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Extreme, continuous variation in an island snail: local diversification and association of shell form with the current environment

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On Rosemary Island, a small continental island (11 km^2) in the Dampier Archipelago, Western Australia, snails of the genus *Rhagada* have extremely diverse morphologies. Their shells vary remarkably in size and shape, with the latter ranging from globose to keeled-flat, spanning the range of variation in the entire genus. Based primarily on variation in shell morphology, five distinct species are currently recognized. However, a study of 103 populations has revealed continuity of shell form within a very closely-related group. A phylogenetic analysis of specimens from Rosemary Island, and other islands in the Dampier Archipelago, indicates that much of the morphological variation has evolved on the island, from within a monophyletic group. Within the island, snails with distinct shell morphologies could not be distinguished based on variation in mitochondrial DNA or their reproductive anatomy. The shell variation is geographically structured over a very fine scale, with clines linking the extreme forms over distances less than 200 m. Although there is no evidence that the different forms have evolved in isolation or as a consequence of drift, there is a strong association between keeled-flat shells and rocky habitats, suggesting that shell shape may be of adaptive significance. *Biological Journal of the Linnean Society*, 2011, **104**, 756–769.

ADDITIONAL KEYWORDS: continental island – geographic variation – land snail – spire index.

INTRODUCTION

An understanding of reproductive relationships is a fundamental precursor to detailed evolutionary studies because patterns of reproductive continuity determine the potential for genetic exchange and divergence. Despite their significance in the evolutionary process, patterns of reproductive continuity can be difficult to resolve. Land snails are a particularly challenging group. Their shells vary dramatically in size, shape, sculpture (Goodfriend, 1986), and colour and banding pattern (Jones, Leith & Rawlings, 1977; Johnson, Murray & Clarke, 1993), both within and among species. Although shell variation can provide useful taxonomic characters (Schander & Sundberg, 2001; Jordaens *et al*., 2009), levels of within-species variation may in some cases exceed those between species or even within entire genera (Gould & Woodruff, 1978; Teshima *et al*., 2003). In addition to highlighting the potential complexity of reproductive relationships among species distinguished by their shells, detailed studies of closely-related groups of land snails, including *Partula* (Murray & Clarke, 1980), *Cerion* (Gould & Paull, 1977; Gould & Woodruff, 1978), and *Ainohelix* (Teshima *et al*., 2003), have demonstrated the fine-scale sampling required to distinguish among-population variation from that distributed among reproductively isolated species.

The present study focuses on an extremely diverse, but poorly understood group of land snails, genus *Rhagada*, inhabiting Rosemary Island, one of the 46 islands in the Dampier Archipelago in Western Australia. Although the genus is morphologically conservative across its mainland distribution (approximately 200 000 km^2), the *Rhagada* of the Dampier Archipelago have undergone extensive diversification in shell form, including size, shape, and sculpture

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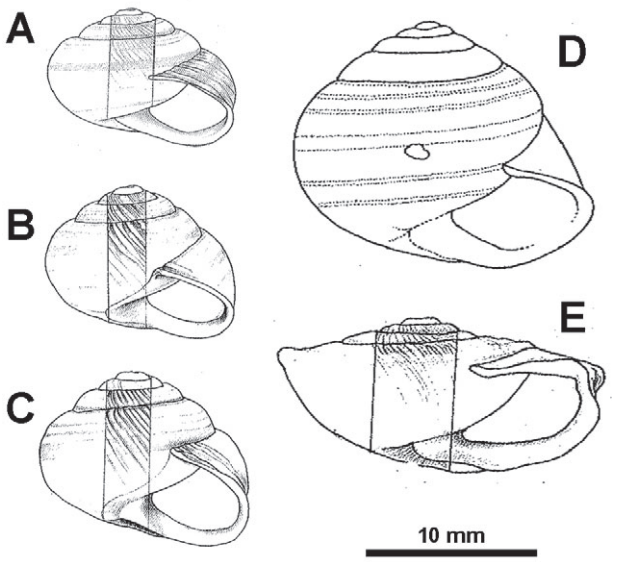

Figure 1. The *Rhagada* land snails of Rosemary Island *sensu* Solem (1997). A, *Rhagada minima*. B, *Rhagada intermedia*. C, *Rhagada elachystoma*. D, *Rhagada perprima*. E, *Rhagada dampierana*.

(Solem, 1997; Johnson *et al*., 2004). Within the Archipelago, Rosemary Island is especially rich in variation (Fig. 1). Although shell size varies considerably, variation in shape is most striking, with shells ranging from globose to flat with a distinct keel at the periphery. Because the keeled-flat form is known only from Rosemary, this small island (11 km^2) contains the entire range of overall shell shape variation known in the genus, which exceeds that in most genera of land snails.

Based mainly on variation of shell morphology, five species of *Rhagada* are recognized on Rosemary Island, four of which also occur on other islands in the Dampier Archipelago (Solem, 1997) (Fig. 1). Although the variation in size and shape far exceeds the levels typically expected between congeneric species, in some ways justifying the description of several taxa, the taxonomy is based on limited, opportunistically collected material. There have been no formal surveys of the island, and the geographic distributions of the different forms are unknown.

As a basis for understanding the range of morphological diversity on such a small island and the relationships among the described forms, a detailed population study of morphological, anatomical, and molecular variation in the *Rhagada* of Rosemary Island was conducted. Specifically, the present study focussed on the reproductive relationships between snails with different shell forms, their evolutionary histories and geographic distributions. Also, given that variation in shell shape is almost certainly of

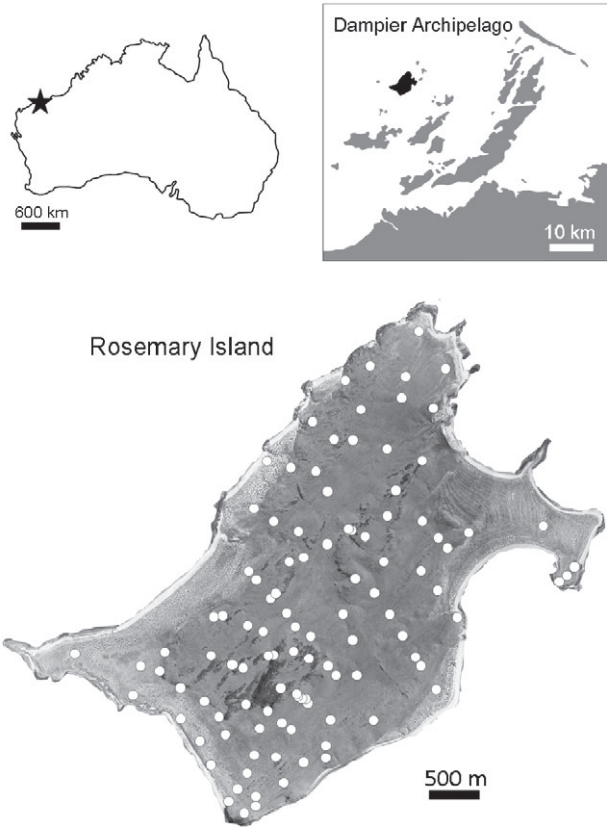

Figure 2. The location of Rosemary Island and the 103 sites where *Rhagada* were collected.

adaptive significance (Cain, 1977; Okajima & Chiba, 2009), a search was made for associations between features of the environment and the distribution of keeled-flat shells.

MATERIAL AND METHODS

Study location and samples

Rosemary Island (20°28'47' S, 116°35'36' E) is the fourth largest island in the Dampier Archipelago (Fig. 2). The climate in the region is semi-arid, with consistently high temperatures and low, unreliable rainfall (Kreiwaldt, 1964).

The island has been in its current form for 6 to 8 kyr. For approximately 120 000 kyr prior to that, the islands of the Dampier Archipelago were connected, forming part of continental Australia. Based on historical estimates of global sea levels, there have been four cycles of fragmentation and connection in the last million years (Bintanja, van de Wal & Oerlemans, 2005), which is the period of time that *Rhagada* are assumed to have inhabited the region (< 1 my; Johnson *et al*., 2004). Approximately 130 000 kya, during the Eemian Historical Highstand

(Vezini, Jones & Ford, 1999), rising sea levels fragmented Rosemary into two islands: a large main island and a small satellite island, which were separated for approximately 6–8 kyr (Lambeck & Chappell, 2001).

Rhagada snails were collected from 103 locations during October 2008 (Fig. 2). At each site, up to ten live adults were collected, as defined by the presence of a reflected lip on the shell aperture, which marks the end of shell growth (Solem, 1997). If unable to collect ten live adults, empty shells were collected that had retained their colour and texture. The material at each site was collected within a radius of 15 m, an area smaller than the neighborhood size estimated for the closely-related congener *Rhagada capensis* (referred to as *Rhagada convicta*; Johnson & Black, 1991). Thus, it is reasonable to assume that each of the 103 samples was collected from a single panmictic unit, and they are referred to as populations in the present study. Live, aestivating snails were transported to the laboratory, activated by exposure to moisture so that tissue could be harvested, and stored at –80 °C.

Adult shells were also obtained from the Western Australian Museum, including all specimens collected from Rosemary Island, except for a small number preserved in alcohol. The material examined comprised: *Rhagada dampierana* (Solem, 1997), 49 specimens, including the holotype (WAM 766.87) and paratypes (WAM 221.74, WAM 10.87, WAM 12.87, and WAM 1549.70); *Rhagada minima* (Solem, 1997), 42 specimens, including the holotype (WAM 763.87) and paratypes (WAM 32.74, WAM 252.74, WAM 14.87, and WAM 22.87); *Rhagada intermedia* (Solem, 1997), eight paratypes (WAM 24.87); *Rhagada elachystoma* (von Martens, 1878), eight shells examined by Solem (1997) but not involved in the description of the species (WAM 327.74 and WAM 329.74).

Despite efforts to locate material, three specimens of *Rhagada perprima* (Iredale, 1939), the holotype and two paratypes, could not be recovered. Because these were the only specimens of *R. perprima* known from Rosemary Island, it was not possible to examine historical material from only four of the species recognized by Solem (1997).

Analysis of shell size and shape

The existing taxonomy of *Rhagada* was based on simple shell measurements, including width and the spire index (shell height divided by shell width). In the present study, a geometric morphometric approach was used to quantify the variation in shell form. Now routinely used in morphological studies of biological structures (Zelditch *et al*., 2004), procrustes-based geometric morphometric methods rely on geometric information captured by sets of Cartesian coordinates (configurations), which correspond to biological landmarks. Geometric morphometric methods have several advantages over 'traditional' methods. These include fewer a priori assumptions about what should be measured, size-independent estimates of shape, and greater statistical power. A description of the methods used in the present study is provided in Zelditch *et al*. (2004).

Each shell was photographed at the same magnification and in the same orientation (columella parallel to the *y*-axis) using a digital camera mounted on a dissecting microscope. Landmarks were digitized using TPSDIG2 (Rohlf, 1998a; Fig. 3A). Fixed landmarks were placed at the apex of the shell spire (L1), where the top of the shell aperture meets the shell wall (L5) and where the shell aperture crosses the base of the shell (L12); these were the only three features that could be recognized across all the specimens as a result of the large variation in shape. The remaining landmarks were designated as semi-sliding. Unlike fixed landmarks, which are placed on the same features for all specimens, the precise locations of semi-sliding landmarks are not important. Rather, groups of semi-sliding landmarks can be placed along curves or contours that are homologous among specimens, in the present case, the shell outline between the spire and shell aperture (L2–L4), the outer edge of the shell aperture (L6–L11), and the entire left-hand edge of the shell (L13–L20).

Nonshape variation in the landmark coordinates (i.e. variation associated with the position, orientation and size of specimens) was removed by generalized procrustes analysis in TPSRELW (Rohlf, 1998b). The resulting superimposition was analyzed with TPSRELW, which calculated a consensus shape, and then partial warp scores, which describe the deviation in the shape of each sample from the consensus shape. TPSRELW was used to conduct relative warps analysis on the partial warp scores. Relative warps analysis is a means of data reduction analogous to a principal components analysis, so that each consecutive relative warp explains a decreasing percentage of the total variation in shape. Three relative warps analyses were conducted. The first was conducted on the museum specimens, enabling a test of the ability of relative warps analysis to discriminate the groups recognized by Solem (1997); multivariate analysis of variance (MANOVA) was conducted on these scores using JMP, version 5 (SAS Institute). The second analysis was conducted on 941 new specimens. From these, the mean scores were calculated for each population. The third relative warps analysis was conducted after pooling the

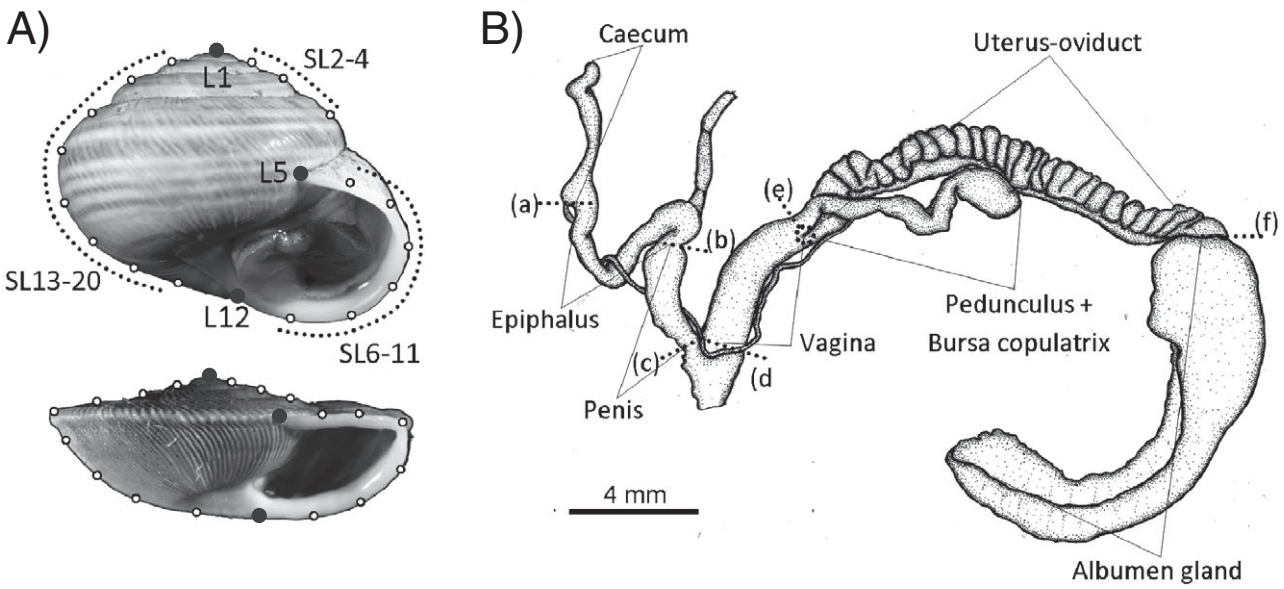

Figure 3. A, the positions of the 20 landmarks on a globose and a keeled-flat shell. Landmarks 1, 5 and 12 were fixed landmarks (black circles), while the remainder were semi-sliding landmarks (white circles) that traced the shell outline. B, a drawing of the reproductive tract and the measured structures. The dashed lines represent the boundaries between structures: (a) where the vas deferens joins the epiphalus; (b) the narrow junction between the penis and epiphalus; (c, d) the junction where the penis and vagina meet; (e) the junction of the uterus-oviduct, vagina and pedunculus; and (f) the junction between the uterus-oviduct and the albumen gland.

museum specimens and the new specimens, totalling 1048 shells.

Estimates of error associated with digitization and specimen orientation were calculated based on ten specimens that represented the range of variation in the pooled samples. Digitizing error was determined by digitizing a single image of each specimen ten times, and orientation error by repositioning and photographing each specimen ten times. The mean Procrustes distance was calculated for each specimen using TPSSMALL (Rohlf, 2003), averaged across specimens, and expressed as a percentage of the mean Procrustes distance in the whole data set. These estimates revealed that digitizing and orientation error were negligible.

TPSRELW was used to estimate the centroid size of each shell. Centroid size is a landmark-based estimate of the size of a structure, independent of shape, and is calculated as the square root of the summed square distances between each landmark and the centroid of the structure being measured. Centroid size was used as the measure of shell size for both the museum specimens and the new samples.

Anatomical analysis

Solem's taxonomy of *Rhagada* also included an assessment of the reproductive system, the assumption being that variation in reproductive structures, particularly the genitalia, causes or reflects speciation. As his assessment was qualitative, and included only three of the species recognized from Rosemary Island, the present study tested whether morphologically distinct shells could be distinguished based on features of their reproductive system.

Four distinct populations were selected, representing the extreme combinations of shell size and shape variation (small flat, $N = 7$; large flat, $N = 7$; small globose, $N = 10$; large globose, $N = 9$). When examining reproductive structures in land snails, it is important to consider the reproductive status of the animal, as condition may vary seasonally, even in fully mature adults. Unlike species of camaenid that inhabit areas with seasonal rainfall, which exhibit significant change in the size of reproductive structures in anticipation of extended periods of high or low activity (Solem & Christensen, 1984), *Rhagada* in the region of the Dampier Archipelago maintain an active reproductive system year round, presumably as an adaptation to maximize reproduction after sporadic rainfall (Solem, 1997). Recent activity, access to food and state of hydration may also influence reproductive condition. As a precaution against these potential effects, snails were activated and maintained with food and water for 14 days before dissection. Snails were then drowned in tap water for 24 h,

and the reproductive tract was removed and photographed under a dissecting microscope.

Rather than performing an exhaustive evaluation of all the features of the reproductive tract that may vary, the analysis was restricted to structures that varied among the species from Rosemary Island, according to the taxonomic descriptions, as well as some additional structures that distinguished other species of *Rhagada* (Solem, 1997). These included the: (1) albumen gland, (2) uterus-oviduct, (3) pedunculus-bursa copulatrix, (4) vagina, (5) penis, (6) epiphalus, (7) epiphalic caecum (Fig. 3B), and (8) penial verge. As the Rosemary Island species were distinguished based on variation in the relative lengths of the above structures (Solem, 1997), with no mention of variation in width, the present analysis was also restricted to length variation. Structures were measured from photographs using Image J (Abramoff, Magalhaes & Ram, 2004), with the boundaries between structures demarcated according to Figure 3B. With the exception of the penial verge, which was expressed as a proportion of the length of the penis, the absolute length of each structure was expressed as a proportion of the total length of the reproductive tract, which was determined as the sum of the length of the all the structures, with the exception the albumen gland. The albumen gland was excluded from the estimate of total length because variation in this large organ had a dramatic effect on the relative length of other shorter structures, potentially reducing the sensitivity of subsequent analyses.

The relationships between the absolute length of the genital tract (minus the albumen gland) and the width and relative height of shells were examined using linear regression. To determine whether the four populations could be distinguished based on the relative lengths of reproductive structures, discriminant analysis was performed on the arcsine-transformed data using JMP, version 5. Discriminant analysis was chosen because, unlike factor analyses such as principal components, it is efficient at finding differences between a priori groups, even when many variables vary within groups. Thus only one variable needs to differ consistently for accurate discrimination among groups, in this case the four morphologically distinct populations. Two discriminant analyses were performed. The first was a stepwise analysis that used the Wilks' lambda criterion to determine the influence of each variable on the separation of the groups, and *F*-statistics to test the significance of the change in the Wilks' lamda following each step (maximum partial *F*-to-enter = 3.84; minimum partial *F*-to-remove = 2.71). The second analysis was performed with all the variables entered, regardless of their individual power to discriminate between the groups.

The interior penial wall was also examined, noting whether pilasters were present. Although known from only a single mainland species of *Rhagada*, *R. torulus*, basal pilasters are considered important taxonomic characters that may be directly involved in reproductive isolation (Solem, 1997).

Phylogenetic analysis

Phylogenetic relationships were inferred between the different shell forms based on variation in DNA sequence. Total DNA was extracted from foot tissue taken from one individual from each population, using Qiagen DNeasy blood-tissue kits in accordance with the manufacturer's instructions. Phylogenetic analysis was based on four gene fragments that vary in land snails at a range of evolutionary timescales. The first two, the nuclear gene ITSII and the mitochondrial gene 18S are normally highly conserved in land snails, whereas the mitochondrial DNA markers 16S and COI are known for rapid rates of divergence (Thomaz, Guiller & Clarke, 1996; Chiba, 1999). Primer sequences for ITSII, 16S (16SAR and 16SBR), and COI (COIH and COIR) were obtained from White *et al*. (1990), Palumbi (1996) and Folmer *et al*. (1994), respectively. The 18S fragment was amplified with the primers 18SF (5′-GGTGATCCTGCCAG-3′) and 18SR (5′-AGGCTCCCTCTCCGAATCGAA-3′). Each 25 μL polymerase chain reaction (PCR) reaction contained 50 mM KCl, 20 mM TrisHCl (pH 8.4), 0.2 mM of each DNTP, 0.3 mM of forward and reverse primer, 1 U of *Taq* DNA polymerase, either 0.75 mM (18S) or 1 mM (ITSII, 16S & COI) $MgCl_2$, and 20 ng of DNA. Cycling conditions for ITSII comprised an initial denaturation period of 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s (denaturation), 53 °C for 30 s (annealing), and 72 °C for 1 min (extension), with a final extension period of 72 °C for 1 min. The mitochondrial genes were amplified using an initial denaturation period of 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s (denaturation), 51 °C for 30 s (annealing), and 72 °C for 1 min (extension), with a final extension period of 72 °C for 5 min. PCR products were purified using UltraCleanTM PCR Clean-UP Kits (MO BIO Laboratories Inc.). Sequencing reactions were conducted with Big Dye, version 3.1 (Applied Biosystems) chemistry and sequenced on an ABI 3730 capillary machine. Sequences were edited and aligned with SEQUENCHER, version 4.6 (Genecodes).

In addition to the sequences obtained as part of the present study, sequences from *Rhagada* on other islands in the Dampier Archipelago were provided by M. S. Johnson, Z. Hamilton, R. Teale & P. Kendrick (unpubl. data). These included all the described *Rhagada* from the Dampier Archipelago (Solem, 1997), and several undescribed forms. An appropriate

outgroup, *Rhagada angulata*, was determined from a broader analysis of the genus (M. S. Johnson, Z. Hamilton, R. Teale & P. Kendrick, unpubl. data).

Phylogenies were constructed using two methods. The first was the Neighbour-joining method implemented in MEGA, version 4.0 (Tamura *et al*., 2007). Support for each node was determined via 1000 bootstrap replicates. The analysis was performed on each gene separately, and then on a concatenated alignment comprising all variable gene fragments. The second was a Bayesian analysis, as implemented in MrBayes (Huelsenbeck & Ronquist, 2003). The software JMODELTEST (Guindon & Gascuel, 2003; Posada, 2008) and the Akaike information criterion were used to determine the most likely model of nucleotide substitution for each alignment. Runs in MrBayes consisted of four Markov chains; 10–20 million generations with sampling every 1000 generations. Convergence was assessed by examining the SD of split frequencies and plots of log likelihood values across a minimum of five runs for each alignment. Fifty-percent majority-rule consensus trees were constructed from post-burn-in genealogies.

RESULTS

Morphological variation in the museum specimens

The first two relative warps accounted for 87.5% of the total variation in shell shape (76.6 and 10.9%, respectively). RW1 reflected variation in the relative height of shells, and was almost perfectly correlated with the measure of shape used in the existing taxonomy, the spire index ($r^2 = 0.979$: spire index = 1.3481*RW1 + 0.6419). Thus, RW1 and the spire index can be considered analogous measures of shape, both reflecting variation in relative shell height. RW2 reflected variation in the position that the top of the aperture joined the shell wall. The variation in shape associated with subsequent relative warps was too subtle to be visualized via the thin-plate spline deformations.

A one-way MANOVA conducted on the first ten relative warps, accounting for 99% of the total variation in shape, revealed a significant difference among the four previously described species (Wilks' lambda 0.0592, approximate $F = 14.778$; d.f. = 30,273.65; $P < 0.0001$). A second MANOVA, conducted without RW1 (Wilks' lambda 0.651, approximate $F = 1.628$; d.f. = 27,278.09; $P = 0.0287$) and third without RW1 and RW2 (Wilks' lambda 0.825, approximate $F = 0.795$; d.f. = 24,279.03; $P = 0.742$) revealed that the first two relative warps were the source of the variation among the four species.

Of the four species, only one was distinct, based on variation in shell shape (Fig. 4A). A plot of the RW1 and RW2 scores revealed two distinct clusters that were separated based on variation in relative shell height. The cluster at the positive end of the RW1 axis comprised the 49 shells from the keeled-flat species, *R. dampierana*. The shells in the other distinct cluster, which ranged in shape from globular to subglobular, belonged to the other three species. There was considerable overlap between the clusters of *R. minima* and *R. intermedia*, which were recognized as distinct based on relative shell height. RW2 did not contribute to the separation of any species.

In agreement with the taxonomy, two of the species that could not be separated based on shape were distinct based on shell size (Fig. 5). *R. elachystoma* and *R. minima* were the largest [3955 (SD 182.83)] and smallest [3152 (SD 329.21)] of the four species, with almost no overlap between their centroid size-frequency distributions. Overall, three distinct groups were identified in the museum specimens based on the geometric morphometric estimates of shell size and shape (Fig. 6).

The new collections

By contrast to the taxonomic expectation, distinct morphological groups were not evident in the samples collected in the present study. As with the museum specimens, RW1 explained the majority (67.1%) of the variation in shape, and reflected variation in relative shell height (Fig. 4B). Rather than the two distinct clusters observed in the museum specimens, there was a continuum of variation, ranging from globose, to keeled-flat, with a subglobular shape most common. The third relative warps analysis, conducted on the pooled samples, demonstrated that the museum collection contained the extremes of variation uncovered by the comprehensive population samples but not the intermediate shapes that connected the subglobular and keeled flat forms (Fig. 4C). The detailed sampling also revealed a continuum of shell size, with the range of variation slightly exceeding that observed in the museum specimens (Fig. 5).

Continuity was also observed in several other shell characters that were used to distinguish the previously recognized species (Fig. 7). In the museum specimens, the peripheral keel was observed only in shells from the flattest species, *R. dampierana*. This was not the case in the new specimens. Although a strong keel was always present in the flattest shells and absent in the tallest, shells of intermediate height varied in the degree to which they were keeled. This was true not only for the presence of the keel, but also for the strength of the keel, which varied from just visible to thickened. Similar patterns were

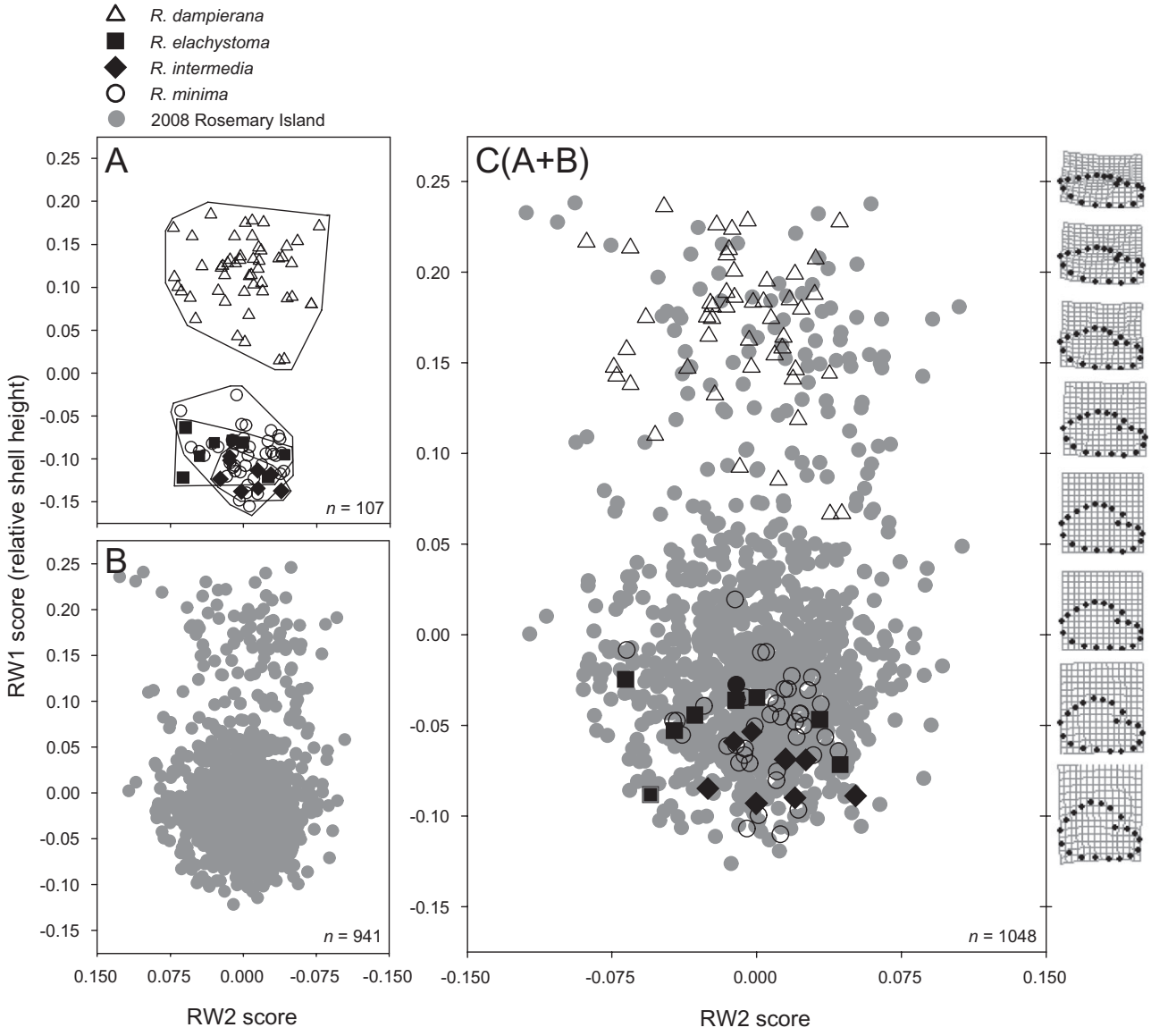

Figure 4. Two-dimensional plots of shell shape (RW1 and RW2) extracted from relative warps analysis. A, shape variation in the museum specimens (RW1 = 76.6%; RW2 = 10.9%). B, shape variation in the samples collected during the present study (RW1 = 67.1%; RW2 = 13.8%). C, (A) and (B) combined (RW1 = 67.2%; RW2 = 13.9%). The thin plate spline deformation plates (C) illustrate the change in shape associated along the RW1 axis (RW2 score of 0).

also observed for the pattern of sculpturing, which was most prevalent in the flattest shells, and banding, with taller shells generally being more banded (Fig. 7).

There was no relationship between shell centroid size and RW1 score ($r = 0.047$; $P = 0.150$). Instead, all combinations of size and shape were present. This was a striking result because the keeled-flat shape was associated only with larger shells in the analysis of the museum specimens. Thus, the new samples extended the combinations of size and shape to include keeled-flat, small shells, which were not present in the museum collections.

Population-level patterns of shell shape (RW1) and size were similar to those observed at the individual level (Fig. 8). The mean values for the 103 populations were distributed fairly continuously along both morphological dimensions, although subglobular populations were most common and intermediate sized populations least common. The variation in size and shape was distributed among populations, with mean RW1 scores ranging from

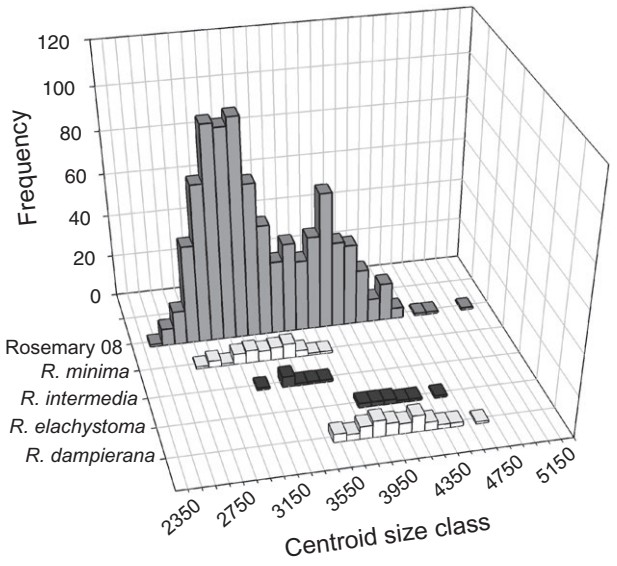

Figure 5. Shell size (centroid size) frequency distributions for the museum specimens (coded by species) and the samples collected in the present study (coded Rosemary 08).

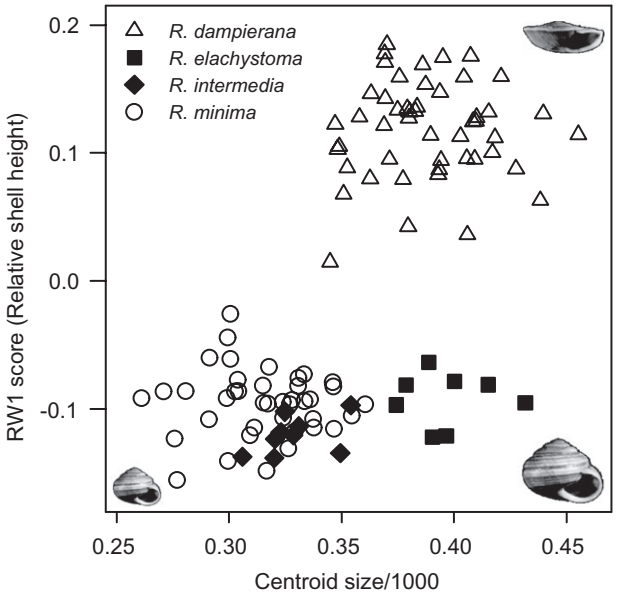

Figure 6. RW1 and centroid size scores for the museum specimens.

0.206 [SE 0.030 (flattest shape)] to –0.091 [(SE 0.016 (tallest shape)], and mean centroid size scores ranging from 2690.0 [SE 144.70 (smallest)] to 4154 [(SE 288.97 (largest)]. Plotting population variation in two dimensions revealed less morphological continuity than was observed among individuals. Nevertheless, the four extreme combinations of the variation were observed, with no significant relationship between centroid size and the RW1 score ($r = 0.107$; $P = 0.274$).

Figure 7. Shell variation in *Rhagada*. The shells are arranged in ascending relative shell height. Note also variation in the peripheral keel, as well as sculpture and banding pattern. With the exception of the flattest shell and two tallest shells, all the specimens were collected at a single locality.

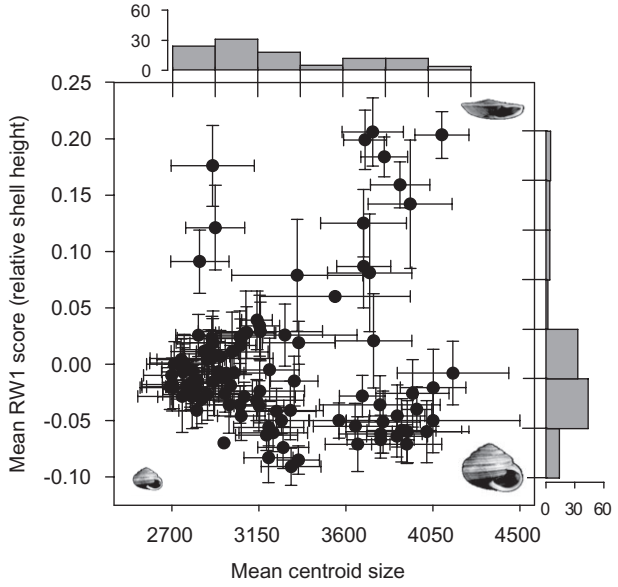

Figure 8. Mean RW1 and centroid size scores for the 103 populations. The vertical and horizontal bars represent the standard deviations of the means for RW1 and centroid size scores, respectively. The histograms on the *x*- and y-axes show the distributions of populations among seven shape and size classes used in Figure 10.

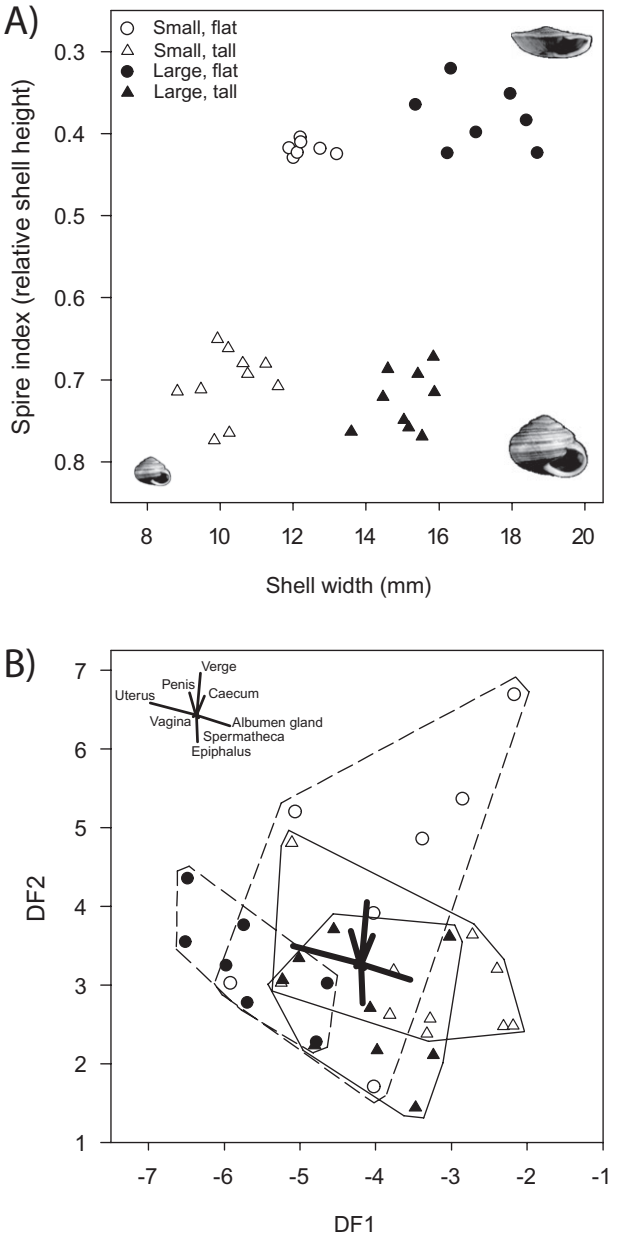

Figure 9. Variation in shell shape and genital anatomy in four populations of *Rhagada*. A, size and shape variation within and among the populations. B, plot of the first two discriminant functions extracted from analysis of reproductive measurements. The canonical vectors show the relative contribution of each reproductive structure to the discriminant functions.

Anatomical variation

The four populations selected for anatomical analysis were distinct, based on shell size and shape (Fig. 9A). There was a significant, linear relationship between the length of the reproductive system and shell width ($r^2 = 0.41$; $P \leq 0.000$) but no relationship with relative shell height ($r = 0.03$; $P = 0.845$). The morphology of the penial wall did not vary in any of the specimens.

Although there was variation in the relative lengths of the reproductive structures examined, the majority of the variation was distributed within the four morphological groups. All the structures were poor individual discriminators, and none met the criteria for entry into the stepwise analysis. In the second analysis, in which all variables were entered regardless of their discriminatory power, over one-third of individuals (34.4%) were misclassified. Of the 24 individuals that were correctly classified, only two were classified with a level of confidence exceeding 95% (mean confidence for correct classifications was 72.9% (SD 0.19).

The lack of anatomical distinctness among the populations was illustrated in the first two dimensions of the discriminant space (Fig. 9B). The canonical biplot arrays showed that the lengths of the uterus and spermatheca contributed most to the separation of points along discriminant axis one, whereas discriminant axis two mainly reflected variation in the length of the penial verge and epiphalic caecum. The remaining variables made little contribution to the separation on either discriminant axis. Although some variation was exclusive to each group, there was clear and considerable overlap between the distinct shell groups in the anatomical discriminant space.

Phylogenetic relationships and molecular varaition

The Hasegawa–Kishino–Yano (HKY+G) model with gamma-distributed rates was the best fitting model of sequence evolution for both mitochondrial fragments, whereas the nuclear gene ITSII and the mitochondrial gene 18S, were invariant. The same model was favoured for the concatenated 16S/COI alignment, with the addition of a proportion of invariable sites (HKY+G+I). The Neighbour-joining and Bayesian phylogenetic reconstruction methods recovered very similar topologies, as did both gene fragments.

Within the samples of *Rhagada* collected from the surrounding Dampier Archipelago, the samples from Rosemary Island formed a well supported monophyletic clade, which, according to the phylogeny, is most recently derived (Fig. 10). Within this clade, the most divergent samples were from Rosemary Island's easternmost point, which was isolated as a separate island during the Eemian Historical Highstand. The three samples formed a distinct clade, as a sister group to the samples from the main part of the island.

Within the island, 16S and COI were moderately variable (7.3% and 14.7% of sites variable, respectively). Mean pairwise divergence was 0.53% [SD 0.57 (range 0–2.5%)] for 16S and 1.94% [SD 1.22 (range 0–5%)] for COI. By contrast to the taxonomic expectation, there was very little concordance between morphological and molecular variation (Fig. 10). Rather than forming exclusive monophyletic clades, as would be expected under the present taxonomy, samples with the same extreme shell morphology were distributed among several well-supported clades, and grouped with samples that were morphologically distinct.

Geographic variation

Plotting the morphological variation on a map revealed clear but complex patterns of geographic variation (Fig. 10). Three main features were apparent. First, shell shape and size varied over an extremely fine spatial scale, with as little as 200 m separating the extremes of shell form. Second, there were several areas where morphologically distinct populations were connected by clines of variation. Third, although there were some clear geographic associations between populations of the same form (e.g. large shelled populations were clustered together in the south of the island), populations of similar shape or size were also found at several, independent locations, rather than clustering as a single cohesive geographic group.

These tendencies were observed in the geographic distribution of keeled-flat snails, which were found at two locations near the island's centre (Fig. 11). Each of these areas, which were separated by over 350 m, coincided with a massive rock outcrop, the larger of which had an area of approximately 90 000 m^2. The association was illustrated by plotting the mean shape (RW1) score for each population against the minimum straight-line distance to the edge of the closest outcrop. Populations located further than 250 m (–250 to –2500 m) from a rocky outcrop were of a fairly consistent globular to subglobular shape. Variation in shape was highest within the narrow zone (–250 to 0 m) surrounding the rocky outcrops, spanning the full range of variation on the island, from globose to keeled-flat. Variation was lowest on the rocky outcrops, with all populations of a relatively flat shape. A four-parameter sigmoid function explained 56% of the variation between shape and distance to the edge of the nearest outcrop ($r^2 = 0.560$; $P \leq 0.000$).

DISCUSSION

The present study has revealed patterns of morphological variation far more complex than were expected from the existing taxonomy. Although the re-analysis of the museum specimens confirmed the presence of several distinct morphological groups, largely agreeing with those recognized by Solem (1997), those distinct groups were not apparent in the comprehensive samples collected during the study. Instead, a continuum of morphological variation was observed, with little evidence for discontinuity among the previously recognized forms. From a taxonomic perspective, the discontinuity in the museum specimens is compatible with the hypothesis that there are several species. However, within the context of the new collections, it is possible to conclude that the discontinuities are an artefact of inadequate sampling. Thus, the present study adds to those that demonstrate the need for detailed geographic sampling when establishing or testing taxonomic relationships (e.g. Gould & Woodruff, 1978; Murray & Clarke, 1980; Teshima *et al*., 2003), and also demonstrates the potential limitations of material obtained from selective sources, such as museums or the fossil record.

The range of morphological variation on Rosemary Island, particularly of shell shape, far exceeds what is observed in most land snail species, and even genera. Nevertheless, the morphological continuity provides evidence for reproductive continuity between the different forms, suggesting that they may a single biological species (Gould & Johnston, 1972; Endler, 1977). The molecular and anatomical results are compatible with this view, although they could also be consistent with recent speciation between the

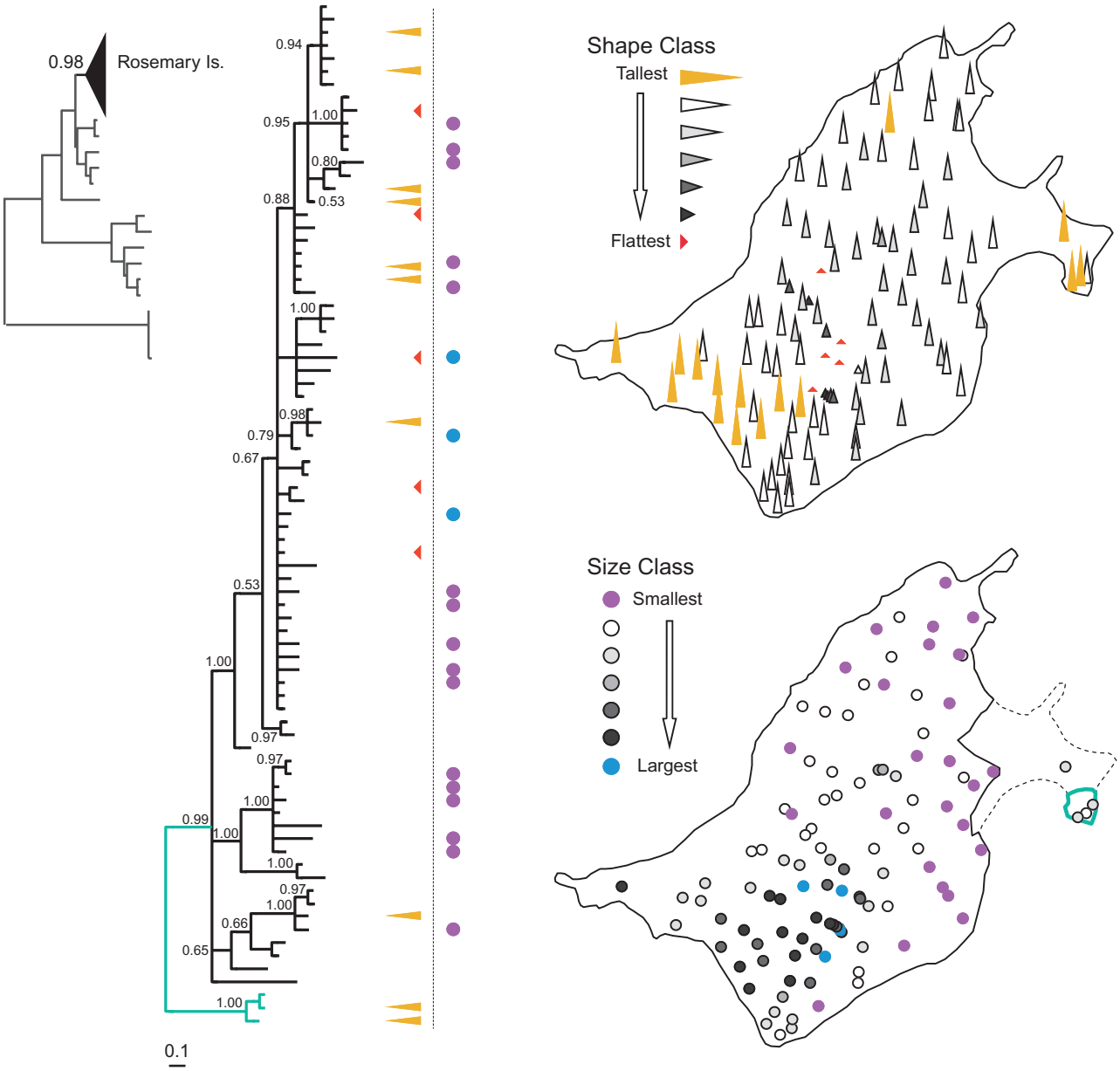

Figure 10. Phylogenetic and geographic distributions of shell shape (relative shell height) and (centroid) size. Populations were sorted into seven shape and size classes (Fig. 8) represented by the triangles and circles, respectively. The phylogeny in the upper left shows the relationship between the samples from Rosemary Island and *Rhagada* from other islands in the Dampier Archipelago. The distributions of extreme shape and size classes are shown on the main phylogeny, which is a 50% majority-rule Bayesian phylogram of concatenated 16S and COI sequences (779 bp). The numbers on the phylogeny represent the Bayesian posterior probabilities for each node. The green outlined area on the bottom map was fragmented from the main island during the Eemian Historical Highstand when sea levels were higher than current levels (see also corresponding clade).

different forms. For example, it is possible that reproductive isolation has developed so recently that it has not yet affected patterns of mitochondrial diversity, as a result of incomplete lineage sorting (Avise, 2000). Similarly, many closely-related species of land snail, for example species of *Partula* on Moorea (Murray & Clarke, 1968, 1980), had very similar reproductive systems and genitalia, yet were reproductively isolated from one another.

Although the morphological evidence for reproductive continuity is compelling, it should be treated as preliminary because intermediate phenotypes can be

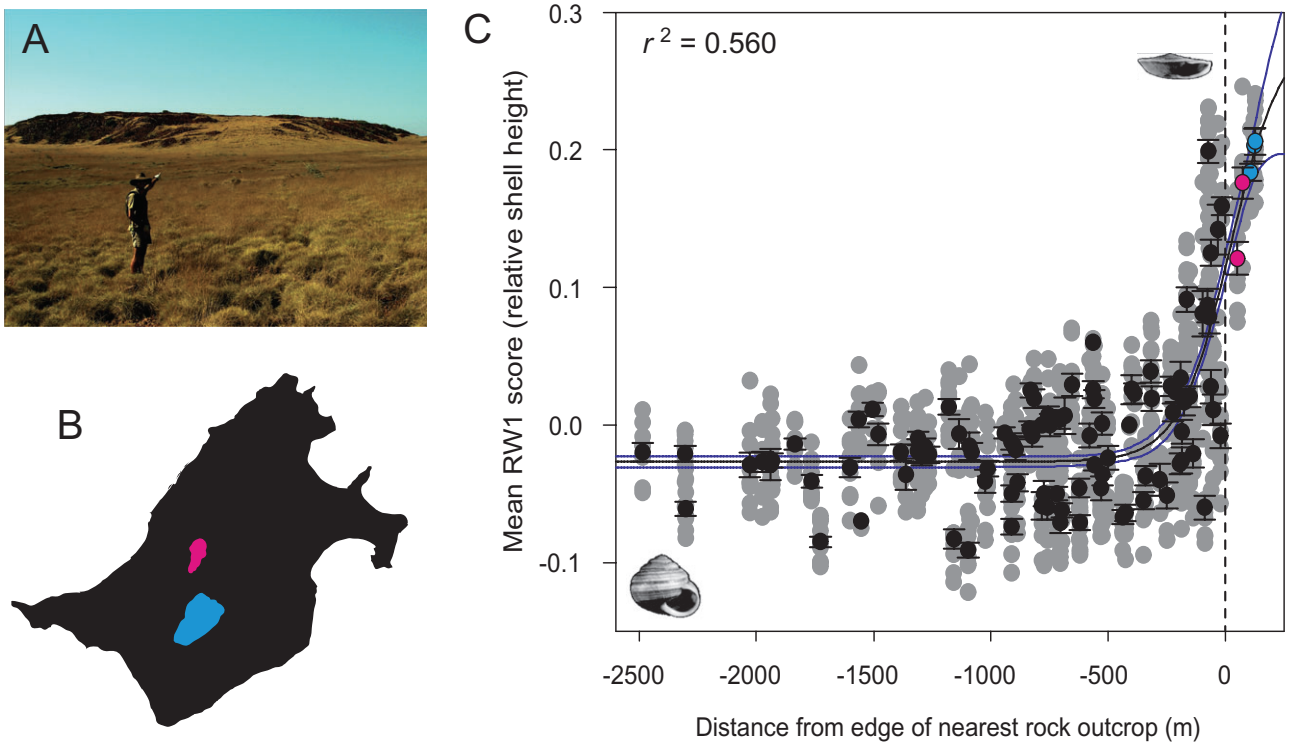

Figure 11. Geographic variation of shell shape in relation to landscape features. A, one of the two rock outcrops occupied by keeled-flat populations of *Rhagada*. B, the position of both rock outcrops on Rosemary Island (indicated by the coloured areas). C, the relationship between the distance of each population from the edge of the nearest rock outcrop and mean relative shell height. The Dashed line at '0 m' represents the edge, so that populations on an outcrop have positive distance scores, and those off an outcrop have negative distance scores. Coloured symbols indicate outcrop of origin, as in (B). The grey symbols are individual scores. The vertical bars represent the standard error of the mean. The regression line ($r^2 = 0.560$) is a four-parameter sigmoid function of the form: $y = y_0 + a/(1 + \exp[-(x - x_0)/b])$. The lines above and below are 95% confidence intervals for the regression.

present in situations where reproductive isolation between distinct populations is almost complete (Jiggins & Mallet, 2000). For example, it is possible that the clines of shell form, particularly the steeper transitions occurring between the extremes of shell size and shape, are hybrid zones. Within a hybrid zone, the production of even a sterile F_1 hybrid generation could result in a spread of intermediate morphologies, either in a polygenic trait, or where the phenotype is determined by interaction between the genotype and the environment. Even if there is evidence for reduced fitness in later generation hybrids, such as reduced viability or strong selection against intermediate phenotypes, it may be more accurate to view this group as a species complex, rather than as a species *sensu stricto*. Detailed studies of the transition zones, including surveys of rapidly evolving nuclear markers, are required to test these alternative possibilities.

Regardless of the extent of reproductive continuity between the different shell forms, the presence of intermediate morphologies suggests that they are very closely related. The phylogenetic evidence strongly supports this interpretation. Within the *Rhagada* collected from throughout the Dampier Archipelago, the snails from Rosemary form a well supported monophyletic group, and are most recently evolved. The closeness of the group is unexpected, given that four of the five species are assumed to occur on several other islands (Solem, 1997). This may suggest that some shell forms in the Dampier Archipelago, currently recognized as the same species, may have evolved several times. Concurrently, the monophyletic ancestry of the group suggests that much of the variation, including the endemic keeled-flat form, has evolved on Rosemary Island.

Within the island, the origins of the complex patterns of geographic variation can be explained in terms of historical processes and events or current selection (Davison, 2002). One possible historical explanation is that the different shell forms evolved

in physical isolation, as has been inferred from studies of other species (Johnson, 1976; Gould & Woodruff, 1990; Davison & Clarke, 2000; Goodacre, 2001). Although it is almost impossible to reject historical isolation as a potential cause of geographic variation (Endler, 1977), there is no phylogenetic or geographic evidence that the different shell forms on Rosemary Island have ever been isolated from one another. The population from the island's easternmost point, which was isolated as a separate island during the Eemian, provides some confidence that the mitochondrial genes are useful for identifying populations that have evolved in relatively short periods of isolation (approximately < 8 kyr), and that the signal can persist for a significant period after secondary contact (approximately 120 000 kyr). However, much shorter periods of isolation, such as those associated with bottleneck or founder events, may have had much less impact on patterns of mitochondrial diversity, and thus may have gone unnoticed. Weak associations between molecular and morphological variation may also be broken down quite rapidly after secondary contact (Endler, 1977).

Genetic drift, acting on gene frequencies within individual populations, can also generate and maintain complex patterns of geographic variation (Endler, 1977). Drift alone, however, is unlikely to have produced these patterns, particularly because *Rhagada* exists as a series of continuous populations, and that some morphs occupy areas that are several orders of magnitude larger than the neighbourhood size estimated for a congeneric species (1100 m^2; Johnson & Black, 1991). Instead, based on the scale and complexity of the geographic patterns, and the phenotypic, reproductive, and phylogenetic continuity within the group, a selective explanation appears more likely.

In the case of shell shape, there is a strong association between the geographic patterns of variation and landscape features, suggesting that shell shape is of adaptive significance. The association between keeled-flat shells and rocky habitats, observed in two separate locations, has also been observed in other morphologically diverse species of land snails, including *Ainohelix editha* (Teshima *et al*., 2003) and *Iberus gualtieranus* (Elejalde *et al*., 2008), adding support to the selective interpretation. Although some possible selective mechanisms have been proposed to explain the association (Goodfriend, 1986; Teshima *et al*., 2003), more work is required to understand the evolution of this unusual shell form. As an extremely diverse, yet cohesive group, these snails provide an excellent opportunity to study the functional aspects of shell morphology, insipient divergence and the forces that shape geographic variation.

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