

¹*Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia;* ²*Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia;* ³*Section of Conservation Biology, University of Basel, Basel, Switzerland;* ⁴*National Institute of Water and Atmospheric Research, Hamilton, New Zealand*

Possible speciation with gene flow in tropical cave snails

M. SCHILTHUIZEN¹, A. S. CABANBAN² and M. HAASE^{3,4}

Abstract

In this paper, we present a newly-discovered troglobitic species of the snail genus *Georissa* from a limestone cave in Borneo. Molecular phylogenetic analysis of 16S mitochondrial DNA sequences shows that its ancestor is the local epigeal population of *Georissa saulae*, living in the rainforest directly at the cave entrances. A multivariate analysis of shell characters reveals that the troglobite has diverged morphologically, but is connected to its epigeal ancestor by a population of intermediate morphology in the twilight zone of the cave. The molecular data further indicate ongoing gene flow between the epigeal population and the troglobite, via the intermediate population. We suggest that the troglobite may have diverged from its ancestor without prior isolation.

Key words: Gastropoda – parapatric speciation – caves – troglobites – mitochondrial DNA – conchometry – Borneo – Sabah – 16S – principal component analysis

Introduction

Most generally accepted modes of speciation require complete isolation of populations, as gene flow is considered to prevent genetic divergence under all except a few special cases (Mayr 1999, 2001). However, various developments in the past decade, particularly a critical reassessment of laboratory experiments with *Drosophila* (Rice and Hostert 1993), empirical studies on insects and fish (Schliewen et al. 1994, 2001; Flichak et al. 2000) and advanced theoretical models and computer simulations (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999; Gavrilets 2003) have lent increasing support to the prevalence of speciation modes in which ongoing genetic exchange is not prohibitive to genetic divergence (Schilthuisen 2000a). Such models are collectively termed 'divergence with gene flow' (Rice and Hostert 1993). In this paper, we are concerned with one particular category of speciation by divergence with gene flow, namely parapatric speciation, which takes place along an environmental gradient or ecotone.

Endler (1977) and Rice and Hostert (1993) reviewed evidence suggesting that parapatric speciation may happen if an ecotonal gradient is steep relative to migration distances and divergent selection is strong and multifarious. Reproductive isolation could then evolve via cycles of pleiotropy or hitch-hiking. Recently, Doebeli and Dieckmann (2003) expanded on this model by simulating evolutionary branching in an ecotone under frequency-dependent selection because of reduced competition between dissimilar genotypes. Nevertheless, only little field evidence for parapatric divergence with gene flow is available as yet (Schilthuisen 2000b). Smith et al. (1997) and Schneider et al. (1999) showed morphological differentiation in spite of considerable gene flow across tropical rainforest edges in a bird and a lizard, respectively, and comparable data are available for parapatric morphs of intertidal snails on European coasts (Johannesson et al. 1995; Wilding et al. 2001).

In the present paper, we examine the possibility of parapatric speciation across the ecotone that exists between the above-ground (epigeal) and the cave (hypogean) environment. It is well established that these differ dramatically in environ-

mental conditions. In general, temperature and evaporation rate are lower and relative humidity higher in a cave, although the exact ranges depend strongly on the epigeal conditions (Poulson and White 1968). Furthermore, light is absent, food availability is poor, and the ecosystem complexity is much reduced, and these changes take place over an ecotone that may be as narrow as a few tens of metres (Culver 1982). In troglobites (organisms restricted to caves), adaptations to these extreme conditions are often drastic. For example, in troglobitic arthropods, the modifications usually involve the absence of light reception, sensory compensation by extension of limbs, increased size of the digestive system, and a shift from reproductive *r*-strategy to *K*-strategy (Wilkens et al. 2000).

It may thus be expected that populations that straddle the ecotone between epigeal and hypogean environments, are likely candidates for parapatric speciation. Yet troglobites have usually been considered the opposite: classical examples of allopatric speciation after isolation of small groups of founders in the deep recesses of a cave (Culver 1982; Barr and Holsinger 1985; Sbordoni et al. 2000). This is probably partly because of the fact that troglobites mostly have been studied in temperate caves, where their above-ground ancestors have disappeared because of the severe effects of glaciation. Recently, however, biospeleological work in the Tropics has increased and many examples of troglobites with existing epigeal sister species have been discovered (Peck and Finston 1993; Holsinger 2000). Some authors have suggested parapatric speciation as an explanation for such cases (Wilkens and Hüppop 1986; Peck and Finston 1993), but very few detailed studies have been done.

Here, we present a troglobitic species within the saxicolous land snail genus *Georissa* (Hydrocenidae). The (undescribed) species, *Georissa* n. sp., inhabits a cave in an isolated limestone outcrop in western Sabah, Malaysian Borneo. On limestone in the rainforest surrounding the outcrop and on the outside of the cave entrances lives the related species *Georissa saulae*. The cave species differs from *G. saulae* in having a broader shell that has lost its sculpture. Also, its body and shell are largely unpigmented (Fig. 1). Using morphometrics and sequencing of

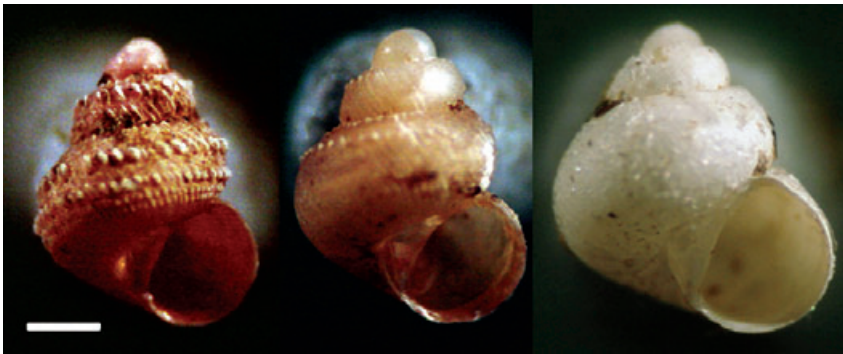


Fig. 1. Representative shells. Left: *Georissa saulae* (OUT-1); Center: *Georissa* n. sp. cave species (IN-1); Right: *Georissa* n. sp. cave species (IN-2). Scale bar equals 0.5 mm

mtDNA from populations of both species, we attempt to establish whether a parapatric speciation scenario is possible for the given situation. The results show that the cave species has descended from the local population of *G. saulae* and that the two species experience gene flow via an intermediate population in the ecotone. The geographic distance between the cave species and *G. saulae* is not more than 120 m. Consequently, the situation suggests that the two forms may have diverged parapatrically.

Materials and Methods

Location and sampling

Batu Sanaron is located in the Sepulut valley in the Interior Province of Sabah, Malaysian Borneo (GPS-derived coordinates: 04°42.052'N, 116°36.016'E), at 450 m above sea level. It is a karstic outcrop of Oligocene limestone (Yin 1985), measuring about 600 by 300 m. The hill contains a system of cave passages, which has not yet been fully explored (Francis 1987). A small stream runs in northerly direction through part of the cave system. The surrounding vegetation of Batu Sanaron consists of logged lowland dipterocarp rainforest on gently sloping ground. The outside of the limestone itself is covered in characteristic (Vermeulen and Whitten 1999) calcareous vegetation.

Populations of *Georissa* n. sp. were sampled near two entrances to the cave (Fig. 2). 'IN-1' was located approximately 60 m from a cave entrance, in an area between the 'twilight zone' and the 'transition zone' (Howarth 1987). 'IN-2' was located approximately 120 m from a different cave entrance, in the 'deep zone' of the cave. We decided to sample only these two cave populations, since the snails were not seen in sufficient density in other parts of the cave. In addition, two samples of the epigeal *G. saulae* (van Benthem Jutting 1966), 'OUT-1' and 'OUT-2' were taken from the hill surfaces closest to the locations of IN-1 and IN-2, respectively. Voucher specimens of these samples have been deposited in the *BORNEENSIS* collection at Universiti Malaysia Sabah under reference numbers BOR/MOL/2663–2667.

For use in the molecular analyses, we included several other samples of *G. saulae* and related species: two individuals of *G. saulae* from Batu Sinobang, 10 km north of Batu Sanaron (04°48.040'N, 116°37.035'E; BOR/MOL/34); two individuals of *Georissa scalinella* (van Benthem Jutting, 1966) from Batu Sanaron (BOR/MOL/39); one individual of *Georissa monterosiana* Godwin Austen and Nevill, 1879 from Bukit Sagu in Pahang, Peninsular Malaysia (BOR/MOL/22); and one individual of *Georissa semisculpta* Godwin Austen and Nevill, 1879 from Merapoh in Pahang, Peninsular Malaysia (BOR/MOL/47). *G. saulae* and *G. scalinella* are closely related species (Thompson and Dance 1983), and the only species of the *hosei*-group present in Sabah. *G. semisculpta* and *G. monterosiana* are more distantly related and may be considered an outgroup.

Biometrics and multivariate analysis

For biometric analysis, we selected mature specimens from all four Batu Sanaron samples: IN-1 (*Georissa* n. sp., 15 individuals), IN-2 (*Georissa* n. sp., 8 individuals), OUT-1 (*G. saulae*, 16 individuals), and

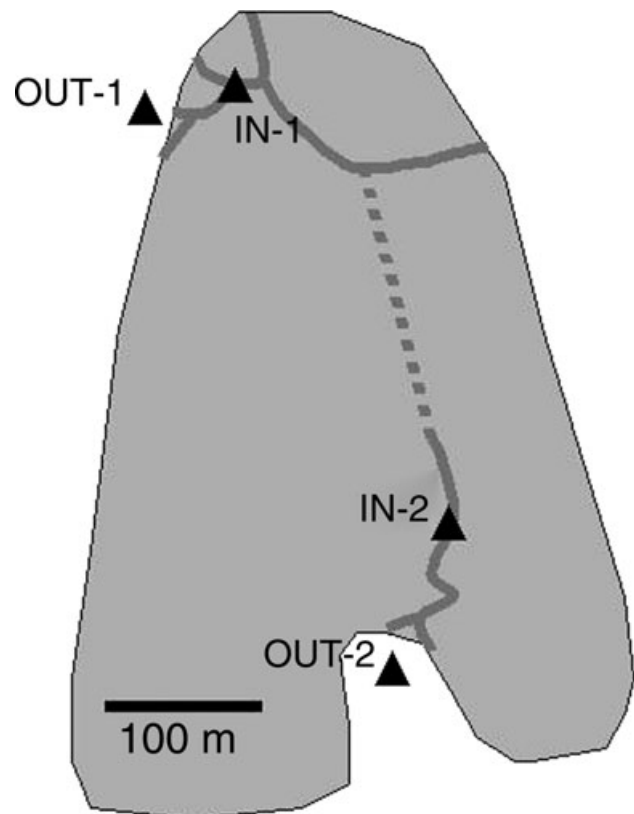


Fig. 2. A map of Batu Sanaron (light grey) and its cave system (dark grey with unexplored parts dashed) and sampling localities (black triangles) for IN-1, IN-2, OUT-1, and OUT-2

OUT-2 (*G. saulae*, 14 individuals). Each individual was scored for overall shell pigmentation (0, white; 1, yellow; 2, orange; 3, red) and measured for the following morphometrics: shell height; shell width; aperture height (measured at the inside of the aperture, from the basal-columellar corner to the columellar-parietal corner); aperture width (measured at the inside of the aperture, along the furthest distance between the columellar and the palatal sides); number of discernable spiral bands of granules on the ultimate whorl in apertural view; granule length for the primary, secondary, and tertiary spiral bands (arranged according to prominence), each averaged over five randomly selected granules each.

We subjected the nine biometrics above to a principal component analysis (PCA). First, variables were checked for normality. As not all were normally distributed, they were log-transformed in PC-ORD for Windows version 4.14 (MJM Software, Gleneden Beach, OR, USA). A subsequent analysis in PC-ORD revealed no outliers. The transformed data were then subjected to a factor analysis in SPSS for

Windows, version 11.0.0 (SPSS, Chicago, IL, USA). The first two components extracted 89% of total variance; 60% of all correlations lay between 0.30 and 0.75; Bartlett's test of sphericity was significant ($p < 0.001$) and the Kaiser–Meyer–Olkin measure of sampling adequacy was >0.6 (viz. 0.865); communalities were all above 0.7. Thus, the most important conditions for carrying out a PCA were satisfied (Jolliffe 1986).

MtDNA sequencing and phylogenetic analysis

Genomic DNA was extracted, following the method of van Moorsel et al. (2000) from 45 separate individuals of *Georissa*, viz: *G. saulae* (OUT-1: 10 individuals; OUT-2: nine individuals; Batu Sinobang: two individuals), *Georissa* n. sp. (IN-1: 10 individuals; IN-2: 10 individuals), *G. scalinella* (Batu Sanaron: two individuals), *G. monterosatiiana* (Bukit Sagu: one individual), and *G. semisculpta* (Merapoh: one individual). For each extraction, we amplified a c. 510 bp fragment of 16S mtDNA, using the primers LR-J-12887 (5'-CCGGTCTGAACT-CAGATCACGT-3') and LR-N-13398 (5'-CGCCTGTTAACA-AAACAT-3') from the University of British Columbia's universal insect mtDNA primer set (Nucleic Acid-Protein Service Unit; J.B. Hobbs). Polymerase chain reactions were carried out in 50 μ l reaction volumes, using 0.5 μ l of undiluted template, 10 pmol of each primer, a final Mg^{2+} concentration of 4.5 mM, and otherwise standard reaction conditions. The amplifications were done on a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA) using the temperature profile: 5 min denaturation at 95°C, followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 60 s; the programme was terminated with a final extension step of 5 min at 72°C. PCR products were purified on Roche spin columns and cycle-sequenced directly on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using LR-J-12887 as sequencing primer.

The sequences from IN-1, IN-2, OUT-1 and OUT-2 were aligned manually in the BioEdit Sequence Alignment Editor version 5.0.9 (T. Hall, North Carolina State University). A representative of each of the recognized haplotypes was then aligned with the sequences of *G. saulae* (Batu Sinobang), *G. scalinella*, *G. semisculpta* and *G. monterosatiiana*, using default settings in ClustalW version 1.8 (Thompson et al. 1994). We refrained from any subsequent manual adjustments. The data set was subjected to maximum-parsimony (MP) and maximum-likelihood (ML) tree construction in PAUP*4.0 version b10 (Swofford 1998). Under MP, we performed an exhaustive search, treating gaps as a fifth character state. Under the ML optimality criterion, we assumed the two-parameter model of Hasegawa et al. (1985), with transition–transversion ratio and nucleotide frequencies estimated by ML. A branch-and-bound search with tree-bisection–reconnection branch-swapping was performed. ML and MP trees were rooted with the sequences of the Peninsular–Malaysian outgroup species, and the stability of branches was tested with 500 bootstrap replicates. The sequences were deposited in Genbank under accession numbers AY547380–AY547389. Using statistical parsimony (Templeton et al. 1992) in the computer programme TCS version 1.13 (M. Clement et al., Brigham Young University), we created a network of all haplotypes present in IN-1, IN-2, OUT-1, and OUT-2.

Results

We scored and measured nine shell characters in two samples of the *Georissa* n. sp. cave species, IN-1 (in the twilight zone of the cave) and IN-2 (in the deep zone of the cave), and two samples of *G. saulae* outside the cave, OUT-1 and OUT-2. The first two axes of the PCA extracted 89% of total variance. The groupings in the PCA show that OUT-1 and OUT-2 fully overlap, whereas both are completely separated from IN-2, which has broader and larger shells, reduced pigmentation, and reduced sculpture. IN-1, which occupies the twilight zone of the cave, forms a well-defined group with intermediate morphology, occupying the morphospace in between IN-2 and OUT-1 + OUT-2 (Fig. 3).

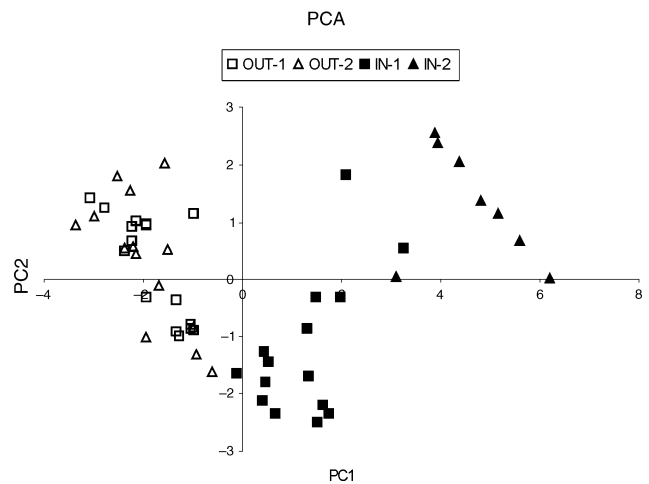


Fig. 3. Principal component analysis. Axes 1 and 2 (together representing 89% of all variability) are shown

We performed ML and MP phylogenetic analysis on the five 16S mtDNA haplotypes recognized in IN-1, IN-2, OUT-1, and OUT-2, sequences from the *G. saulae* population from Sinobang (10 km distance from Sanaron) and three related species from Borneo and the Malay Peninsula. Both ML and MP produced a single tree, the topologies of which were identical (MP: length 213 steps; Retention Index = 0.86; ML: $-\ln L = 1607.66$; Fig. 4). The phylogenetic reconstruction shows that the cave species *Georissa* n. sp. is most closely related to the local population of *G. saulae* (bootstrap values of 95 and 79% for MP and ML, respectively).

Within the four samples OUT-1, OUT-2, IN-1, and IN-2, we found five different haplotypes (Table 1), which were clearly divided into a *Georissa* n. sp. cave species group (haplotypes c and d) and a *G. saulae* epigeal group (haplotypes a, b, and e). In the network (Fig. 5), the nearest nodes of both groups were separated by six mutational steps. Population IN-1 contained haplotypes typical of both the epigeal and the cave groups, suggesting ongoing gene flow between the

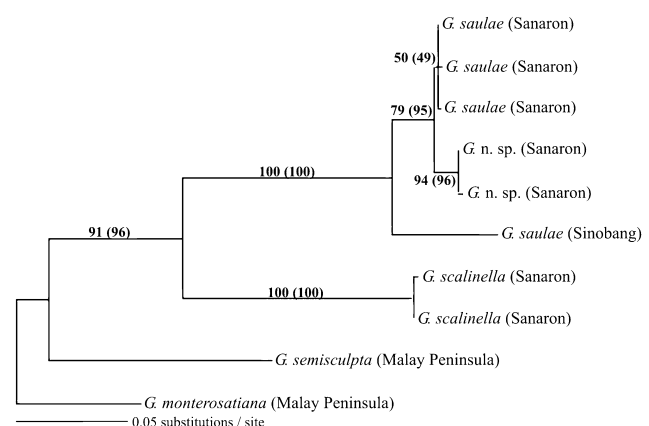


Fig. 4. Maximum-likelihood phylogeny reconstruction ($-\ln L = 1607.66$) based on 16S mtDNA. The substitution model used was HKY85, with ML-estimated base frequencies and transition/transversion ratio. The tree was rooted with the two species from Peninsular Malaysia

Table 1. Haplotype frequencies in samples of the epigeal *G. saulae* (OUT-1 and OUT-2) and the cave *Georissa* n. sp. (IN-1 and IN-2)

	IN-1 (n = 10)	IN-2 (n = 10)	OUT-1 (n = 10)	OUT-2 (n = 9)
Haplotype a	0.00	0.00	0.40	0.45
Haplotype b	0.20	0.00	0.60	0.33
Haplotype c	0.70	1.00	0.00	0.00
Haplotype d	0.10	0.00	0.00	0.00
Haplotype e	0.00	0.00	0.00	0.22

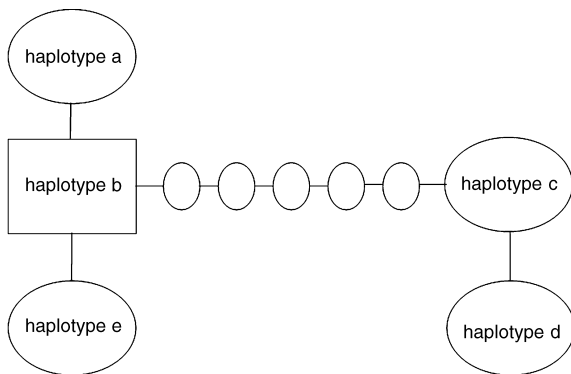


Fig. 5. Network of the five 16S mtDNA haplotypes recognized. Haplotypes a, b, and e are mostly found in OUT-1 and OUT-2, whereas haplotypes c and d are restricted to IN-1 and IN-2

epigeal *G. saulae* and the cave species *Georissa* n. sp. The calculation of Nm from the haplotype frequencies is not appropriate in this case, as such calculations depend on the existence of a drift/mutation/gene-flow equilibrium, which will not be established in situations where molecular markers are likely to hitch-hike on strong selection (Bossart and Pashley Prowell 1998; Whitlock and McCauley 1999; Mallet 2001).

Discussion

Our phylogenetic analysis suggests that the cave species has descended from the epigeal population of *Georissa saulae*, and has diverged in shell proportions, pigmentation, and ornamentation. Almost nothing is known of the ecology of any *Georissa*, but most species, including *G. saulae*, appear to be saxicolous, foraging on algae and lichens of vertical rock surfaces (Berry 1961). The snails are often exposed and active in full sunlight. Therefore, although the cave species was also seen feeding on the film of microorganisms covering the cave walls, it may be assumed that the shift from an epigeal to a hypogeal mode of life has involved several major changes in morphology and physiology, which remain to be investigated.

The conchological traits for which the cave species has diverged are known to be heritable in other prosobranch land snails (Grahame and Mill 1993; Johannesson and Johannesson 1996), and are not likely to be ecophenotypic in this case. Broader shells in snails are often associated with a less vertical substrate (Cain 1977). The cave snail was mostly seen foraging on wet spots on the cave floor, which might explain the shift to a broader shell habitus. Ornamentation in land snail shells has been variously viewed as defensive structures or sexual signals (Schilthuisen 2003) so its loss could indicate a change in either

mate choice strategies or predation; both are plausible in a cave environment. Shell depigmentation, finally, is one of the few characteristics that all troglobitic snails share. Otherwise, and in contrast with other groups of cave-organisms, cave snails do not have any derived morphological traits in common (Gittenberger 1985).

The presence of a morphologically intermediate population (IN-1) and the evidence of both epigeal and cave haplotypes in this population indicate that gene-flow is ongoing or has recently been ongoing between the cave and the outside population. Dispersal in land snails is known to be very limited, in the order of several metre per generation at most (Schilthuisen and Lombaerts 1994; Baur and Baur 1995; Pfenninger et al. 1996). The distance between IN-1 and the nearest epigeal population is c. 60 m, so that active dispersal may be insufficient to effect this amount of gene-flow. However, given the topography of the terrain, with the cave entrance steeply sloping downward, it is perhaps more likely that the major contribution to gene-flow comes from passive dispersal with rainwater.

In summary, our data show a situation where a presumably cave-adapted and morphologically derived species has descended from its above-ground ancestor, but remains genetically connected with it via a population of intermediate morphology at the cave entrance. Two distinct scenarios may explain this situation. First, the cave species may have diverged in allopatry from founders isolated in the deep recesses of the cave, and then, after expansion, secondarily established genetic contact with its epigeal ancestor. Alternatively, the intermediate population may be primary in origin, and cave-adaptation may have taken place with ongoing gene-flow, representing a stage in parapatric speciation.

Without further investigations of multilocus clines, our data do not allow a formal rejection of either hypothesis, but circumstantial evidence does favour the parapatric scenario. The geography and size of the limestone outcrop make it unlikely that a population could be fully isolated inside the cave for a long period of time. The outcrop is just a few hundred metre in diameter, and the cave entrances are many and descend steeply; dense populations of the epigeal *G. saulae* occur directly at the cave entrances, and at least one stream runs through the cave system, so passive dispersal must be continuous and substantial. It is, therefore, likely, that the cave species has evolved while remaining genetically connected with its above-ground ancestor.

We suggest that the *G. saulae* system has potential for demonstrating a parapatric speciation scenario in a troglobite. Few detailed studies of troglobites and their epigeal relatives are available. Recently, Strecker et al. (2003), using mtDNA and microsatellites, cast doubt on a system that had for some time been viewed as a possible candidate for parapatric speciation in a cave, viz. the blind Mexican cave fish *Astyanax* and its eyed surface-dwelling relatives. The biospeleological literature offers descriptions of many systems that appear similar to the one analysed for *G. saulae* and *Georissa* n. sp. Peck and Finston (1993), for example, list no fewer than 10 cases of troglobites from lava-tube caves in the Galápagos archipelago, with extant surface-dwelling sister species living near the cave entrance. They also mention several more possible cases from temperate regions. We suggest that these and other cases be studied in more detail. The evolution of troglobites involves strong selection pressures in geographically and ecologically simple settings, providing ideal case studies for the investigation of speciation modes.

Acknowledgements

This work was financially supported by research grants from Universiti Malaysia Sabah, and the Society for the Advancement of Research in the Tropics, Amsterdam (Treib-Foundation). For help during the field work, we are grateful to Jaap Vermeulen (Singapore Botanic Gardens), Somsak Panha, Chirasak Sutcharit and Sakboworn Tumpeesuwan (Chulalongkorn University, Bangkok, Thailand), Zalaluddin Latipi (Universiti Malaysia Sabah), and Bronwen Scott (Victoria University, Melbourne, Australia). The people of Kampung Labang are gratefully acknowledged for their hospitality and help in logistics. Permission to do research in the Sepulut Forest Reserve was given by Dr Sining Unchi of the Sabah Forestry Department. We also wish to thank Jaap Vermeulen, Henry Bernard, Edi Gittenberger, and Jim Mallet for their contributions to discussions on this work.

Zusammenfassung

Mögliche Divergenz mit Genfluß bei einer tropischen Höhlenschnecke

In dieser Arbeit stellen wir eine neu entdeckte Höhlenart der Schneckengattung *Georissa* aus einer Kalksteinhöhle in Borneo vor. Die molekulare phylogenetische Analyse von mitochondrialer 16S rDNS zeigt, daß ihre Stammform die lokale epigäische Population von *G. saulae* ist, die im Regenwald direkt vor dem Höhleneingang lebt. Eine multivariate Analyse von Schalenmerkmalen zeigt, dass die Höhlenpopulation morphologisch divergiert, aber über eine Population mit intermediärer Morphologie, die in der Zwielichtzone der Höhle lebt, mit der epigäischen Stammform verbunden ist. Die molekularen Daten lassen auch auf weiterhin bestehenden Genfluß zwischen der Oberflächen- und Höhlenart über die intermediäre Population schließen. Unsere Daten legen nahe, dass die Höhlenart ohne vorherige Isolation von ihrer Stammform abzweigt ist.

References

- Barr, T. C.; Holsinger, J. R., 1985: Speciation in cave faunas. *Annu. Rev. Ecol. Syst.* **16**, 313–337.
- Baur, B.; Baur, A., 1995: Habitat-related dispersal in the rock-dwelling land snail *Chondrina clienta*. *Ecography* **18**, 123–130.
- Berry, A. J., 1961: The habitats of some minute cyclophorids, hydrocenids and vertiginids on a Malayan limestone hill. *Bull. Natl. Mus. Singapore* **30**, 101–105.
- Bossart, J. L.; Pashley Prowell, D., 1998: Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends Ecol. Evol.* **13**, 202–206.
- Cain, A. J., 1977: Variation in spire index in some coiled gastropod shells, and its evolutionary significance. *Phil. Trans. R. Soc. Lond. B* **277**, 377–428.
- Culver, D. C., 1982: *Cave Life: Evolution and Ecology*. Cambridge, MA: Harvard University Press.
- Dieckmann, U.; Doebeli, M., 1999: On the origin of species by sympatric speciation. *Nature* **311**, 354–357.
- Doebeli, M.; Dieckmann, U., 2003: Speciation along environmental gradients. *Nature* **421**, 259–264.
- Endler, J. A., 1977: *Geographic Variation, Speciation, and Clines*. Princeton, NY: Princeton University Press.
- Flichak, K. E.; Roethele, J. B.; Feder, J. L., 2000: Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. *Nature* **407**, 739–742.
- Francis, C. M., 1987: *The Management of Edible Bird's Nest Caves in Sabah*. Sandakan: Sabah Forest Department.
- Gavrilets, S., 2003: Models of speciation: what have we learned in 40 years. *Evolution* **57**, 2197–2215.
- Gittenberger, E., 1985: Beiträge zur Kenntnis der Pupillacea. XI. *Speleodentorcula beroni* gen. & spec. nov. (Mollusca: Gastropoda: Orculidae) aus einer Höhle in Euboea, Griechenland. *Zool. Meded. Leiden* **59**, 221–228.
- Grahame, J.; Mill, P. J., 1993: Shell shape variation in rough periwinkles: genotypic and phenotypic effects. In: Aldrich, J. C. (ed.) *Quantified Phenotypic Responses in Morphology and Physiology*. Ashford: Japaga, pp. 25–30.
- Hasegawa, M.; Kishino, H.; Yano, T., 1985: Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **21**, 160–174.
- Holsinger, K., 2000: Ecological derivation, colonization, and speciation. In: Wilkens, H.; Culver, D. C.; Humphreys, W. F. (eds) *Subterranean Ecosystems*. Amsterdam: Elsevier Science Publishers, pp. 399–415.
- Howarth, F. G., 1987: The evolution of non-relictual tropical troglobites. *Int. J. Speleol.* **16**, 1–16.
- Johannesson, B.; Johannesson, K., 1996: Population differences in behaviour and morphology in the snail *Littorina saxatilis*: phenotypic plasticity or genetic differentiation? *J. Zool. Lond.* **240**, 475–493.
- Johannesson, K.; Rolán-Alvarez, E.; Ekendahl, A., 1995: Incipient reproductive isolation between two sympatric morphs of the intertidal snail *Littorina saxatilis*. *Evolution* **49**, 1180–1190.
- Jolliffe, I. T., 1986: *Principal Component Analysis*. New York: Springer.
- Kondrashov, A. S.; Kondrashov, F. A., 1999: Interactions among quantitative traits in the course of sympatric speciation. *Nature* **311**, 351–353.
- Mallet, J., 2001: Gene flow. In: Woiwod, I. P.; Reynolds, D. R.; Thomas, C. D. (eds) *Insect Movement: Mechanisms and Consequences*. Wallingford: CAB International, pp. 337–360.
- Mayr, E., 1999: Introduction. In: Mayr, E. (ed) *Systematics and the Origin of Species from the Viewpoint of a Zoologist; With A New Introduction*. Cambridge, MA: Harvard University Press, pp. xiii–xxxv.
- Mayr, E., 2001: *What Evolution Is*. New York: Basic Books.
- van Moorsel, C. H. M.; van Nes, W. J.; Megens, H. J., 2000: A quick, simple, and inexpensive mollusc DNA extraction protocol for PCR-based techniques. *Malacologia* **42**, 203–206.
- Peck, S. B.; Finston, T. L., 1993: Galapagos islands troglobites: the question of tropical troglobites, parapatric distributions with eyed-sister-species, and their origin by parapatric speciation. *Mém. Biospéol.* **20**, 19–37.
- Pfenninger, M.; Bahl, A.; Streit, B., 1996: Isolation by distance in a population of a small land snail *Trochoidea geyeri*: evidence from direct and indirect methods. *Proc. R. Soc. Lond. B* **263**, 1211–1217.
- Poulson, T. L.; White, W. B., 1968: The cave environment. *Science* **165**, 971–981.
- Rice, W. R.; Hostert, E. E., 1993: Laboratory experiments on speciation: what have we learned in 40 years? *Evolution* **47**, 1637–1653.
- Sbordoni, V.; Allegrucci, G.; Cesaroni, D., 2000: Population genetic structure, speciation and evolutionary rates in cave-dwelling organisms. In: Wilkens, H.; Culver, D. C.; Humphreys, W. F. (eds) *Subterranean Ecosystems*. Amsterdam: Elsevier Science Publishers, pp. 453–477.
- Schilthuizen, M., 2000a: Dualism and conflicts in understanding speciation. *Bioessays* **22**, 1134–1141.
- Schilthuizen, M., 2000b: Ecotone-speciation prone. *Trends Ecol. Evol.* **15**, 130–131.
- Schilthuizen, M., 2003: Sexual selection on land snail shell ornamentation: a hypothesis that may explain shell diversity. *BMC Evol. Biol.* **3**, 13, Available at: <http://www.biomedcentral.com/1471-2148/3/13> via the Internet. Accessed 24 February 2004.
- Schilthuizen M.; Lombaerts, M., 1994: Population structure and levels of gene flow in the Mediterranean land snail *Albinaria corrugata* (Pulmonata: Clausiliidae). *Evolution* **48**, 577–586.
- Schliwien, U. K.; Tautz, D.; Pääbo, S., 1994: Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature* **368**, 629–632.
- Schliwien, U. K.; Rassmann, K.; Markmann, M.; Markert, J.; Tautz, D., 2001: Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol. Ecol.* **10**, 1471–1488.
- Schneider, C. J.; Smith, T. B.; Larison, B.; Moritz, C., 1999: A test of alternative models of diversification in tropical rainforests: ecological gradients vs. rainforest refugia. *Proc. Natl Acad. Sci. USA* **96**, 13869–13873.

- Smith, T. B.; Wayne, R. K.; Girman, D. J.; Bruford, M. W., 1997: A role for ecotones in generating rainforest diversity. *Science* **276**, 1855–1857.
- Strecker, U.; Bernatchez, L.; Wilkens, H., 2003: Genetic divergence between cave and surface populations of *Astyanax* in Mexico. *Mol. Ecol.* **12**, 699–710.
- Swofford, D. L., 1998: PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods). Sunderland: Sinauer.
- Templeton, A. R.; Crandall, K. A.; Sing, C. F., 1992: A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619–633.
- Thompson, F. G.; Dance, S. P., 1983: Non-marine mollusks of Borneo; II Pulmonata: Pupillidae, Clausiliidae; III Prosobranchia: Hydrocenidae, Helicinidae. *Bull. Florida State Mus. Biol. Sci.* **29**, 101–152.
- Thompson, J. D.; Higgins, D. G.; Gibson, T. J., 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**, 4673–4680.
- Vermeulen, J. J.; Whitten, T., 1999: Biodiversity and Cultural Property in the Management of Limestone Resources; Lessons from East Asia. Washington, DC: The International Bank for Reconstruction and Development.
- Whitlock, M. C.; McCauley, D. E., 1999: Indirect measures of gene flow and migration: $F_{st} \neq 1/(4Nm + 1)$. *Heredity* **82**, 117–125.
- Wilding, C. S.; Butlin, R. K.; Grahame, J., 2001: Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *J. Evol. Biol.* **14**, 611–619.
- Wilkens, H.; Hüppop, K., 1986: Sympatric speciation in cave fishes? Studies on a mixed population of epi- and hypogean *Astyanax* (Characidae, Pisces). *Z. Zool. Syst. Evolutionsforsch.* **24**, 223–230.
- Wilkens, H.; Culver, D. C.; Humphreys, W. F., 2000: Subterranean Ecosystems. Amsterdam: Elsevier Science Publishers.
- Yin, E. H., 1985: Geological Map of Sabah, 3rd edn. Ipoh: Geological Survey of Malaysia.
- Authors' addresses:* Menno Schilthuisen (for correspondence), Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Malaysia. E-mail: schilthuisen@yahoo.com; Annadel S. Cabanban, Borneo Marine Research Institute, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Malaysia; Martin Haase, National Institute of Water and Atmospheric Research, Hamilton, New Zealand.