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The evolution of extreme shell shape variation in the land snail *Ainohelix editha*: a phylogeny and hybrid zone analysis

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Abstract

***Ainohelix editha* from Hokkaido, Japan, exhibit great geographical variation in their shell morphology. In particular, *A. editha* in two quite separate locations, Shimamaki and Samani, are striking because they are extremely flat and have a sharp keel, whereas at adjacent sites the shells are globular or depressed-globular. We used mitochondrial 16S rRNA and nuclear ITS-2 sequences to infer a phylogeny among 47 snails from 29 locations. Snails from the two keeled-flat populations clustered separately in the phylogeny, suggesting that this unusual shell form could have evolved independently. A morphological analysis of shells collected along a transect between keeled-flat and globular snail sites showed a cline for shell shape and the angle of the keel. Two different mtDNA lineages were found across the transect, with a cline for an ITS-2 single nucleotide polymorphism. Together, the results may suggest a lack of reproductive isolation between keeled-flat and globular snails, with possible introgression by hybridization.**

Keywords: introgression, ITS, keel, mitochondrial DNA, morphological evolution, parallel evolution

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Introduction

The functional aspects of shell morphology in land snails have been of great interest, especially since the observation of Cain (1977) that widely separate and taxonomically-distinct land snail faunas have a bimodal distribution of shell shape. Snails tend to be either high spired or discoidal, with few globular forms between. Each type is often associated with a particular habitat (Goodfriend 1986; Heller 1987; Cameron & Cook 1989; Cook 1997). Shell shape variation has been less well studied within and among closely-related species. Exceptions are in *Albinaria*, a clausiliid genus from the eastern Mediterranean, and *Cerion* from the Caribbean (Nordsieck 1977; Mylonas *et al*. 1987; Gould & Woodruff 1990; Gittenberger & Menkhorst 1992). Sometimes, morphological variation may result from selection in different environments (Engelhard & Silk 1994; Welter-Schultes 2000), whereas in other species the history of the populations is implicated (Gould & Woodruff 1990; Davison & Clarke 2000).

Characters such as shell colour, size, banding pattern, whorl expansion rate, aperture size and presence or absence of an umbilicus are commonly used to describe variation between land snails. Many species show variation in the form of the periphery, whether the edge of the shell is rounded or angular. In the extreme case, an angled periphery becomes a 'keel'. Gould (1969, 1971) suggested that a peripheral keel evolved as a paedomorphy, that is, the retention of a juvenile character. Cook & Pettitt (1979) suggested that keeled shells may be more resistant to crushing than rounded forms, and Solem & Climo (1985) found that development of a peripheral keel is associated with sheltering in open ground under deep, wet litter. In some groups of snails, keeled shells are associated with limestone substrates (Alonso *et al*. 1985).

Ainohelix editha (Adams) (Pulmonata, Bradybaenidae) from Hokkaido, Japan, have an extremely variable shell morphology, with much of the variation being among snails from different locations (Katakura *et al*. 1990; Fig. 1). *Ainohelix editha* in two geographically-separate populations, Shimamaki and Samani, are especially striking because in

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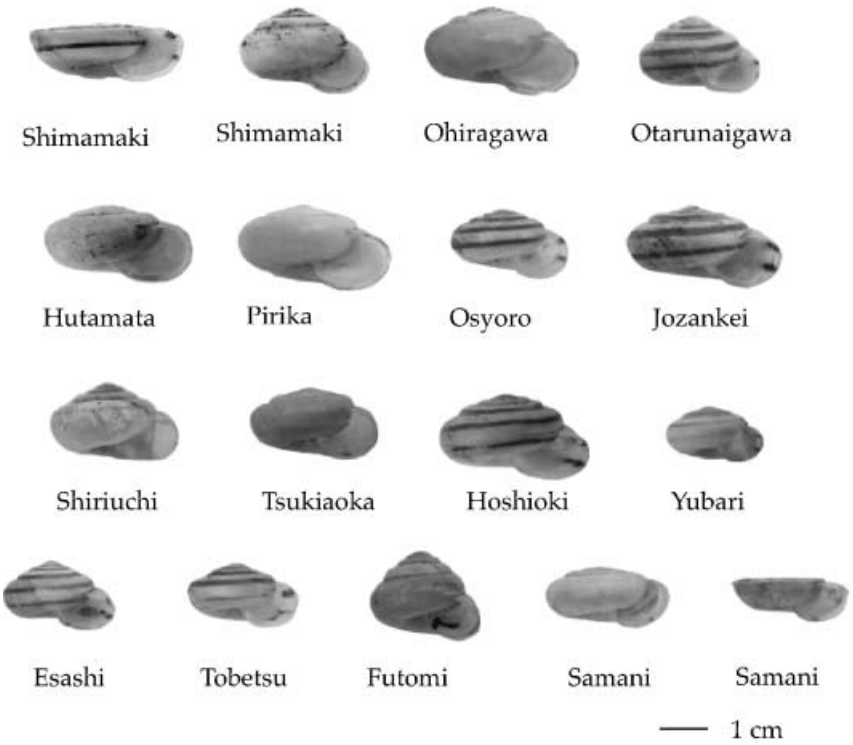

Fig. 1 Morphological variation of *Ainohelix editha* shells. Globular or depressed-globular and keeled-flat snails are found in both Shimamaki and Samani.

both there are extremely flat and sharp-keeled snails, whereas in adjacent populations the snails are globular or depressed-globular, respectively (Fig. 1). Individuals from different regions of Hokkaido tend to have discoidal to globular shells with a rounded periphery and great variation in shell height, shell size, banding patterns and shell surface (hirsute or not hirsute; Katakura *et al*. 1990; Fig. 1). Intermediate forms between keeled-flat shells and other types have not been reported, nor have the two discrete phenotypes been found sympatrically.

In an earlier study, Habe (1977) synonymized five taxa previously described under the name of *Ainohelix*. Like Habe, we have no reason to suspect that the separate shell forms are separate species because no differences in genitalia or radulae have been detected among the different types (Katakura *et al*. 1990; Y. Kuwamura, unpublished), and they mate and produce viable offspring in the laboratory (Ogimura *et al*., unpublished). However, it would be wise to be sceptical about the species status until a more detailed examination of the genitalia has been carried out.

The purpose of this paper is twofold. First, by inferring phylogenetic relationships, we examine whether the keeled-flat snails found in separate populations are likely to have evolved independently. Second, we report the discovery of a putative hybrid zone between globular and keeled-flat populations, and investigate any cline in the morphology, gene flow between populations and degree of reproductive isolation between different types.

Materials and methods

Samples

Ainohelix editha is widely distributed in Hokkaido, Japan. Samples used for the phylogenetic analysis were collected from 29 sites (Table 1; Fig. 2), including both Shimamaki and Samani where the shells are keeled and flat at some locations. Shimamaki and Samani are separated by about 250 km apart, and the underlying geology is limestone in both places. *Acusta despecta* from Amami Island, Japan, was used as an outgroup. A separate analysis using a variety of bradybaenids confirms that this outgroup is reasonable (Davison *et al*., unpublished).

Fine-scale sampling of snails was carried out across a newly discovered, putative hybrid zone between globular and keeled-flat snails in Shimamaki. Samples were collected along a road from the Chiwase river to the Tomari river, using sampling intervals of 620–1470 m (sites SH1 to 8, Shimamaki transect; Fig. 2b).

DNA extraction, PCR amplification and RFLPs

Total DNA was isolated using the procedure of Doyle & Doyle (1987) with some modifications as follows. Foot tissue was homogenized in 300 µL of 2á× CTAB (cetyltrimethyl ammonium bromide) solution and 20 µL of 10 mg/mL proteinase K, incubated at 60 °C for approximately 1 h, extracted once with phenol/chloroform/

Table 1 Sampling locality and number of individuals used in phylogenetic, morphological and PCR-RFLP analysis

	Number of individuals				
	Phylogenetic analysis			PCR-RFLP	
Locality	mtDNA	ITS-2	Morphological analysis	mtDNA	ITS-2
SH1	1	5	12	27	16
SH2	2	2	10	10	9
SH3	—		3	6	5
SH4	6	2	11	33	16
SH5	2	2	15	19	5
SH6	2	9	20	34	25
SH7	—	7	4	6	7
SH8	2	4	10	32	22
SA1	5	3			
SA2	1	4			
SA3	3	1			
Shiriuchi	1				
Obiragawa	3				
Futamata	3				
Pirika	2	3			
Osyoro	1	2			
Jozankei	1				
Otaruanigawa	1				
Hoshioki	1				
Sapporo	1				
Urakawa	1	2			
Yubari	4	2			
Futomi	1	1			
Toubetsu	1				
Tsukigaoka	1				
Esashi	1				
Urahoro		1			
Atsuma		1			

isoamyl alcohol (25 : 24 : 1, v : v : v) and precipitated with two volumes of ethanol. The DNA pellet was then washed in 70% ethanol, air-dried for approximately 30 min and dissolved in 50 μL of TE.

A 16S ribosomal RNA (16S rRNA) mitochondrial DNA (mtDNA) gene fragment of approximately 900 bp was amplified by polymerase chain reaction (PCR) with primers 16Scs1 (5′-AAACATACCTTTTGCATAATGG-3′) and 16Scs2 (5′-AGAAACTGACCTGGCTTACG-3′; Chiba 1999). Also, an approximately 900 bp region of the nuclear rRNA gene cluster was amplified by PCR, including the 3′ end of the 5.8s gene, the complete internal transcribed spacer (ITS)-2 region and the 5′ end of the large subunit (LSU; 28s) gene, using the primers LSU-1 (5′-CTAGCT-GCGAGAATTAATGTGA-3′) and LSU-3 (5′-ACTTTC-CCTCACGGTACTTG-3′; Wade & Mordan 2000). Both PCR reactions used Takara rTaq™ (Takara Biomedicals, Japan) and buffers, with annealing temperatures of 50 °C and 55 °C, respectively.

Cycle sequencing was carried out with both forward and reverse primers, using about 80–100 ng of PCR product in the reaction and the DYEnamic™ ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech) or the BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA sequences were electrophoresed on a 373A DNA Sequencer or 310 Genetic Analyser (both Applied Biosystems). ITS-2 heterozygotes, which were easily recognizable, were scored using the IUPAC-IUB Joint Commission on Biochemical Nomenclature ambiguity codes.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses for the 16S rRNA gene and ITS-2 region were used to investigate changes in haplotype frequency in the Shimamaki transect. Sequence analysis showed that of the two mtDNA haplotypes found in Shimamaki, one has two *Cla*I sites and a *Spe*I site, whereas the other lacks all three sites. Therefore, for the mtDNA RFLP, PCR products were digested with one of the restriction enzymes for 1 h, then electrophoresed on a 1.5% agarose gel.

Preliminary analysis of the ITS-2 region found a G/C polymorphism, corresponding to presence/absence of a *Hin*dIII restriction site, as well as a *Pst*I site in all sequences. These restriction enzymes enabled us to carry out an ITS-2

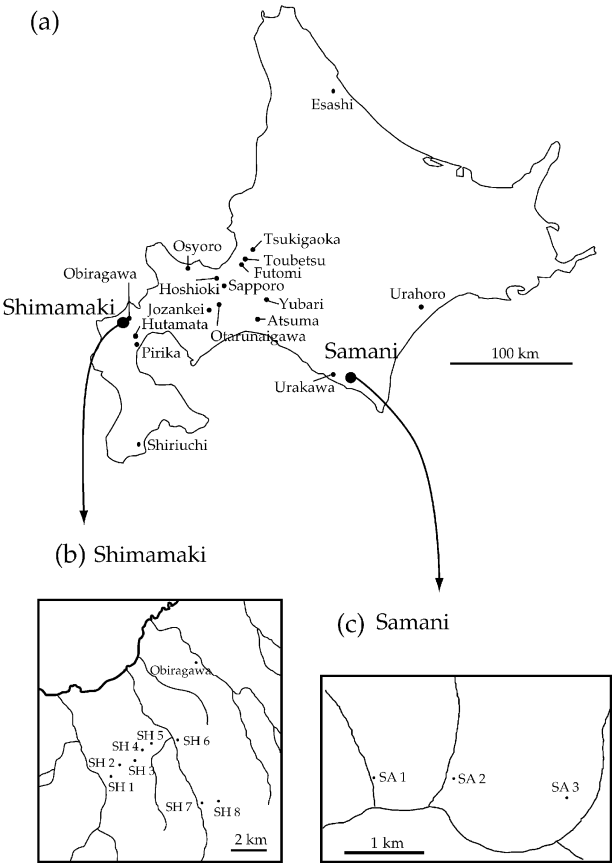

Fig. 2 Map showing the sampling localities of *Ainohelix editha* in (a) Hokkaido, Japan (b) Shimamaki (c) Samani.

RFLP, when combined with ABI fragment analysis and GENESCAN® Analysis Software v3.1.2. In this way, the frequency of the ITS-2 single nucleotide polymorphism could be estimated in each snail. ITS-2 PCR was carried out using a 6-FAM-labelled LSU-1 primer. The PCR product was then digested with *Hin*dIII and *Pst*I, and run on a 310 Genetic Analyser. DNA sequences with a *Hin*dIII site produced a 180 bp band, otherwise the band was 480 bp. The ratio of the area under each peak was used to estimate the proportion of each haplotype in the PCR product.

Phylogenetic analyses

Sequences were aligned using the CLUSTALX software, and results were then checked manually to minimize the total number of insertions and deletions (indels). All indel sites were removed from the alignment before phylogenetic analysis.

Phylogenetic relationships were analysed using two methods: neighbour-joining (NJ) and maximum-likelihood (ML), both with PAUP*4.0b8 (Swofford 2001). The transition : transversion ratio was fixed at 2 : 1. Kimura's 2-parameter method was used for the calculation of the distance

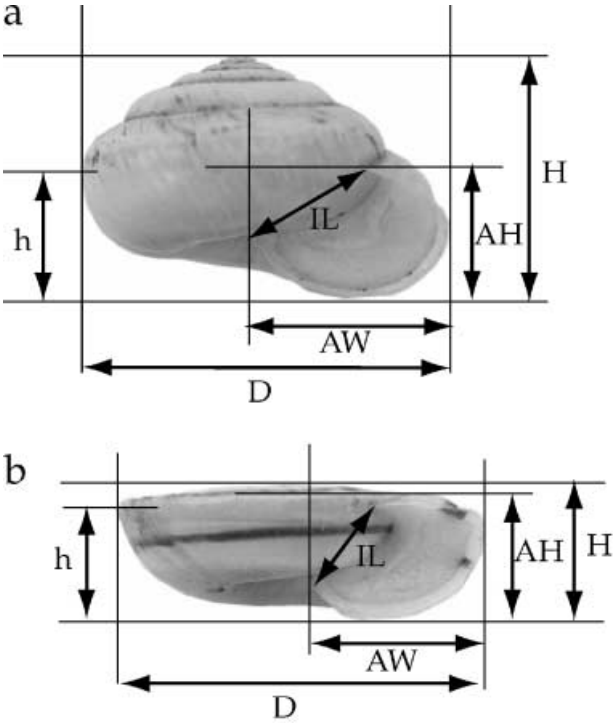

Fig. 3 The morphological character measurements (see methods for explanation).

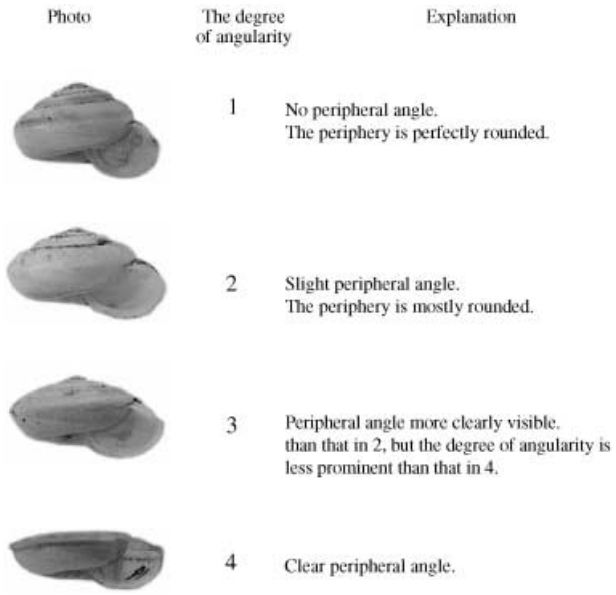

Fig. 4 Four degrees of angularity.

matrix for the NJ analysis, and the F84 model was used for the ML analysis. Bootstrap re-sampling used 1000 replications for the NJ analysis and 100 replications for the ML analysis.

The relationship between ITS-2 haplotypes was visualized using a minimum spanning network, with the sequences as nodes of a network instead of the terminal

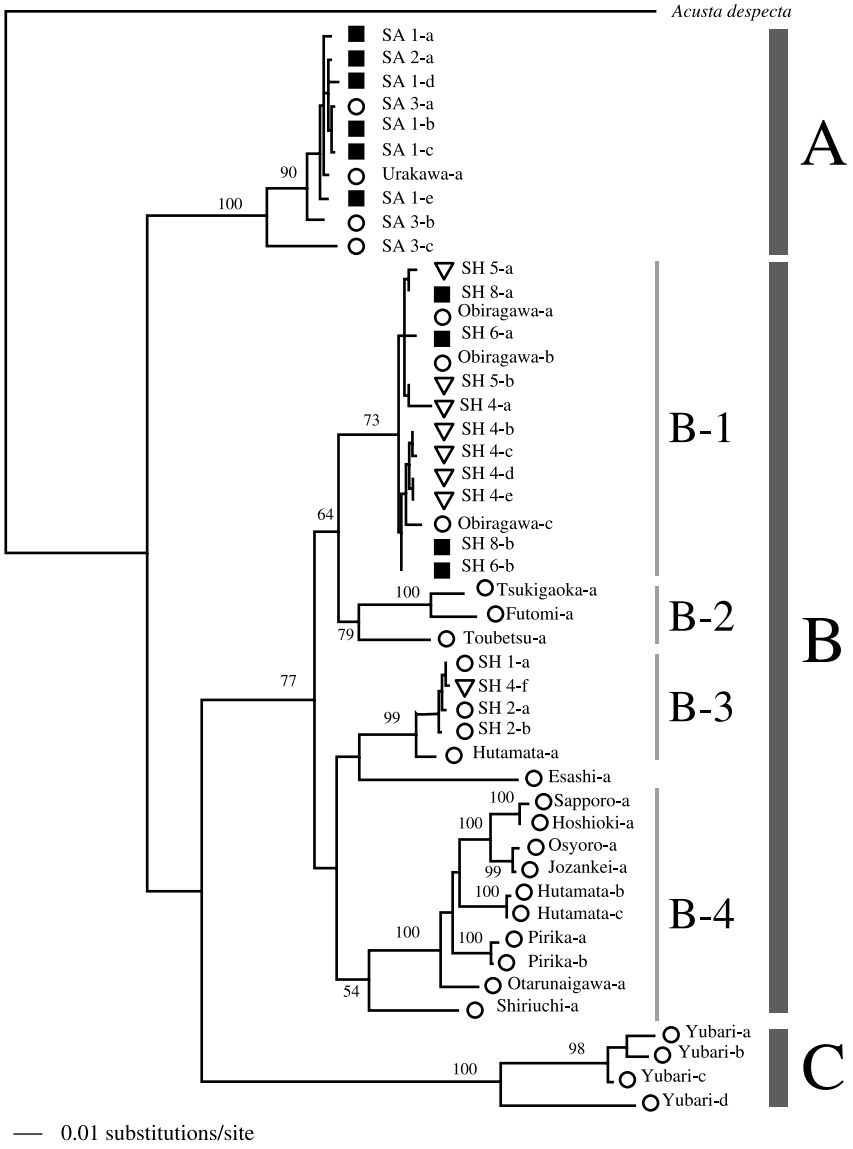

Fig. 5 Neighbour-joining tree based on 16S rRNA sequences. Numbers above nodes are the bootstrap support for that clade (values < 50% not shown). Different characters, a, b, c, d and e, with the location name indicate different haplotypes from the same site. Open circles and filled squares indicate globular or depressed-globular and keeled-flat snails, respectively. Triangles are possible intermediate snails from SH4 and 5. SH1 to 8 are sample sites of the Shimamaki transect.

tips of a tree. Networks are useful when many of the sequences may be derived from the same ancestral genotype. The algorithm used was MINSPNET within ARLEQUIN 2.0 (Schneider *et al*. 2000).

Morphological analysis in the hybrid zone

Six shell morphology characters were measured by digital image analysis: D (width), H (height), h (height to periphery or mid-point), AW (aperture width), AH (aperture height) and IL (inner lip length; Fig. 3). H/D and h/H were calculated as indices of 'globularity' and 'flatness', respectively. It was not possible to measure accurately the angle of the keel, so we chose four qualitative categories (Fig. 4). The digital images were then scored by 10 persons for 'angularity' and the mean used as an index of angularity (K).

Unfortunately, the number of sample sites was too small to estimate the cline centre and width accurately. Thus, our arguments on cline shape are qualitative rather than quantitative.

Results

Genetic variation and phylogeny

As expected from studies of other land snails (Thomaz *et al*. 1996; Watanabe & Chiba 2001), high levels of sequence divergence were found within and among populations. In total, 47 16S rRNA haplotypes were found in 47 snails (GenBank accession numbers AY137531 to AY137578). Sequence divergence among the haplotypes ranged from 0.14% to 23%.

The inferred phylogenetic relationship between the haplotypes is shown in Fig. 5. Three monophyletic groups, A,

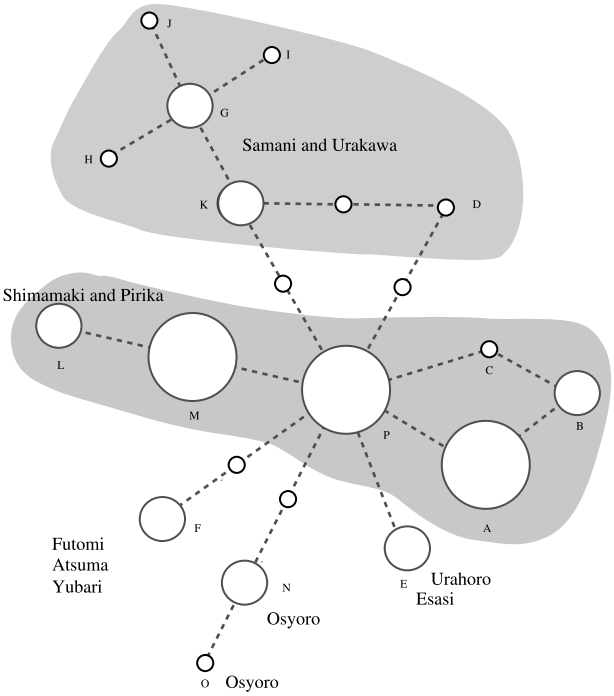

Fig. 6 Minimum spanning network constructed using nuclear ITS-2 sequences, excluding indels. There were 18 haplotypes, but only 16 excluding indels. All haplotypes differ by single steps. Circle size is related to haplotype sample size: small, $n = 1$; medium $n = 2$–9; large, $n > 10$. There wyere five unsampled haplotypes, indicated by the small unlabelled circles.

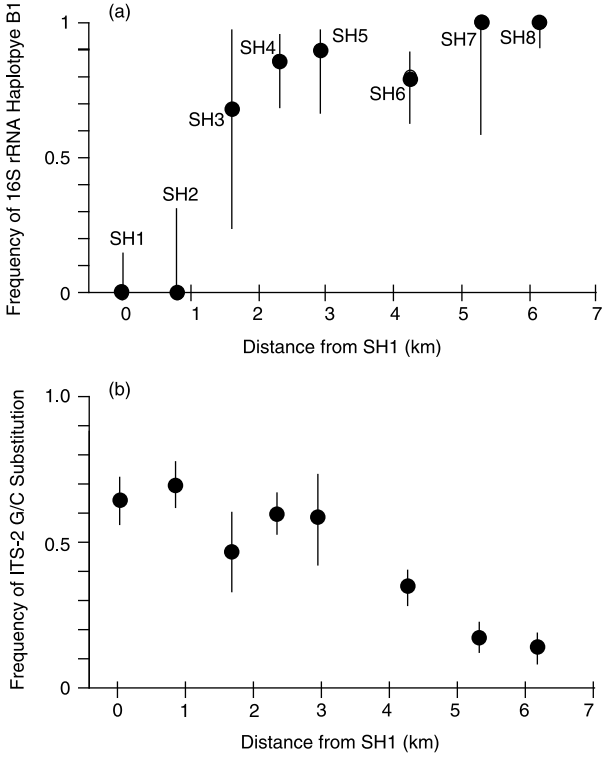

Fig. 7 Changes in the frequency of 16S rRNA haplotype B1 (a) and ITS-2 G/C substitution from SH1 to 8 (b). Vertical bar indicates 95% confidence limits.

B and C, are present, with bootstrap support values higher than 70% using both phylogeny methods. The relationship between groups A, B and C is uncertain because there was no support for the A(BC) branch. Both ML and NJ trees consistently showed that group B haplotypes divide into four subgroups (B1, B2, B3 and B4). Phylogenetic relationships among the haplotypes within each subgroup were mostly similar between the NJ tree and the ML tree, but there were some inconsistencies, probably because of different sensitivities to 'long-branch attraction' (Hillis *et al.* 1997). For this reason, the 'true' relationship between groups A, B and C is uncertain, as well as the placement of the haplotype Esashi-a.

Group A haplotypes were found in the southeastern parts of Hokkaido (Samani and Urakawa; Fig. 2). Group B haplotypes were found in the western and northernmost part of Hokkaido. Group C haplotypes were found in the Yubari area. Geographical patterns in the distribution of subgroup B1–B4 haplotypes were not clear. For example, both of the subgroup B1 and B3 haplotypes were found in the Shimamaki area, and subgroup B4 haplotypes were widely distributed in the western ranges from Sapporo to Shiriuchi.

Whatever the method used, snails with keeled-flat shells from Samani and Shimamaki were in separate groups (groups A and B; Fig. 5). Samani keeled-flat snail haplotypes (sites SA1 and SA2) grouped with depressed-globular snail haplotypes from an adjacent population (SA3) or nearby (Urakawa), whereas Shimamaki globular and keeled-flat snails fell into subgroup B1.

Variation within the ITS-2 region was low, as expected (GenBank accession numbers AY159486 to AY159512). Eighteen variable base positions were detected in 18 haplotypes, with at least a further seven indels. Samani haplotypes (D, G, H, I, J, K) are separated from Shimamaki haplotypes (A, B, C, L, M, P) by a single step in the minimum spanning network (Fig. 6).

Haplotype frequencies across the transect

In the populations from Shimamaki, keeled-flat snails mostly had subgroup B1 haplotypes, whereas globular snails mostly had the subgroup B3 haplotype (Figs 5 and 7). PCR-RFLP analysis using the mitochondrial 16S rRNA gene confirmed this. The frequency of subgroup B1 haplotypes increased from zero to 100% from SH1 to 8 with a sharp change in frequency centred around SH3 (Fig. 7a), although the estimation of the mean frequency is not significantly different from either. Populations from SH4 to 6 had subgroup B3 haplotypes at low frequency, whereas SH7 and 8 were fixed for B3.

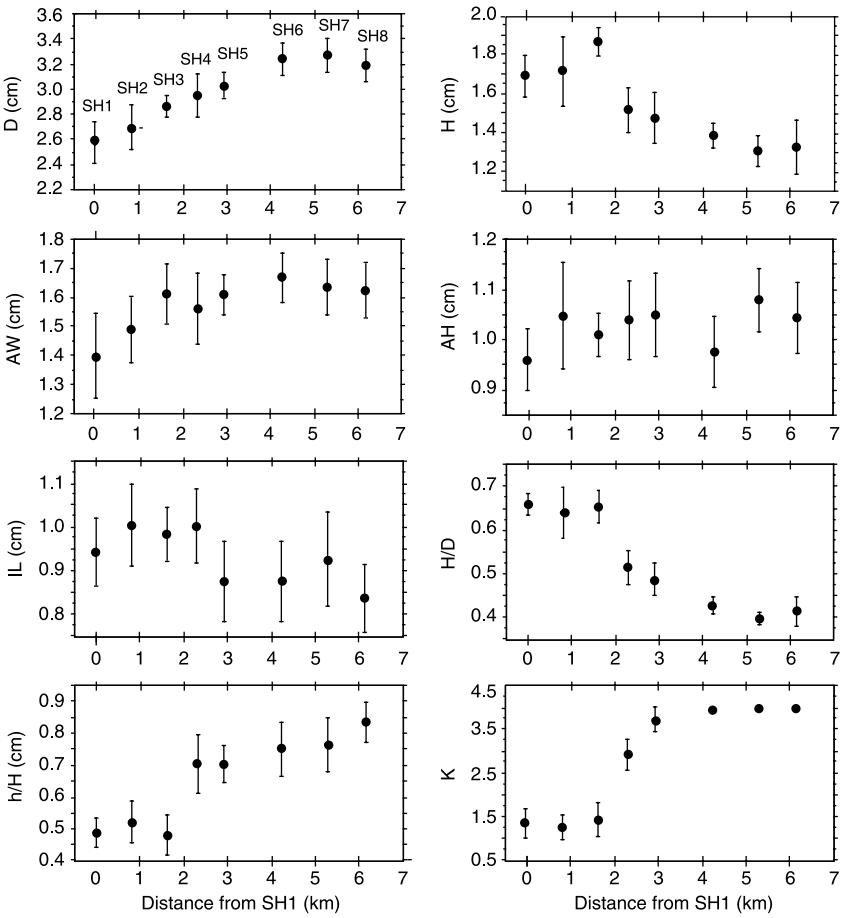

Fig. 8 The change in eight measurements of shell morphology across the Shimamaki transect, showing mean value with standard error bars.

Similarly, ITS-2 haplotypes A and B were more common in globular shells (16/34 haplotypes), whereas L, M and P were more common in keeled-flat shells (27/29 haplotypes). PCR-RFLP analysis using the ITS-2 region confirmed this. ITS-2 haplotypes with a *Hin*dIII site were more common in globular snails (around 70%), especially from SH1 to 5, falling to around 10% in SH7 and 8 (Fig. 7b).

Morphological changes across the Shimamaki transect

The change in the mean values for eight shell variables from SH1 (globular shell) to 8 (keeled-flat shell) are shown in Fig. 8. There was probably a cline in size between SH1 and SH8, as measured by D and H, though the two measures are correlated. Likewise, a cline was also found for H/D. Using these three intercorrelated measures, globular snails (high H, low D, high H/D) were found in SH1 to 3, with not-globular snails in SH6 to 8 (low D, high H, low H/D). Intermediate snails were found in SH4 and 5. The measure of degree of flatness, h/H, may be a stepped cline. Again, for the degree of angularity, K, SH4 was intermediate compared with SH1 to 3 and SH5 to 8. For IL, AH and AW, there may have been one or two groups with or without intermediates. It is clear that further sampling on a finer scale is required to establish the exact structure across the transect.

As sample sizes were small, we pooled SH1 to 3 (globular snail samples), SH4 to 5 (putative intermediate samples) and SH6 to SH8 (keeled-flat samples). The sample means were significantly different among the three groups for all character measurements shown in Fig. 8 (ANOVA $P < 0.0001$ except IL, $P = 0.0004$), except for AH ($P = 0.112$). Critically, the character mean for shells from SH4 and 5 was always intermediate and significantly different from either globular or keeled-flat shell sites, except for the character IL.

Figure 9(a) shows the relationship between the degree of angularity (K) and flatness (h/H). The flatness ratio of snails at SH1 to 3 was mostly from 0.4 to 0.6 (globular), rising to 0.6 to 1.0 for the snails at SH6 to 8 (flat). The degree of angularity (K) of snails at SH1 to 3 was less than 2 (no peripheral angle or slight peripheral angle), whereas at SH6 to 8 it was 4 or nearly so (clear peripheral angle or keel). Snails from SH4 and 5 had intermediate degree of flatness, particularly using the H/D measure (not shown) and angularity.

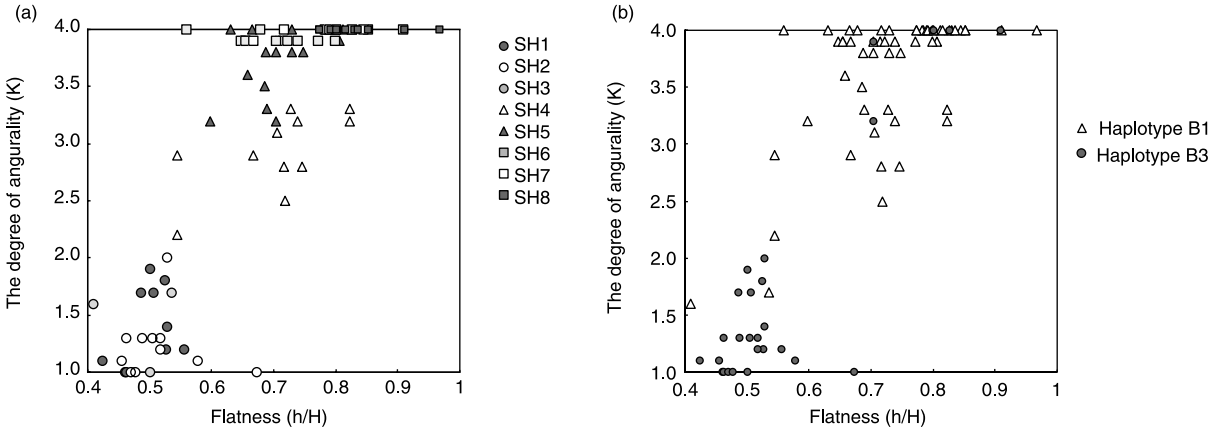

Fig. 9 Scatterplot showing the relationship between the degree of angularity (K) and flatness (h/H). (a) Circles are snails from SH1 to 3 (all globular); squares are snails from SH6 to 8 (all keeled-flat); triangles are putative intermediate snails from SH4 and 5. (b) The same figure showing the mtDNA haplotype.

Snails with the keeled-flat and intermediate shell mostly had mtDNA haplotype B1, whereas snails with globular shells had mtDNA haplotype B3. However, a few individuals with globular shells had haplotype B1 and several individuals with keeled-flat shells had haplotype B3.

Discussion

Phylogeny

The evidence from mitochondrial DNA and, to a lesser extent, ITS-2 suggests that the histories of keeled-flat *A. editha* snails from the two geographically-separate populations, Shimamaki and Samani, are independent, implying that this shell form may have arisen by convergent evolution (Schluter 2000). As juvenile snails sometimes have a keel, it is easy to imagine how a juvenile character could be retained to adulthood (paedomorphy) (Gould 1969, 1971). However, there is no direct evidence that the keel evolved via a paedomorphy, especially since the juvenile snails of globular adults do not usually have a keel. The main alternative explanation is a single evolution of keeled-flat snails, where some populations have acquired their mtDNA and ITS-2 sequences from adjacent populations of different shape snails by hybridization and introgression (Davison 2002).

The results reported here and in Katakura *et al*. (1990) are iconoclastic in terms of the range of the intraspecific shell variation, so it would be wise to consider other possibilities. Further studies on the genitalia and radulae, as well as microsatellite data, may reveal that *A. editha* is not one, but a complex of species. If so, then the evolution of the keeled and globular forms is interesting nonetheless, and ultimately, decisions on whether they have reached species status may depend on taxonomic definitions.

Hybrid zone

Several results across the Shimamaki transect point to a hybrid zone between the two types of snails. First, there were clines in most of the measured characters (Fig. 8). In particular, the characters that we were especially interested in, the angularity (K) and globularity (H/D), were intermediate near the centre. Second, the mtDNA frequency changed, and there was an ITS-2 cline with the centre around SH3, 4 or 5 (Fig. 7). Third, laboratory studies have shown that keeled-flat and globular snails in Shimamaki can mate and produce viable offspring (Ogimura *et al.* unpublished), and the different populations of snails have identical genitalia (Katakura *et al*. 1990; Y. Kuwamura, unpublished).

There are three hypotheses that could explain the existence of the putative hybrid zone. First, the populations of snails with globular and keeled-flat shells met only recently. Second, shell morphology is an adaptation to current habitat and environment; thus, intermediate shells in the centre of the hybrid zone have the highest fitness (bounded hybrid superiority). Finally, hybrid snails could have lower fitness, so the hybrid zone is maintained by the balance between selection against the hybrids and gene flow (tension zone; Barton & Hewitt 1985).

Snails in Obiragawa (Fig. 2), which is very near the Shimamaki keeled-flat sample sites but to the north, have the subgroup B1 haplotype, the same as that found in keeled-flat shells from Shimamaki. Unfortunately, we were unable to sample a transect between the Obiragawa and Shimamaki populations because of the extreme topography (also, between depressed-globular and keeled-flat populations in Samani). It is possible that snails in the Obiragawa population have subgroup B1 haplotypes because of introgression from keeled-flat snails, or *vice versa*.

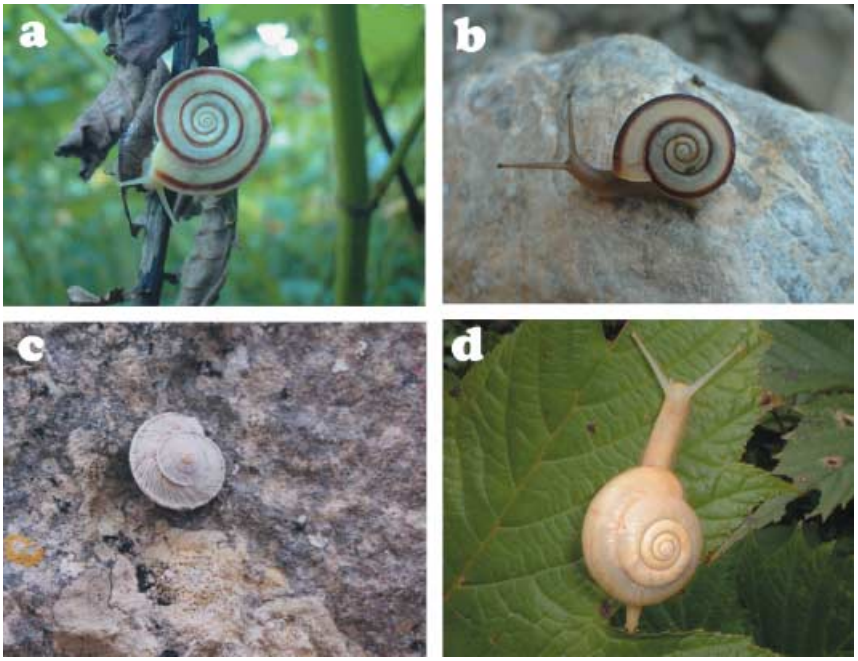

Fig. 10 Possible convergent evolution in limestone environment? (a) Keeled-flat *Ainohelix editha* from Shimamaki, (b) *Euhadra murayamai*, Niigata prefecture (left coiling), (c) *Helicigona lapicida* from Sicily (d), globular *A. editha* from Shimamaki (photos: A. Davison except c, E. d'Amato).

The evolution of keeled-flat shells

There are several explanations as to why shells might be keeled and flat. The present study suggests that *A. editha* keeled-flat shells could have evolved independently in two different locations. Similar shells, though not so extreme in form or variation, are *Euhadra murayamai*, a bradybaenid snail that is confined to a single limestone mountain in Niigata prefecture, Honshu, Japan, and *Helicigona lapicida*, a European helicid, also predominantly found on limestone (Fig. 10). It is possible that keeled shells are an adaptation to limestone substrates (Alonso *et al*. 1985), but we are unaware of any phylogeny-controlled comparative studies that have tested whether an association exists, so the function remains speculative.

Snails like *A. editha* that dig into the ground when inactive (during winter or dry weather), or roam over the ground when active, require a large foot and consequently, a large-mouthed shell to accommodate it, resulting in a discoidal to globular form. Cook & Jaffar (1984) and Heller (1987) have proposed that snails that climb up vertical vegetation may require a large shell, but that a globular shell would be at a disadvantage because of torque, which would tend to pull the snail off the surface. Similarly, *A. editha* might require a flattened shell, either because they climb (snails of both types are often found in shrubs and trees, but we do not have comparative frequency data) or because it is an adaptation to movement through rock crevices on hard substrata. Alternatively, Cain & Cowie (1978) suggested that a flat shell is an adaptation for crawling on horizontal surfaces. It is clear that there are a great variety of possible theories to explain the keeled-flat shells, but which are impossible to discount. Obviously, further studies are needed to understand the evolution of this shell form.

Acknowledgements

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Hiroaki Teshima and Hideo Ogimura completed this work as part of their masters research projects at Tohoku University. Yashuhiro Kuwamura is working on morphological variation in land snails. Masakado Kawata, Jun Yokoyama and Satoshi Chiba are interested in evolutionary theory, land snail and plant evolution. Angus Davison was a visiting JSPS research fellow, concentrating on speciation in Mandarina land snails.
