknown. Compared to results from phylogenetic signal test, the presence of a~~ dark ~~ring band~~apertural band was higher in *L. pellucidum* compared with *L. sericatum*. We also observed that dark apertural band is generally present in ~~the shell aperture would not be a reliable~~shells with thickened outer lip (gerontic shell) and a high abundance of shells with that ~~such~~ character can be ~~to distinguish between~~found in the Tabin Wildlife Reserve area. This might indicate ~~d~~ a longer life span of *Leptopoma* species ~~due to geographically induced morphology variations~~in ~~from~~ the area, though this requires proper investigation. Overall, our findings here suggest that although this character shows a strong phylogenetic signal, it is not an appropriate character for species-level identification in *Leptopoma*.

Shell colour patterns are usually used as one of the key determinants to discriminate between species in traditional ~~morphology~~taxonomic classification. One of the sister taxa of *Leptopoma*, the species in genus *Cyclophorus*, was distinguished unambiguously based on shell patterns that were also supported by molecular data (Nantarat *et al*., 2014b). Compared to genus *Cyclophorus*, shell colour patterns of the ~~genus *Leptopoma*, particularly in *L.*~~*L. sericatum* and *L. pellucidum*, exhibited a weak phylogenetic signal. This case of shell colour pattern polymorphisms of the two *Leptopoma* species is similar to other well-known land snails ~~namely~~such as *Cepaea nemoralis* and *C. hortensis* (Owen & Bengtson, 1972; Ozgo & Schilthuizen, 2012; Cameron & Cook, 2012; Cameran, 2013). However, unlike *Cepaea* land snails that have been studied extensively, the causal mechanism for the *Leptopoma* land snail's

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diverse shell colour patterns is still unknown. This study also revealed that the *Leptopoma* species in Sabah exhibits idiosyncratic differences between locations in the degree of shell ~~polymorphisms~~colour patterns polymorphism. For example, the *Leptopoma* population at Balambangan Island has more shell colour patterns as compared to the population at Kinabatangan. As a result, the geographically~~- induced~~associated variations in shell colour patterns and weak phylogenetic signal strongly ~~suggest~~indicate that ~~shell patterns should not be used~~this character is unreliable as a diagnostic character for the ~~genus~~identification of the *Leptopoma* species considered in this study.

Significant inter- and intra-specific variations in quantitative shell characters ~~within or between species were often detected~~have been noted both in family Cyclophoridae (Lee *et al*., 2012; Nantarat *et al*., 2014b) and in other gastropods (Kameda *et al*, 2007; Desouky & Busais, 2012; Hirano *et al*., 2014). In Vermeulen's~~From the phylogenetic signal test, only shell height produced a significant signal. In the Vermeulen~~ (1999) ~~description~~descriptions of *L. sericatum* and *L. pellucidum*, the ratio ~~between~~of shell height ~~and~~to width of *L. sericatum* is slightly smaller than *L. pellucidum*. However, intermediate in ratio of shell height to width between the two species occur and lead to weak phylogenetic signal in this character. From the phylogenetic signal test, only shell height produced a significant signal. ~~This~~Nevertheless, this study revealed a high degree of geographical variations in the quantitative shell characters; for example, both *Leptopoma* species from Balambangan Island were larger than the same species found in Kinabatangan. Previous studies suggested that land snails found on islands tend to undergo extensive morphological diversification (Johnson & Black, 2000; Stankowski, 2011). In view of this, quantitative shell characters are thus not advisable as a diagnostic indicator ~~for~~to delimit among these two *Leptopoma* species ~~delimitation~~in Sabah due to the strong influence of geographical variations.

While our findings reflected the reliability of 'hard' character (i.e. shell sculpture) over colour patterns and banding, and revealed considerable geographical variation in some shell characters, the findings from this study were ~~only reflected the case for~~based exclusively on *Leptopoma* species from Sabah. For future work~~s~~ to improve the taxonomy of this genus, the study needs needs to be extended to include ~~to be conducted with~~ larger numbers of specimens from a larger geographical area~~extent~~. In addition, more genetic markers and examination of reproductive characters are required to elucidate comprehensive phylogeny and morphological variation among the *Leptopoma* species~~To broaden the application of our findings on the genus, improved sampling effort and experiment design are needed. For example, increased number of specimens in the study, involved larger geographical ranges, additional molecular marker, and considered reproductive characters are required to elucidate comprehensive phylogeny and morphological variation among the *Leptopoma* species.~~

CONCLUSION

This study has ~~only~~ revealed partial information on the ~~phylogenetic~~phylogeny and morphology variations of all *Leptopoma* species in their entire distribution range. ~~However,~~ D~~d~~espite its small geographical scale, the study has resolved taxonomic uncertainties of three *Leptopoma* species ~~from~~in Sabah and revealed notable variations in both the quantitative and

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qualitative shell characters for the species. From the findings, it is ~~clear~~suggested that any future revisionary attempt of the taxonomy ~~works~~ on the rest of *ca*. 100 *Leptopoma* species ~~in the future~~ should consider the possible caveats in using the shell characters as the sole evidence~~s~~ and should ~~instead~~ include molecular markers~~phylogeny~~ in the study. Further studies that include more samples from a wider geographical reach are strongly recommended.

~~**CONCLUSION**~~

~~This study represents the first attempt to conduct phylogenetic investigation into the genus *Leptopoma* and provides phylogenetic assessment of the genus in Sabah. The results unambiguously separate *L. pellucidum*, *L. sericatum* and *L. atricapillum* into three distinct monophyletic groups, and highlight substantial congruence among the traditional morphological classifications based on spiral ridges and molecular phylogeny of the *Leptopoma* species in Sabah. After performing the phylogenetic signal tests, it can be stated that all quantitative and many qualitative shell characters are not reliable diagnostic indicators for discriminating between the *Leptopoma* species due to the considerable geographical variations in shell form. This study represents an attempt to resolve the taxonomy conundrum for the remaining 100 little known *Leptopoma* species from other distribution regions. Further studies that include more samples from a wider geographical reach are recommended.~~

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SUPPLEMENTARY FILES

SUPPLEMENTARY FILE 1. Raw data for shell morphological analysis.

SUPPLEMENTARY FILE 2. Table S1. ~~Specimens~~Specimen information; Table S2. ~~Shell~~ Description of shell colour patterns ~~description~~, Table S3 & S4. Results of the normality tests and homogeneity of variances tests prior to ANOVA; Table S5 & S6. Frequency data ~~of~~for shell qualitative characters used ~~for~~in chi-square tests.

SUPPLEMENTARY FILE 3. R script for shell morphological data and phylogenetic signal analysis.

SUPPLEMENTARY FILE 4. FASTA file for the concatenated DNA sequences ~~alignments~~dataset for ~~concatenated data of~~ COI, 16S, and ITS-1.

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Figure 1: Distribution map of four *Leptopoma* species in Sabah based on the records from *BORNEENSIS* Mollusca collection, Institute of Tropical Biology and Conservation, Universiti Malaysia Sabah collected between 2000-2016 and localities of molecular samples.

Each circle represents a collection lot for a single *Leptopoma* species with the size of circle indicat~~e~~sing the number of specimens in the lot (smallest circle = 1 individuals; largest circles = 140 individuals). The insets (A) and (B) show the sympatric populations of *L. sericatum* and *L. pellucidum* (i.e. on Balambangan Island and in Kinabatangan), which were used for shell morphological analysis. Orange triangles show localities of specimens for molecular study.

Figure 2: Qualitative and quantitative shell characters included in the study were assessed on the basis of the shell apertural view.

(A) The five shell quantitative measurements: SH - Shell height, SW - Shell width, AH - Aperture height, AW - Aperture width, SpH - Shell spire height. (B) Spiral ridges: Left – Strong, Right – Weak. (C) The eight distinct shell colour patterns identified in the study. (D) Dark apertural band: Left – Presence, Right – Absence.

Figure 3: Bayesian inference tree of *Leptopoma* spp. based on concatenated dataset of 16S, COI and ITS-1.

Support values on branches are Bayesian posterior probability (BI) followed by maximum likelihood (ML) bootstrap value. Internal branches with ML bootstrap value = 100% and PP value =100 were not represents in the figure. The number shown beside each specimen of Sabah *Leptopoma* is the relevant specimen number (Table S1 in Supplementary File 2), and the specimens with asterisk are non-Borneo's *Leptopoma* species (i.e sequences obtained from Genbank). Clades A, B and C indicate the three Sabah species of focal interest. Scale bar for branch length = 0.1 substitutions per site.

Figure 4: Shell quantitative and qualitative shell characters as mapped on to the phylogenetic tree.

Tree as in Figure 3, which only 14 adult of the three *Leptopoma* species were included, whereas the juvenile specimens together with other outgroup taxa were dropped from the tree. Different categories of the three qualitative shell characters: spiral ridges, shell colour patterns and dark apertural band (Figures 1A, 1B, 1C respectively) were represented by different colour of the squares; and the six shell quantitative measurements: shell height, shell width, aperture height, aperture width, shell spire height and ratio of shell height to width were represented by the size of the circle.

Figure 5: Boxplots show the differences of the six quantitative measurements of shell for the two sympatric species (*L. pellucidum* and *L. sericatum*) in each of the two locations (Balambangan Island and Kinabatangan region).

Grey boxplot indicated sample from Balambangan Island (BI) and white boxplot indicated sample from Kinabatangan (K). Sample sizes for each dataset were: BI-pellucidum (n=45); K-pellucidum (n=90); BI-sericatum (n=46); K-sericatum (n=68).

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