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Phylogenetic relationships between isolated populations of the limestone-dwelling microsnail *Gyliotrachela hungerfordiana* (Gastropoda: Vertiginidae)

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Abstract

The vertiginid species *Gyliotrachela hungerfordiana*, an obligate limestone-dwelling microsnail, is relatively widespread and is found on a large number of isolated limestone hills in Peninsular Malaysia. To elucidate the pattern of colonization of these hills, we conducted a molecular phylogenetic analysis on *G. hungerfordiana* subpopulations from 15 separate limestone outcrops. As outgroups, we also included five related Peninsular Malaysian Vertiginidae (*Gyliotrachela frequens*, *Gyliotrachela transitans*, *Gyliotrachela salpinx*, *Gyliotrachela depressispira* and *Paraboydsidia tarutao*), one population each. A combined analysis of nuclear (internal transcribed spacer 1) and mitochondrial (cytochrome *c* oxidase 1) sequences showed that (1) *G. hungerfordiana* is monophyletic; (2) there is a clear geographical pattern in the phylogenetic relationships between *G. hungerfordiana* populations, with genetic distances increasing with geographic distance; (3) this pattern is disturbed by a few long-distance (north-west to south-east and north to south) colonizations.

Key words: Long-range dispersal – mtDNA – nrDNA – Pupillidae – South-east Asia – Mollusca – molecular phylogenetics

Introduction

Organisms with specialized habitat requirements and low dispersal capabilities tend to show highly fragmented populations and strong phylogenetic structure with little or no ongoing gene flow among the subpopulations (Gillespie and Roderick 2002). Such patterns are seen, for example, in flightless longhorn beetles on desert mountain tops (Smith and Farrell 2005) and in lizards in patches of scrub vegetation (Clark et al. 1999). In these situations, haplotypes are usually not shared among populations and tend to coalesce within, rather than among subpopulations. For these reasons, a phylogenetic rather than a phylogeographic framework of analysis is appropriate.

In this study, we adopt such an approach for studying a gastropod species restricted to the limestone hills in the Malay Peninsula. These hills are small and scattered over the landscape, most measuring less than a kilometre in diameter (Fig. 1). Although some belong to the same, larger limestone deposit (Paton 1961), each hill is separated from the next by non-calcareous alluvial deposits, creating a very fragmented and isolated habitat type, and this is associated with high rates of endemism (Clements et al. 2006, 2008), especially for organisms with high calcium requirements and low dispersal abilities, like snails (Tongkerd et al. 2004; Schilthuisen et al. 2005; Rundell 2008). In an overview of six mollusc taxa and <10% of the limestone outcrops in Peninsular Malaysia, Tweedie (1961) found that 70 of 106 species were endemic to only one hill. Despite this pattern of high endemism, some calcicolous species are surprisingly widespread. This is the case, for example, in *Gyliotrachela hungerfordiana* (Von Moellendorff, 1891) (Fig. 2), which is found on limestone outcrops throughout the peninsula. Tweedie (1961) suggested that this species may have dispersed so widely by flooding,

while Kirchner et al. (1997) suggest wind dispersal for a Greek vertiginid of similar size and ecology. Still, even if such passive modes of dispersal were important in *G. hungerfordiana*, it does not explain why other, similarly sized and equally calcicolous vertiginid species tend to show single-hill endemism.

Schilthuisen et al. (1999), using only sequences of the First Internal Transcribed Spacer of the rDNA (*ITS-1*), found a strong, positive correlation between geographic and genetic distance in a limited set of *G. hungerfordiana* subpopulations. Given that geological evidence shows that this pattern cannot reflect successive fragmentation of a single limestone plateau (Crowther 1986; Gale 1986), it probably indicates either successive colonization of nearby hills or an isolation-by-distance pattern resulting from a continuous population structure that is not at all restricted to limestone, but exists also in the non-calcareous areas in between limestone outcrops. The aim of this study, therefore, is to obtain a better understanding of the biogeographic history of *G. hungerfordiana* by performing a more extensive analysis of more subpopulations and more molecular data.

Materials and Methods

Sampling

The second author collected vertiginid microsnails on limestone hills in the states of Pahang, Kelantan, Perak, Perlis, and Kedah in Peninsular Malaysia in July 1997 and July 1999. The species and their locations are given in Table S1 and Fig. 1. Most of the limestone hills visited are lower Palaeozoic (540–400 Myr) deposits, except the ones in Kelantan (Middle & Upper Triassic; 245–200 Myr) and the Nakawan range in Perlis, which belong to the 480-Myr-old Setul formation (Gobbett et al. 1973). The snails were placed in 100% alcohol in the field and stored in the laboratory until analysis. The specimens were identified by J.J. Vermeulen, while the material remained in the alcohol. For study, we selected 15 samples of *G. hungerfordiana* and, as outgroups, one sample each of *G. frequens* Van Benthem Jutting 1961; *G. transitans* (Von Moellendorff, 1894), *G. depressispira* Van Benthem Jutting 1949; *G. salpinx* Van Benthem Jutting 1961; and *Paraboydsidia tarutao* (Panha and Burch 2001).

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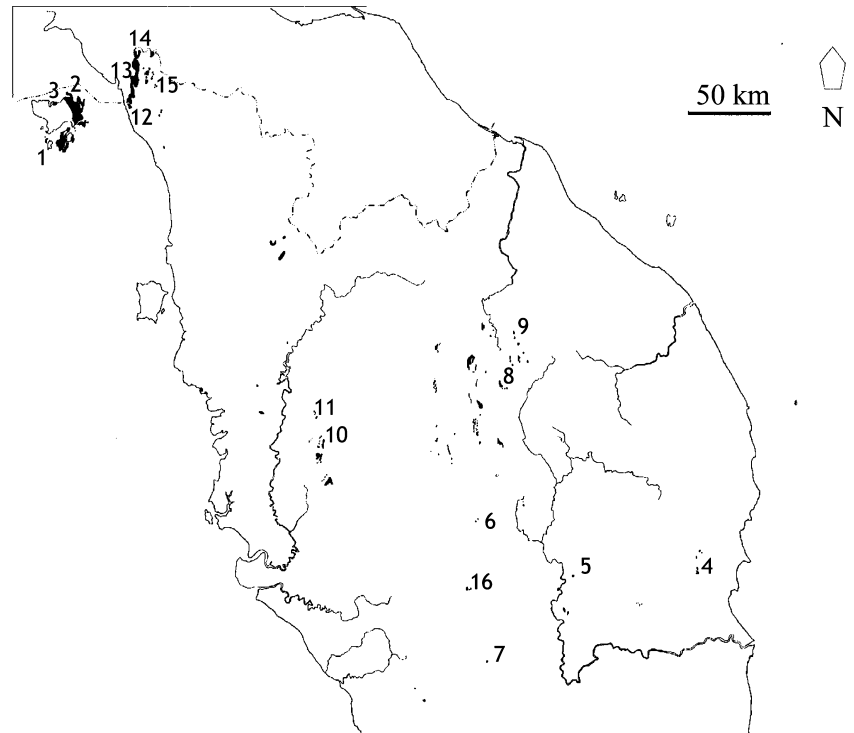


Fig. 1. A map of the northern part of Peninsular Malaysia, with limestone outcrops indicated in black. The names and numbers of collection localities refer to those in Table S1

- | | |
|---------------------------------------|--|
| 1. Kedah: Langkawi: Pulau Singa Besar | 9. Kelantan: 14 km NE of Gua Musang |
| 2. Kedah: Langkawi: Crocodile Farm | 10. Perak: Bukit Tambun |
| 3. Kedah: Langkawi: Sungai Ewa | 11. Perak: Sungai Siput Utara hospital |
| 4. Pahang: Gua Charas | 12. Perlis: 1 km S of Kangar |
| 5. Pahang: Gua Gelanggi | 13. Perlis: 9 km N of Kangar |
| 6. Pahang: Gua Bama | 14. Perlis: Kaki Bukit |
| 7. Pahang: Karak | 15. Perlis: Timah Tasoh |
| 8. Kelantan: Gua Musang | 16. Pahang: Raub |

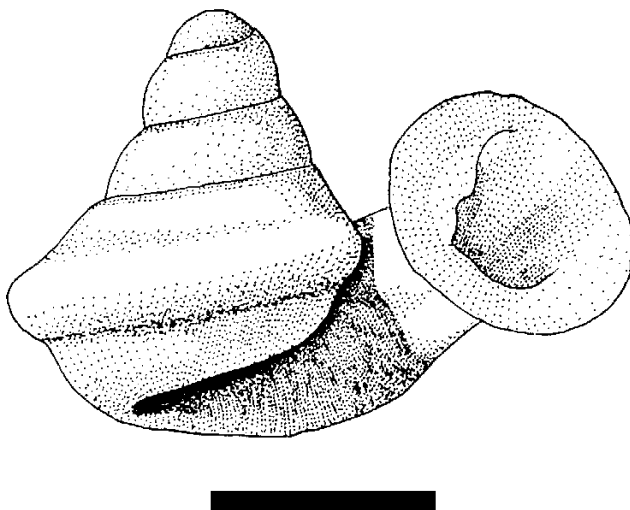


Fig. 2. *Gyliotrachela hungerfordiana* (Von Moellendorff 1891) (Vertiginidae). Scale bar = 1 mm

DNA extraction, PCR amplification and sequencing

From each sample, we selected between one and four individuals for sequencing. The entire specimens were homogenized, and DNA was extracted with the use of the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). For a few samples, only one specimen was available; from these snails, only the apex was used for the DNA extraction to maintain a voucher specimen. A fragment of the cytochrome oxidase 1 (*COI*) gene of the mitochondrial DNA was

amplified with the use of the LCO1490 forward primer (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and the HCO2198 reverse primer (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994). The 25- μ l reaction volume contained 1 μ l 10 mM forward primer, 1 μ l 10 mM reverse primer, 17.75 μ l H₂O, 2.5 μ l buffer (10x, containing 15 mM MgCl₂), 1 μ l 25 mM MgCl₂, 0.5 μ l 2.5 mM dNTPs, 0.25 μ l Taq polymerase and 1 μ l DNA extract. The cycling profile was as follows: a denaturation step of 3 min at 94°C and 40 cycles composed of 15 s denaturation at 94°C, 30 s annealing at 50°C and 40 s extension at 72°C, ending with an extension step of 5 min at 72°C. The first internal transcribed spacer (*ITS-1*) of the nuclear ribosomal DNA was amplified as described in the study by Schilthuizen et al. (1995). The universal primers 5.8c 'silkworm' (5'-GTG CGT TCG AAA TGT CGA TGT TCA A-3') and 18d 'fruitfly' (5'-CAC ACC GCC CGT CGC TAC TAC CGA TTG-3') were used (Hillis and Dixon 1991). The reaction volume was the same as in the *COI* amplification, but without the addition of extra Mg²⁺. The reaction profile contained 35 cycles and an annealing temperature of 57°C; otherwise, it was identical to the one used for *COI* amplification.

The PCR products were purified using a purification kit (Wizard[®]SV Gel and PCR Clean-up System of Promega, Madison, WI, USA). The PCR products were sequenced in both directions by Macrogen (Amsterdam, Netherlands) on an Applied Biosystems ABI3730XL automatic sequencer. All chromatograms were checked, reading errors corrected, and primer sequences removed. The sequences were submitted to Genbank under the accession numbers HM240382–HM240413 and HM243551–HM243578. The *ITS*-sequences were aligned in Bioedit 7.0.9 (Hall 1999) with the alignment in Schilthuizen et al. (1999) as reference. Both *COI* and *ITS-1* alignments [the latter with inclusion of the sequences of Schilthuizen et al. (1999); Genbank accession numbers (see Table S1) AF118000 – AF118016] were converted to nexus files in MacClade 4.08 (Maddison and Maddison 1992).

Phylogenetic and population genetic analyses

We carried out both Maximum Parsimony (MP) and Bayesian (BA) analyses. PAUP*4.0b10 (Swofford 2002) was used for MP. Gaps were treated as missing data, and uninformative characters were deleted. The data set was checked for phylogenetic signal with a permutation tail probability test with 100 replicates ($\alpha = 0.05$), and the protein coding *COI*-sequences were checked for saturation and stopcodons. A partition homogeneity test was carried out to confirm that the *COI* and *ITS-1* data could be combined ($p = 0.41$). The combined data matrix was analysed with a branch and bound search and a bootstrap analysis of 100 replicates (using a heuristic search with 10 addition sequence replicates for each replicate).

The *COI* and *ITS-1* data sets were tested for the best of 24 evolutionary models with MrModeltest v2.3 (Nylander 2004), and the best model was selected with the Akaike's Information Criterion. The HKY model (Hasegawa et al. 1985) + gamma-shaped distribution of rates across sites was the selected model for *COI* and the general time reversible model (Tavaré 1986) + gamma-shaped distribution of rates across sites for *ITS-1*.

Bayesian analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The appropriate evolution models were set, trees were sampled every 50 generations, and the rest of the parameters were left at default (two independent runs each containing four Markov Chain Monte Carlo chains). The analyses were run until the standard deviation of the split frequencies was <0.01 . The potential scale reduction factors for each parameter were also checked to see whether they were reasonably close to 1.0. A 50% majority rule consensus tree was created after discarding the first 25% of the trees.

Pairwise genetic distances were calculated in PAUP and compared with the geographic distances between populations using pairwise regression. To deal with non-independence of distances, a Mantel test was conducted in Arlequin v3.1.1 (Excoffier et al. 2005), on Kimura 2-parameter distances. The genetic differences within and between sample sites were also assessed in an AMOVA (Analyses of Molecular Variance) in Arlequin.

Results

Cytochrome *c* oxidase 1-sequences were obtained for 32 individuals. The length of all *COI*-sequences was 655 bp (excluding primers). This is similar to the lengths for the same fragment in other Vertiginidae (Nekola et al. 2009). Twenty-eight sequences of *ITS-1* were successfully amplified and added to the 17 sequences of Schilthuizen et al. (1999). Their lengths ranged from 703 to 744 bp, but all *G. hungerfordiana* sequences had lengths <722 bp. The optimal MP trees were 733 steps long, and the BA ran for 2 000 000 generations.

All six species (outgroups and ingroup) were well-supported (Figs 3 and 4), mutually monophyletic in both MP and BA analyses. Within the ingroup, *G. hungerfordiana* sequences from the same subpopulation were identical or very similar, which tended for them (especially for the subpopulations in Kedah, Kelantan, and Pahang) to form separate clades. The same was seen in the AMOVA, where most variation (90.2%

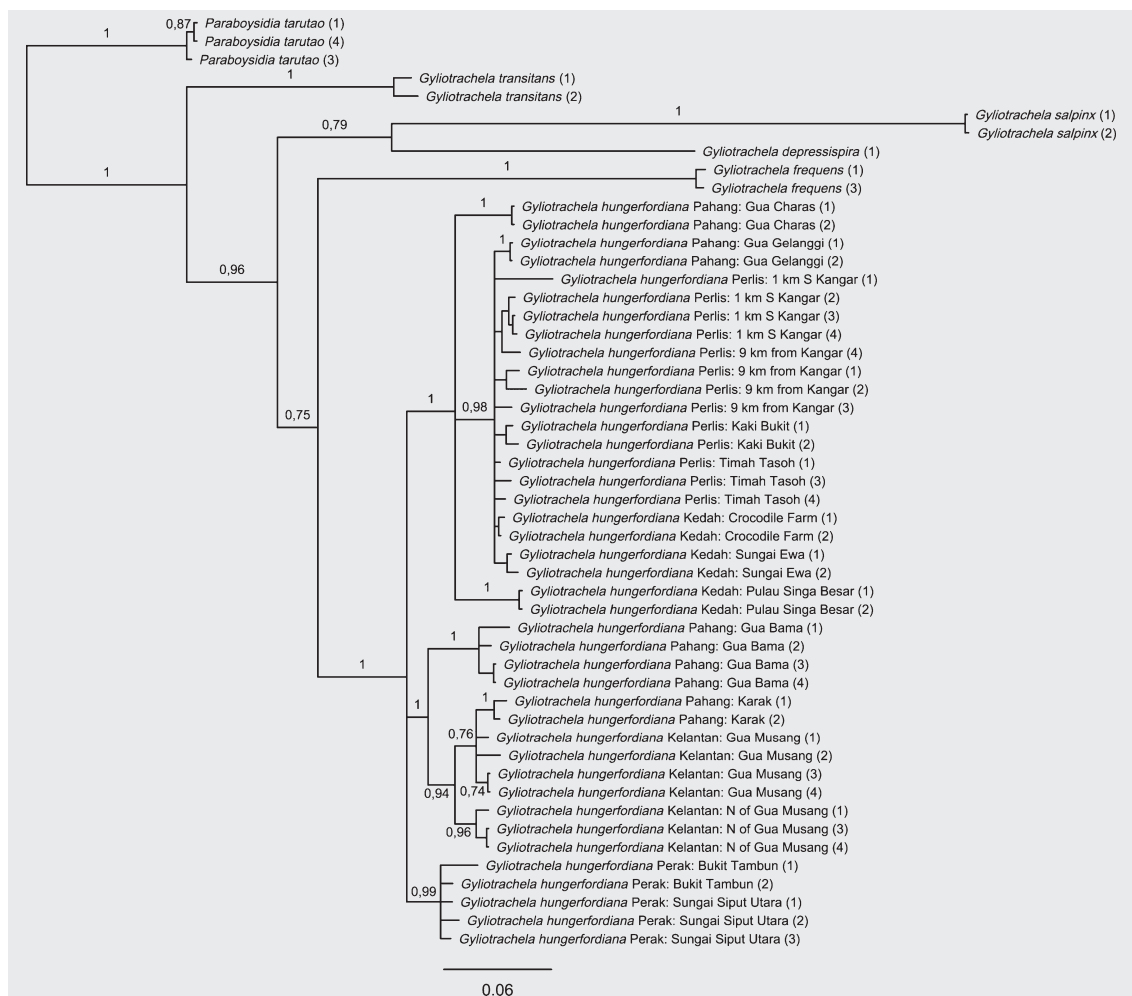


Fig. 3. Tree resulting from the Bayesian analysis of the combined cytochrome *c* oxidase 1 and internal transcribed spacer 1 data set. Where space was available, posterior probabilities have been indicated on the branches

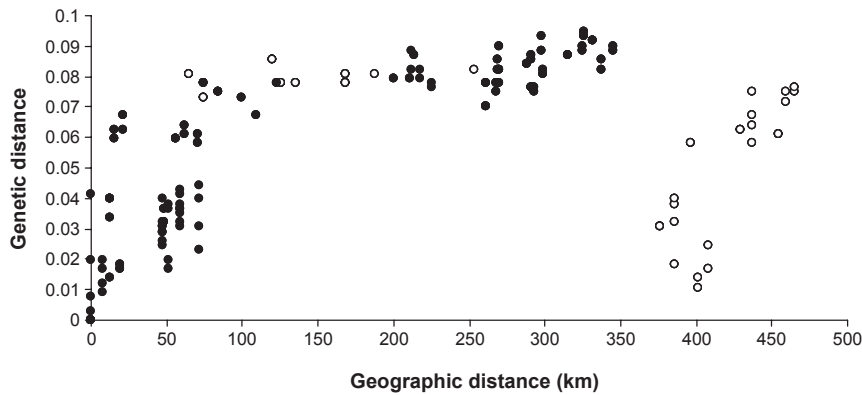


Fig. 5. Pairwise genetic distances for cytochrome *c* oxidase I against pairwise geographical distances. Open symbols indicate data points that include Pahang: Gua Gelanggi or Pahang: Gua Charas

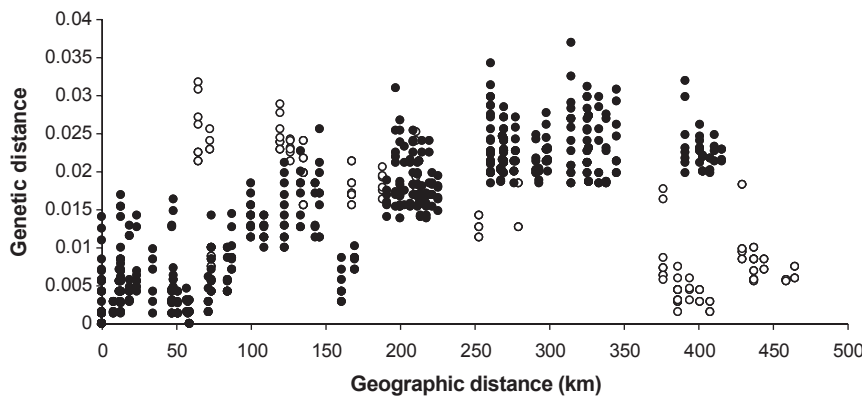


Fig. 6. Pairwise genetic distances for internal transcribed spacer 1 against pairwise geographical distances. Open symbols indicate data points that include Pahang: Gua Gelanggi or Pahang: Gua Charas

However, given the age of the range (165 Myr; Gobbett et al. 1973) and the relatively small genetic divergence between the relevant *G. hungerfordiana* clades (up to 9% for *COI*, up to 3.5% for *ITS-1*), it is not likely [based on the gastropod molecular divergence rates as tabulated by Nekola et al. (2009)] that the uplift of the mountain range has been the driving factor in the biogeographic separation. Rather, it probably was already in place when *G. hungerfordiana* evolved and served as a dispersal barrier.

The correlation found between genetic and geographic distance by Schilthuisen et al. (1999) is, to some extent, also found in this study, with nearby snails from limestone hills being genetically more similar than those from more distant limestone hills. The particularly close similarity among sequences from the Perlis limestone is congruent with the fact that all four subpopulations were collected on the same, continuous limestone massif, which may allow considerable gene flow among these subpopulations. Also, the close relationship between the subpopulations from the Perlis limestone (which reaches to the sea shore) and the ones from the easternmost limestone hills of the Langkawi islands (offshore from Perlis) suggests that the Langkawi and Perlis limestone were connected during sea level regressions in the recent past. The clear geographic pattern is disturbed, however, by the three aberrant subpopulations from Pahang: Gua Charas, Gua Gelanggi and Karak. When sequences from the former two locations are removed from the analyses, a very strong correlation between geographic and genetic distance appears, both in *COI* and *ITS-1*.

Our results thus tend to show two separate patterns: a pattern of successive colonization of ever further limestone outcrops from a starting point possibly located somewhere in

the centre of the current range, and additional long-distance dispersal over distances of hundreds of kilometres. In our present data, at least three independent long-distance dispersal events can be seen: even though the sequences from subpopulations Gua Charas and Gua Gelanggi are both nested in the Perlis–Kedah clade, they are unrelated: the ones from Gua Gelanggi are very similar to Perlis and Langkawi sequences, but the ones from Gua Charas have no close relatives. This does not necessarily mean, however, that the Gua Gelanggi colonization occurred more recently, as we may have missed sampling the source population of Gua Charas. Similarly, the subpopulation from Karak is very closely related to the ones from the vicinity of Gua Musang, in Kelantan, some 200 km to the north.

The data thus suggest two different kinds of between-hill dispersal: short-distance dispersal, in which a positive relationship exists between distance and likelihood of colonization success, and long-distance, sweepstakes dispersal. We have currently no direct evidence for dispersal of obligate limestone-dwelling microsnails, but we could offer the following hypotheses. Severe storms could be responsible for long-distance dispersal. Kirchner et al. (1997) proved that this is possible for *Truncatellina* snails (which are similar in size and ecology to *G. hungerfordiana*). However, the present day prevailing wind directions in Peninsular Malaysia are north-eastern or south-western (thus perpendicular to the direction needed for the longest-distance dispersals from Perlis–Kedah to Pahang) during the monsoons (Malaysian Meteorology Department 2009), but this may have been different in the past. Nearby, distance-dependent dispersal, on the other hand, may be attributed to migrating large mammals, which carry the snails in their fur. Alternatively, there is still the possibility that

numerous low-density, unsampled populations away from limestone exist in between limestone outcrops. In that case, the geographic pattern of phylogenetic relationships may reflect an underlying isolation-by-distance pattern rather than independent colonization events. Terrestrial microsnail sampling in Peninsular Malaysia away from limestone, as has been performed extensively in Malaysian Borneo, might resolve this matter.

An additional question to be solved is why *G. hungerfordiana* is so widespread, whereas related vertiginid microsnails (including all outgroups used in this study) are much more restricted and in many cases endemic to a single hill or cluster of hills. This may mean that either the tendency for morphological differentiation is less in *G. hungerfordiana* or its ability to disperse is greater. More detailed phylogenetic studies of Peninsular Malaysian vertiginid microsnails may resolve this question. The ongoing degradation and disappearance (mostly because of quarrying) of the Malaysian limestone hills (Clements et al. 2006; Schilthuizen and Clements 2008) makes it necessary to carry out these studies soon, while most populations still exist.

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Resumen

Relaciones filogenéticas entre poblaciones aisladas del micromolusco Gyliotrachela hungerfordiana (Gastropoda: Vertiginidae) que habitan en piedra caliza

La especie *Gyliotrachela hungerfordiana* de la familia Vertiginidae, un micromolusco que vive obligatoriamente en piedras calizas, tiene una distribución relativamente amplia y puede ser hallada en un gran número de colinas de piedra caliza aisladas en la península de Malasia. Para dilucidar el patrón de colonización de la especie en estas colinas, realizamos un análisis filogenético molecular de 15 sub-poblaciones de *G. hungerfordiana* separadas por afloramientos de piedra caliza. Como grupos externos, también incluimos una población para cada una de las cinco especies (*G. frequens*, *G. transitans*, *G. salpinx*, *G. depressispira* y *Paraboydsidia tarutao*) de la familia Vertiginidae de la península de Malasia relacionados con *G. hungerfordiana*. El análisis combinado de secuencias nucleares (Internal Transcribed Spacer 1; *ITS-1*) y mitocondriales (Cytochrome C Oxidase 1; *COI*) mostró lo siguiente: (1) *G. hungerfordiana* es monofilética; (2) existe un patrón geográfico claro de las relaciones filogenéticas entre las poblaciones de *G. hungerfordiana*, con un incremento de las distancias genéticas a mayor distancia geográfica; (3) el patrón anterior es interrumpido por algunas colonizaciones de larga distancia (noroeste a sureste y de norte a sur).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. A list of all sequences analysed in this study, including taxonomic identities, collection localities (numbers refer to Fig. 1) and Genbank accession numbers.

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