Morphological differentiation correlates with ecological but not with genetic divergence in a *Gehyra* gecko

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Abstract

Body size affects life history, the ecological niche of an organism and its interactions with other organisms. Resultantly, marked differences in body size between related organisms are often an indication of a species boundary. This is particularly evident in the *Gehyra variegata* species complex of geckos, which displays differential body sizes between genetically divergent species, but high levels of intraspecific morphological conservatism. We report on a *Gehyra* population that displays extraordinary body size differentiation in comparison with other *G. variegata* species. We used morphological and environmental data to show this population is phenotypically and ecologically distinct from its parapatric congener *Gehyra lazelli* and that morphology and ecology are significantly correlated. Contrastingly, mtDNA analysis indicates paraphyly between the two groups, and allele frequencies at six microsatellite loci show no population structure concordant with morpho-/ecotype. These results suggest either ecological speciation or environmentally induced phenotypic polymorphism, in an otherwise morphologically conservative group.

Introduction

Body size is one of the most important ecological and evolutionary attributes of an organism. The size of an organism influences its energetic requirements (Nagy, 2005) and ability to exploit resources (Schluter, 2000), as well as influencing the interactions it will have with other organisms (Schluter, 2010). Resultantly, differences in body size are the predominant way in which related organisms can avoid direct resource competition, thus allowing for assemblages of related organisms to occupy an environment (Dayan & Simberloff, 2005); similarly, size selective predation can be a primary organizing force in a community assembly (Palkovacs & Post, 2009). Body size places important constraints on how an organism interacts with its environment and the magnitude, manner and symmetry of its interactions with other species (Schluter, 2000). While the ecological

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impacts of shifts in body size have implications for macro- and microecological interactions, evolutionary changes in body size can also be an important component of speciation processes.

Differential body size can arise through differential environmental selection, interspecific interactions or intraspecific phenotypic plasticity (Schluter, 1994). Differential body sizes between species are hypothesized to have arisen through two distinct processes (Losos, 1990). The first is character displacement, which is an evolutionary response to divergent selection pressure (Nagel & Schluter, 1998; Pfennig & Pfennig, 2010). The second is through premating selection either through divergent mate selection or reduced hybrid fitness (Rundle & Schluter, 1997; Nagel & Schluter, 1998). As such, divergent body size can lead to the development and subsequent reinforcement of species boundaries following secondary contact of divergent populations that have arisen either in sympatry or in allopatry.

Studies of model organisms such as *Anolis* lizards have shown that rapid morphological change can occur in a very small number of generations when divergent

selection pressure is high (Losos *et al.*, 1997) although the role of phenotypic plasticity in such adaptation is thought to be significant (Losos *et al.*, 2000). In addition, stickleback fishes show both ecologically divergent selection and assortative mating in relation to body size, providing evidence for an adaptive shift in body size being fundamental in recent speciation (Nagel & Schluter, 1998). In species pairs that have undergone recent and rapid divergence, genome-wide divergence would be expected to accumulate at a slower rate, under the 'genomic islands of speciation' model, demonstrated in several recent studies (e.g. *Anopheles* – Turner *et al.*, 2005; *Mus* – Harr, 2006; *Drosophila* – Ting *et al.*, 1998). As such, rapid, recent speciation associated with strong diversifying selection can produce phenotypically distinct species that are not necessarily differentiated when examined using neutrally evolving genetic markers. As a result of the important role that body size can play in the development and maintenance of species boundaries, when a marked difference in body size between populations is observed it is often a robust indicator of the presence of multiple species, particularly when population distributions are adjacent or overlapping (e.g. Sota *et al.*, 2000). While some taxa do display significant intraspecific plasticity in body size within population, this is typically partitioned by sex as a result of selection on mating systems (e.g. male size differentiation in frogs – Smith & Roberts, 2003; lizards – Stuart-Smith *et al.*, 2007; and fishes – Gross, 1984, 1985).

Geckos of the *Gehyra variegata* species complex (King, 1979, 1983; Sistrom *et al.*, 2009) display a number of interspecific body size shifts. Body size (snout–vent length – SVL) of species within the complex ranges from an average of 45 mm in *Gehyra minuta* to 79 mm in *Gehyra xenopus* (see Fig. 1). Pairs of sister species can differ by as much as 17% (*G. purpurascens* and *Gehyra nana*) despite displaying size variation between species, members of the *G. variegata* complex show a narrow range of variation of body size within species, and no member of the genus is known to include obvious multiple size classes (King, 1979). In addition to intraspecific conservatism of body size, members of the *G. variegata* complex historically have proven taxonomically challenging due to conservatism in other morphological characters, particularly body shape and scalation (King, 1979, 1983; Moritz, 1986), despite significant genetic and karyotypic divergence (King, 1979, 1983; Moritz, 1986; Sistrom *et al.*, 2009). As such, body size differences between populations of *Gehyra* are generally a good indicator of species boundaries, especially when populations are sympatric.

As part of a systematic revision of southern Australian *Gehyra*, we discovered a population of exceptionally large and robust *Gehyra* in the far northern Flinders Ranges of southern Australia, where two smaller species *Gehyra lazelli* and *G. variegata* also occur (Fig. 2). As substantial body size differences typically indicate different species in *Gehyra*, we carried out an investigation into the status of
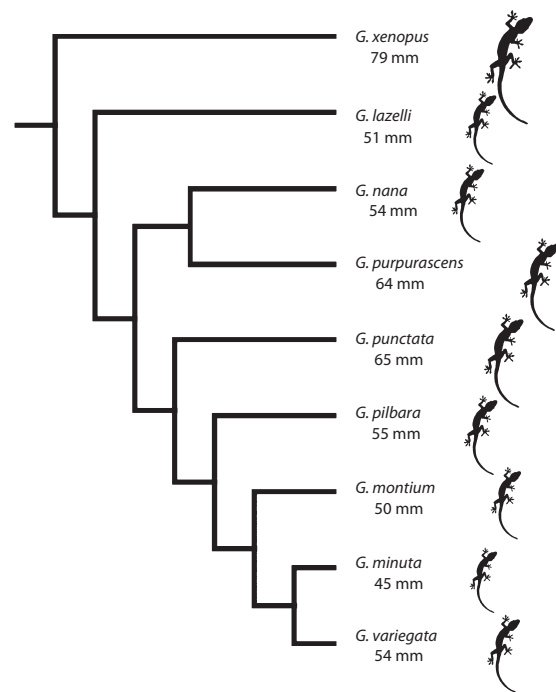
Fig. 1 Phylogenetic tree adapted from the study of Sistrom *et al.* (2009) of the *Gehyra variegata* complex showing body size transitions. Body size measurements represent average snout–vent length of each species (Wilson & Swan, 2010), and silhouettes are to scale.

this large-bodied population (henceforth referred to as LP) using both morphological and genetic data to address the patterns of morphological change. Through extensive field surveys, we sought to determine whether LP and *G. lazelli* occur sympatrically or allopatrically across a broad distributional area centred on known locations where LP occurred. At an early stage, we became aware of substantial discordance between morphological data, which tended to confirm the distinctiveness of LP, and the mitochondrial phylogeny, which indicated no differentiation and in fact seemed to suggest polyphyly of LP compared to *G. lazelli*. In this study, we explore possible scenarios underlying the discordance between genetic and morphological patterns of variation by assessing morphological, genetic and environmental evidence. Specifically, we assess whether morphological divergence is associated with genetic divergence by testing for genetic differentiation between the morphotypes using six microsatellite markers, extended mtDNA screening and karyotype analysis. Further, we determine whether the morphological divergence between the morphotypes is associated with environmental distinction by testing the levels to which morphological variation could be explained by variation in climate, elevation, rock type and vegetation type. We also examine the relative merit

Fig. 2 Representative preserved vouchers of (a) *Gehyra lazelli* [R64427 and R64944], (b) large-bodied population [R58254 and R56408] and (c) *Gehyra variegata* [R59379 and R58593] from the Flinders Ranges, southern Australia, showing the variation in body size and robustness.

of alternative hypotheses for the evolution of this pattern, including allopatric speciation, ecological speciation and phenotypic plasticity.

Materials and methods

Sample selection and field collections

Field surveys of the Terrapinna Springs and surrounding areas were undertaken in the Northern Flinders ranges for a total of 3 weeks over the summer of 2008/2009 and 2009/2010, which resulted in the collection of 22 specimens characteristic of LP morphotype – adults of this form were noticeably larger than surrounding populations of *G. lazelli* and *G. variegata* (Fig. 2), departing from the morphological conservatism typical of the genus (King, 1979; Moritz, 1986; Sistrom *et al.*, 2009), and were only found in granite gorge and rock outcrop habitats. Frozen and alcohol-preserved tissue samples were deposited in the Australian Biological Tissue Collection (ABTC), and whole specimens were deposited at SAMA (see Appendix S1 for specimen details). Populations of *G. lazelli* were at most within 5 km of LP specimens but were never syntopic. We expanded our *G. lazelli* sampling to include specimens collected in the vicinity of the contact with LP, and a representative sampling across the known distribution of *G. lazelli* to make a comparison with the intraspecific diversity of *G. lazelli*.

Karyotyping

Two individuals collected from Terrapinna Springs [R64103 (female) and R64104 (male)], both with the LP morphotype, were karyotyped using standard methods as described in the study of Sistrom *et al.* (2009) to determine the chromosome complement of the LP individuals.

mtDNA molecular protocols

Genomic DNA was extracted using a Puregene™ DNA Isolation Tissue kit D-7000a (Gentra Systems, MN, USA) following the manufacturer's guidelines. The mitochondrial gene *NADH* dehydrogenase *subunit 2* (*ND2*) and partial flanking tRNAs (1136 bp) were used for initial screening to determine the placement of the LP individuals within the broader *Gehyra* phylogeny. Mitochondrial *ND2* fragments were amplified using the primers M112F (5′-AAGCTTTCGGGGCCCATACC-3′) and M1123R (5′-GCTTAATTAAAGTGTYTGAGTTGC-3′). Amplifications were carried out in 25 mL volumes using standard buffer and MgCl$_2$ concentrations, 0.4 mM dNTPs, 0.2 mM each primer, 0.75 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, CA, USA) and approximately 100 ng of genomic DNA. Thermocycler profiles were 9 min at 94 °C, then 45×: 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C with a final extension step of 6 min at 72 °C. The PCR product was purified using a Millipore Montage® PCR384 Cleanup kit (Millipore Corporation USA) following the manufacturer's guidelines. Standard cycle sequencing was carried out according to the standard BigDye Terminator (Applied Biosystems) requirements, and cleaned products were read on an Applied Biosystems 3730xl capillary sequencer.

Phylogenetic analyses

Bayesian and maximum likelihood (M × L) phylogenetic analyses of the *ND2* data were undertaken to ascertain the phylogenetic placement of the LP specimens. The program jModeltest version 0.01 (Posada, 2008) was used to evaluate different models of nucleotide substitution. The *ND2* data were partitioned according to codon position, and corrected Akaike information criterion (AIC) selected the GTR + I + Γ model for all codon positions. M × L analyses were carried out using the RAxML BlackBox

web server (Stamatakis *et al.*, 2008), and branch support was assessed with 1000 bootstrap replicates. Bayesian analyses were undertaken using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). For Bayesian analyses, the data were partitioned for each codon position, as described above, with parameters for each partition unlinked. Four incrementally heated Markov Chain Monte Carlo (MCMC) chains were run for five million generations, sampling every 1000 generations, with the first 10% samples discarded as burn-in. Convergence of posterior probabilities and stationarity of likelihood scores were confirmed through examination of the trace and effective sample sizes of parameters using Tracer version 1.4 (Rambaut & Drummond, 2007).

Microsatellite locus development and genotyping

Given the lack of monophyly for mtDNA, the level of genetic distinctiveness of the LP specimens was examined using microsatellite loci. Microsatellite markers were developed using a next-generation sequencing approach. Total genomic DNA was extracted from a tissue sample from a single *G. lazelli* individual (R52962) using the methods described above. Shotgun sequencing was performed at the Australian Genomic Research Facility in Brisbane, Australia, where samples were prepared according to standard GS-FLX Titanium Library procedure, with the exception that species-specific oligonucleotide adapters (IDT, Iowa, IA, USA) were ligated to the sheared DNA, as multiple species were included in the 454 run. The *G. lazelli* sample occupied 12% of the plate, which resulted in 87 899 individual reads of which 2.18% contained microsatellites.

The program MSATCOMMANDER version 0.81 (Faircloth, 2008) was used to search raw sequences for microsatellites with at least eight repeat units and design appropriate primers. The program MicroFamily (Meglécz, 2007) was used to screen the flanking regions of the reads selected by MSATCOMMANDER for similarities that would prevent successful PCR amplification of the fragments in question. Twenty-four primer pairs were selected for screening across a representative sample of six individuals. Forward and reverse Multiplex-Ready Technology tags were added to the locus-specific primers, and loci were amplified using PCR protocols as specified in the study of Hayden *et al.* (2008). PCR was carried out in 12 µL volumes, containing of 10 ng genomic DNA and 20 nM of forward and reverse locus-specific primers.

A total of eight primer pairs were amplified successfully and were polymorphic in the representative sample, and these loci were used for full screening across 95 individuals (63 *G. lazelli*, 22 LP specimens). *Gehyra lazelli* samples were taken from specimens collected in the area immediately surrounding Terrapinna Springs and extending across the distribution of this species. Electrophoresis of amplified products was carried out using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems) and scored with GENEMAPPER version 3.7 (Applied Biosystems). Two loci proved unscoreable due to a high level of non-amplification. The primers for the remaining six loci used for further analysis are documented (Table 1). The six loci used for analysis were checked for null alleles, large allele dropout and stuttering using MICRO-CHECKER (Oosterhout *et al.*, 2004). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were investigated using Genepop 4.0 (Rousset, 2008).

Genetic clustering methods

An individual-based clustering approach, implemented in STRUCTURE version 2.3.3 (Pritchard *et al.*, 2000), was used to determine how individuals grouped into genetic

Table 1 Summary of microsatellite marker properties and variation.

Locus	Direction/sequence	Length	Repeat unit	N	Ho	He	F_{is}	P	He	F_{is}	P
Geh1	F-ACCTTGAGGGTCCAGTTGTC	178–302	$(GT)_{14}$	70	0.8	0.93	0.14	0.01*	0.91	0.16	0.00*
	R-TCAGGTGGAGATGCCAAGG								0.96	0.09	0.24
Geh2	F-ACCATTAGCTGTTTGTGGATTGC	156–348	$(AC)_{15}$	45	0.89	0.96	0.08	0.57	0.92	0.17	0.00*
	R-CACAGGCTGGTCCCACAG								0.93	−0.09	1
Geh3	F-ATGTATCCTTGGTGTCTCCGC	221–345	$(GT)_{25}$	42	0.76	0.96	0.21	0.00*	0.94	0.21	0.00*
	R-GTGTCTGCCGCTCTTAACC								0.95	0.20	0.00*
Geh4	F-AAAAAGGGGCAGAGCTCAAG	180–338	$(ATCT)_{13}$	76	0.8	0.93	0.14	0.00*	0.91	0.20	0.00*
	R-AATGATCCCCTCCTGCCTC								0.92	0.02	0.11
Geh5	F-AGCTGTTCAAGGAACGAATGC	160–356	$(CTTT)_{14}$	78	0.86	0.94	0.09	0.04*	0.94	0.06	0.00*
	R-TGCAGAGGTGGGTAATGGC								0.94	0.15	0.01*
Geh6	F-ATGACTGGGAGAAAGACAAAGC	195–263	$(ATCT)_{15}$	65	0.78	0.96	0.17	0.00*	0.95	0.17	0.00*
	R-GCAGGATGATCAGTGCAAGC								0.9	0.09	0.08

The first set of summary statistics is for the dataset as whole, and the second represents the dataset split into the large-bodied population specimen cluster (bottom figures) observed for the corrected data and the *Gehyra lazelli* (top figures). N is the number of individuals scored for each locus, H_o is the observed level of heterozygosity, H_e is the expected heterozygosity under Hardy–Weinberg equilibrium, and F_{is} is the F statistic with the corresponding P value resulting from Fisher's exact test implemented in Genepop 4.0 (Rousset, 2008).
*P values significant at 0.05.

clusters. This dataset was run with the inclusion and exclusion of loci for which there was a high degree of missing data. Each STRUCTURE analysis was run for 10 million generations, with the first one million discarded as burn-in at k ranging from 1 to 10 with 20 replicates for each value of *k*. The program HARVESTER (Earl, 2011) was employed to calculate Δk using the approach of Evanno *et al.* (2005). In this way, we determine the number of clusters most likely and generated input files for CLUMPP (Jakobsson & Rosenberg, 2007) so that results from the 20 runs could be combined for visualization using the program DISTRUCT (Rosenberg, 2004).

Morphology and ecology

A total of 83 adult specimens were selected for morphological analysis (see list – Appendix S1), with 19 morphometric and five meristic characters measured. Morphometric data comprised measurements for head length (HL), head width (HW), head depth (HD), internasal width (IN), interorbital width (IO), eye-to-ear distance (EE), ear-to-snout distance (ES), forebody length, axilla–groin length (AGL), humerus length, forelimb length, femur length (FEL), hindlimb length, SVL, tail length (TL), mental scale length (ML), mental scale width (MW), rostral scale height (RH) and rostral scale width (RW). Morphometric measurements were taken to the nearest 0.5 mm using digital callipers. Meristic characters measured included characters traditionally used to assess species boundaries in geckos, including preanal pore counts, and scale counts for supralabial scales (UL), sublabial scales lamellae on fourth rear toe pad and chin shield scales (CS).

All subsequent analyses of morphological and environmental data were conducted using the R statistical package (R Core Development Team, 2011). Each character was tested for sexual dimorphism by regressing values for male and female specimens by SVL (except for SVL which was regressed by HL) using the lm function of the base R package (R Core Development Team, 2011). The slopes of male and female regression lines were compared for significant differences using an F test implemented with the var.test function of the base R package (R Core Development Team, 2011). When slopes were found to not be significantly different, an analysis of covariance (ANCOVA) was carried out on male and female regression lines using the lm function of the base R package (R Core Development Team, 2011) to determine whether sexual dimorphism was present.

Characters that did not show sexual dimorphism were used to conduct a principal component analysis (PCA) using the prcomp function of the base R package (R Core Development Team, 2011). Prior to PCA analyses, data were log-transformed and PCA was undertaken with data both uncorrected and with nonmeristic traits corrected for body size (Lleonart *et al.*, 2000), taking the first principal component (PC) of the uncorrected

analysis as a measure for body size (Marroig & Cheverud, 2009).

Using significant PC axes from both PCA, we undertook both model-based and hierarchical clustering on each of the two datasets (i.e. corrected or uncorrected for body size). This was due to model-based clustering providing an estimate of the most likely number of clusters and hierarchical clustering being able to provide support values via bootstrapping. For Gaussian model-based clustering, we used the mclust function in R package Mclust (Fraley & Raftery, 2006). Mclust implements a Poisson process to handle noisy data, for which an initial noise estimate was obtained using a nearest-neighbour method implemented by the nnclean function in the R package prabclus (Hennig & Hausdorf, 2010). For hierarchical clustering, we used the pvclust function in the R package pvclust (Suzuki & Shimodaira, 2006) using Euclidean distance and the Ward clustering method (Ward, 1963) with 100 000 bootstrap replicates. To determine which morphological characters were most important in the clustering analysis, a discriminant function analysis (DFA) and an ANCOVA were carried out using the clustering of individuals as the independent variable and the morphological measurements as the dependent variables using the R package MASS (Venables & Ripley, 2002). For the ANCOVA, SVL was used as the covariate, except in the case of SVL for which HL was used. In addition, we constructed classification trees to determine the most influential parameters in individual assignment to clusters for both corrected and uncorrected datasets using the cltree function in the R package tree (Ripley, 2010). Homogeneity was measured using the generalized Gini index (Therneau & Atkinson, 2002 – equation 3) to ensure that the precautionary principle applied and that the omission errors are fewer than the commission errors where possible. The recursive partitioning model was run with cross-validation to provide for better accuracy assessments and therefore better final model fit.

To gain some insight into whether the observed morphological differentiation has an ecological basis, an analysis of environmental variables for all of the animals used in the morphological and microsatellite analyses (Appendix S1) was undertaken. ArcGIS version 10 was used to extract values from the 19 climatic variables available through Worldclim (http://www.worldclim.org/), a 90-m digital elevation model available through Diva-GIS (http://srtm.csi.cgiar.org/), categorical surface geology and categorical vegetation type (Geoscience Australia) for each specimen using the Multiple Values to Points tool. Bioclimatic variables and elevation were standardized (by subtracting the mean and dividing by the standard deviation) and reduced to PC scores using the methods described above. The first two principal components were taken as a measure of climatic conditions to avoid autocorrelation between individual climatic variables. As environmental variables included

categorical variables, pvclust cannot be applied due to permutations being conducted by re-estimation of the distance matrix. As an alternative method, the daisy function of the R package cluster (Maechler *et al.*, 2005) was used to produce a dissimilarity matrix of environmental data using Gower's coefficient (Gower, 1971). Hierarchical clustering of the environmental dissimilarity matrix was implemented using the Ward method using the hclust function of the R package cluster (Maechler *et al.*, 2005) – as this is the method implemented by pvclust – meaning the methods are comparable aside the use of bootstrapping. Classification tree construction was carried out using the methods described above.

To evaluate the relationship between morphological and environmental variables, full and partial distance-based redundancy analyses (dbRDA) were undertaken. dbRDA is a multivariate method that allows testing of the influence of environmental factors on values in a linearly dependent dissimilarity matrix (in this case, morphological distance) via permutation testing (Legendre & Anderson, 1999; McArdle & Anderson, 2001). Partial dbRDA allows for the fitting of covariates to take into account the potential confounding effects of these values. In this case, both genetic distance and geographical coordinates have been fitted to account for the influence of genetic structure and isolation by distance on the relationship between environment and morphology in partial dbRDA. Geographical distance matrices were calculated from individual latitude and longitude data using the earth.dist function of the Fossil package (Vavrek, 2010). Values were standardized using logarithmic transformation and converted to a continuous rectangular dataset using principal coordinates analysis via the npcm function of the Vegan package (Oksanen *et al.*, 2011) for further analyses. A genetic distance matrix of Fst scores was calculated from the six microsatellite loci using Genepop 4.0 (Rousset, 2008). Missing values were imputed using the mean Fst value. The influence of each environmental variable (climate PC1, climate PC2, elevation, rock type, vegetation type) on the morphological distance matrix was also tested. All dbRDA were conducted using the 'capscale' function of the R package vegan (Oksanen *et al.*, 2011). Significance of dbRDA was assessed using multivariate *F* statistics with 9999 permutations in the ANOVA function of the base package included in the R statistical Package (R Core Development Team, 2011).

Results

Karyotyping

The diploid number of the two LP specimens karyotyped was $2n = 44$, and chromosome morphology was indistinguishable from that of *G. lazelli* (Sistrom *et al.*, 2009).

As such, LP is not chromosomally differentiated from *G. lazelli.*

Mitochondrial phylogenetic analyses

Results from both Bayesian and ML phylogenetic methods of the *ND2* sequences were congruent. Figure 3 shows the ML phylogram, with asterisks marking nodes with high support from both phylogenetic methods (ML bootstrap values > 70 and Bayesian posterior probability > 0.95). The analysis confirmed that LP specimens are
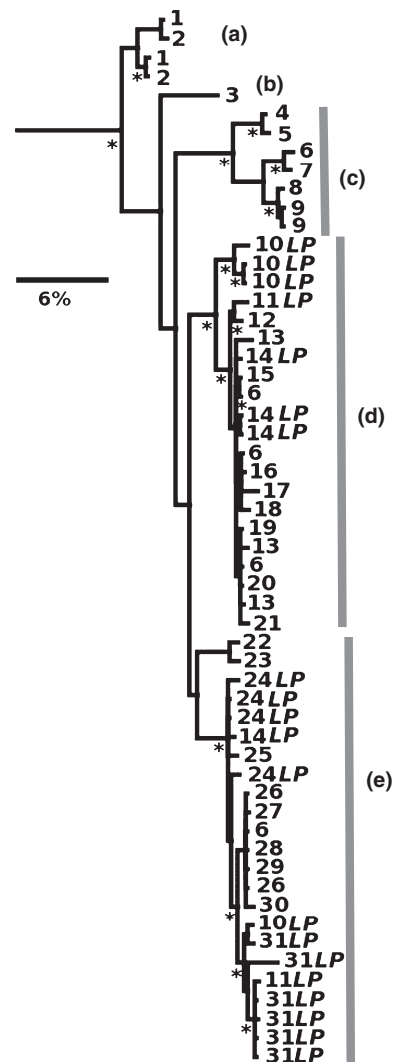

Fig. 3 Phylogenetic ML of preliminary mtDNA screen of large-bodied population (LP) specimens. Stars represent nodes highly supported by both ML bootstrap support (> 70) and Bayesian posterior probability (> 95%). Numbers refer to collection locations (Fig. 2, Appendix S1), and letters designate major clades referred to in the text. Samples labelled 'LP' refer to LP.

polyphyletic within two major *G. lazelli* clades (D and E in Fig. 3). These two clades are distributed broadly throughout the Flinders Ranges and east into western NSW. The southern and western extents of the *G. lazelli* distribution fall into three other distinct clades (A, B and C), which are basal relative to clades D and E. Clades D and E are well supported as distinct; however, the branching order of these clades is poorly resolved by phylogenetic analysis of the mtDNA.

Morphological analyses

A basic overview of morphology is displayed in Fig. 4. LP specimens show a significantly larger and more robust body size in comparison with *G. lazelli* and sympatric *G. variegata*, which are similar in body shape and size in comparison. All three species show considerable intra-specific variation in back pattern; however, fixed differences in colour pattern and meristic measurements
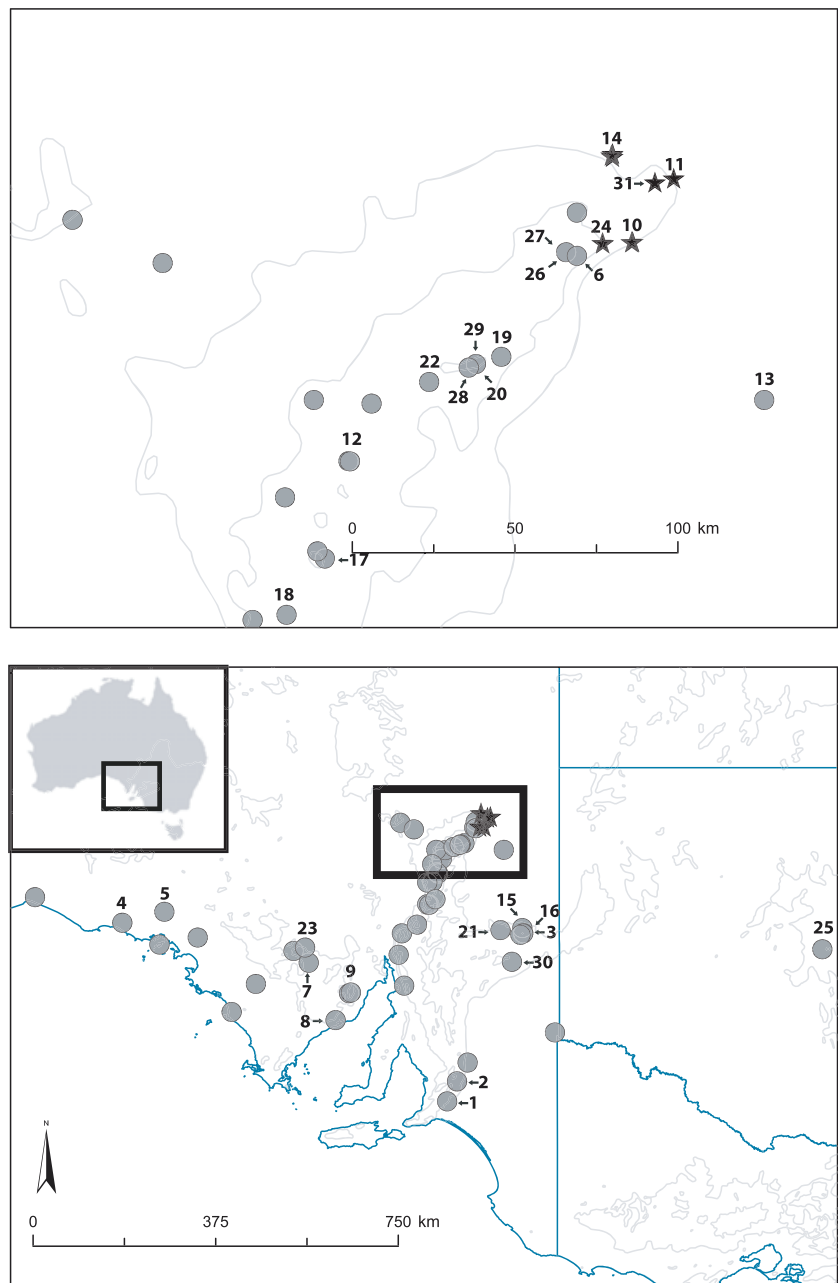

Fig. 4 Collection locations of specimens used for morphological and molecular genetic analyses. Gray circles are collection sites for *Gehyra lazelli* specimens, and black stars are collection sites for large-bodied population specimens. Numbers refer to locality data in Fig. 3, summarized in Appendix S1, and site without numbers represent samples for which only microsatellite and morphological data were collected. Gray contours are at 50-m elevation intervals.

between *G. variegata* and *G. lazelli* are documented in the study of Sistrom *et al.* (2009). The average SVL of predesignated LP specimens based on geographical location and general body size and shape was 62.7 ± 4.83 mm. In comparison, the average SVL of *G. lazelli* samples used in this study was 47.8 ± 5.53 mm (Fig. 2). Less than half of the specimens had intact original tails, and as such, TL was excluded from further analysis. Sexual dimorphism was detected in HW and FEL measurements; as a result, these were also removed from further analyses.

For the PCA on data not corrected for body size, the first and second principal components (PC) accounted for 69% and 10.0% of the variance, respectively, while each of the remaining components explained < 5% of the variance. Hierarchical and model-based clustering both yielded identical individual assignments. As hierarchical clustering provides approximate unbiased bootstrap values as a measure of statistical support for clusters, the results of this analysis is presented (Fig. 5). Model-based clustering yielded two well-supported clusters, with cluster one comprising solely LP individuals and cluster two comprising a mixture of LP (18% of individuals) and *G. lazelli*. Both DFA and ANCOVA indicated a high level of influence due to HL, ES and SVL, which are measurements that would logically be associated with body size (Table 2). Results of the classification tree analysis showed the most accurate number of groups to be two and SVL to be the most important clustering element, with the misclassification error at 0.01 (Fig. 5). These
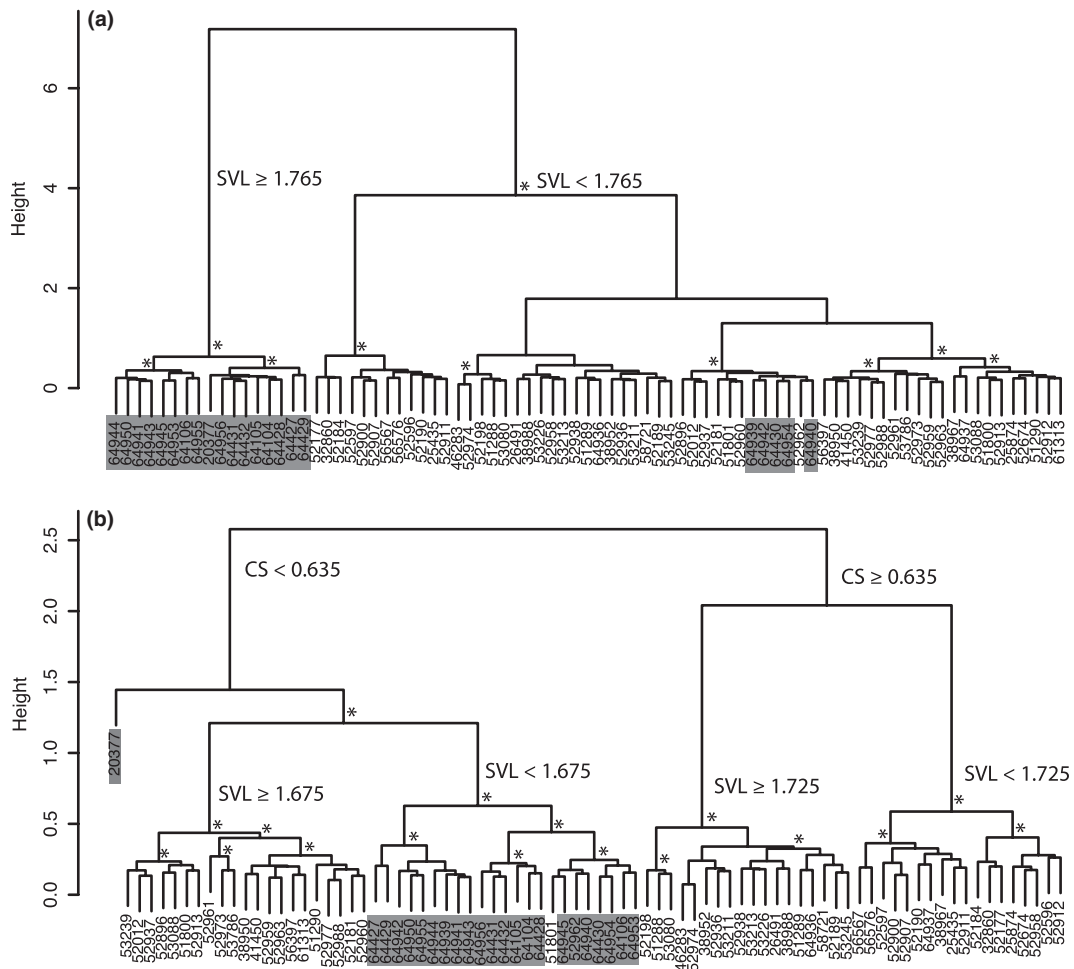
Fig. 5 Dendrograms produced by hierarchical clustering of Euclidean distances from principal component analysis scores of the morphometric data on specimens (a) not corrected for size and (b) corrected for body size. Asterisks indicate nodes with high approximate unbiased bootstrap support (> 70). Gray highlighting designates large-bodied population specimens, with *Gehyra lazelli* individuals unhighlighted. The height scale represents within-dataset Euclidean distance. Notations are the results of classification tree analysis, which looks for the parameter in the dataset, which groups individuals into the designated clusters most accurately, SVL – snout–vent length, CS – chin shield scale count, measurements are based on corrected and scaled values.

Table 2 Summary of morphological analysis.

| | Uncorrected | | | Corrected | | | | |
| | | ANCOVA | | | | | ANCOVA | |
	Root 1	*F*	*P*	Root 1	Root 2	Root 3	*F*	*P*
Wilk's λ	0			0	0	0.004		
Proportion of trace	100			0.6263	0.2458	0.1279		
DF	79			79	79	79		
HL	−2.923	28.176	0.000*	1.333	2.117	−1.991	3.122	0.081
HD	0.356	1.364	0.247	−1.113	−0.412	0.031	0.929	0.338
IO	−0.186	1.585	0.212	0.547	−1.036	0.565	14.546	0.000*
IN	1.237	0.677	0.413	−0.206	−1.235	1.83	17.832	0.000*
EE	0.26	4.628	0.035*	−0.036	0.651	0.287	5.58	0.021*
ED	−0.086	3.237	0.076	−0.357	−0.554	0.111	2.882	0.093
ES	−1.877	23.213	0.000*	0.746	−0.323	−2.072	3.268	0.075
FBL	0.726	3.856	0.053	−0.699	0.045	−0.043	0.397	0.531
AGL	0.817	1.34	0.251	−0.63	−1.74	0.271	2.468	0.12
SVL	2.045	8.204	0.005*	−3.043	0.062	1.956	12.702	0.001*
HU	−0.501	0.563	0.456	0.359	−0.057	−1.218	0.004	0.95
FL	−0.427	13.938	0.000*	0.198	−1.184	0.201	0.778	0.381
HI	0.013	2.308	0.133	−0.804	0.41	−0.47	0.484	0.489
ML	0.341	1.056	0.307	−0.329	−0.224	0.444	6.31	0.014*
MW	0.261	0.02	0.889	0.019	−0.883	0.901	26.057	0.000*
RW	0.137	1.959	0.166	0.794	−0.13	0.529	13.796	0.000*
RH	−0.224	2.976	0.089	1.536	0.253	−0.254	13.837	0.000*
SL	0.083	0.733	0.394	−0.508	1.154	−0.651	0.264	0.609
UL	−0.035	0.052	0.82	0.901	−0.927	−0.485	1.479	0.228
CS	−0.226	7.493	0.008*	0.669	1.824	0.429	2.047	0.157
LL	0.206	0.847	0.36	0.621	0.973	−0.37	0.36	0.55

The first set of data represent the analysis of data uncorrected for body size, and the second set represent results from the analysis of data corrected for size using equation 13 from the study of Lleonart *et al.* (2000), taking the first PC1 of principal component analysis on uncorrected data as a measurement of body size. Numbered roots are standardized coefficients of significant canonical roots resulting from discriminant function analysis. *F* and *P* values are taken from ANCOVA of variables using SVL as the covariant, except in the case of SVL itself for which HL was used as the covariant.
AGL, axilla–groin length; CS, chin shield scales; EE, eye-to-ear distance; ES, ear-to-snout distance; FBL, forebody length; FL, forelimb length; HD, head depth; HL, head length; HU, humerus length; LL, lamellae on fourth rear toe pad; MW, mental scale width; RH, rostral scale height; RW, rostral scale width; SL, sublabial scales; SVL, snout–vent length.
*P values significant at 0.05.

results suggest that two size classes of individuals occur in the dataset corresponding to distinct, but not mutually exclusive groups associated with LP and *G. lazelli*.

When PCA was carried out on morphological data corrected for body size, the first four PCs accounted for 32.2%, 18.1%, 14.4% and 5.5% of the variance, respectively (Fig. 5). Hierarchical and model-based clustering both produced comparable results. Model-based clustering of the size-corrected data yielded four well-supported clusters, one of which contained all of the LP individuals. In the hierarchical clustering, R20377, a sample collected in 1979, was an outlier to all major clusters. In addition, two *G. lazelli* specimens, R52982 and R51801, fell into the LP cluster. Results of the standardized corrected cluster DFA and ANCOVA (Table 2) indicated a high level of influence due to five measurements associated with head shape (mental and rostral scale shape, IO, IN, EE). Results of the classification tree

analysis suggest that the most accurate number of groups is four and that SVL and CS are the most important clustering elements, with a misclassification error of 0.08 (Fig. 5). As all of these metrics are associated with variation in head shape, these analyses indicate head shape significantly differentiates the LP cluster.

Genetic clustering

Microsatellite loci were free of deviation from HWE due to stuttering, null alleles and large allele dropout; however, a heterozygote deficiency was detected in all loci when *G. lazelli* and LP specimens were combined (see Table 1). When samples were separated into two groups based on morphological assignment to group (uncorrected analysis), four of the six loci in the LP group conformed with HWE, but all loci in *G. lazelli* group significantly departed from HWE. This result could be
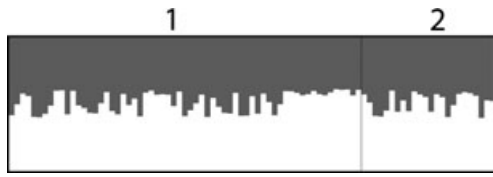
Fig. 6 Structure output when results for *K* = 2 are visualized. Numbers represent specimens of *Gehyra lazelli* characteristic morphology (1) and large-bodied population (2). No structure corresponding with morphology is evident from the analysis.

caused by several genetic populations represented within *G. lazelli* (i.e. a Wahlund effect); however, genetic structure within LP and *G. lazelli* warrants further investigation. The STRUCTURE analysis indicated that a single cluster (Δ*k* = 1) had the highest likelihood. To show the lack of genetic structure corresponding to morphology, STRUCTURE results from the *K* = 2 analysis are visualized in Fig. 6.

The relationship between ecology and morphology

In the PCA of the environmental data, the first two PC scores accounted for 53.4% and 32.0% of the variance, respectively. Clustering of individuals based on ecological data (Fig. 7) yielded four major clusters. LP specimens fell into two of the four clusters, with 17 individuals from nine locations in one cluster (along with four *G. lazelli* individuals from three locations) and five from two locations in a second cluster (along with 14 *G. lazelli* individuals from four locations). Results of DFA and ANCOVA carried out using environmental data (Table 3) show a high level of influence due to climate PC1,

elevation and geology. The classification tree analysis showed the most accurate number of groups to be four with geology, elevation and vegetation type to be the most important clustering elements, and a misclassification error of 0.05 (Fig. 7). In contrast, classification tree analysis using assignment to cluster, based on corrected morphological data as the response variable and environmental dissimilarity as the predictor, found that vegetation type and geology were the most important clustering elements. This contradicted an anecdotal field observation that rock type might be an important factor; however, the misclassification rate was relatively high (0.28). The results of dbRDA showed a significant correlation between morphological distance and climate PC1 – dominated by a mix of precipitation and temperature variables (results not shown), elevation, rock type and vegetation type, which remained significant when genetic distance and geographical distance were used as covariates (Table 4). This result strongly supports a correlation between morphological distance and environmental variables.

Discussion

Gehyra lazelli and the LP are significantly morphologically divergent with both body size and head shape being important distinguishing characteristics. The two morphotypes also utilize different environments, with climate, elevation, vegetation and geology all playing a role in distinguishing their habitats irrespective of geographical or genetic distance. Under the assumption that the morphological variation has a genetic basis, the morphological features together with the evidence that a new distinct habitat has been selected collectively would
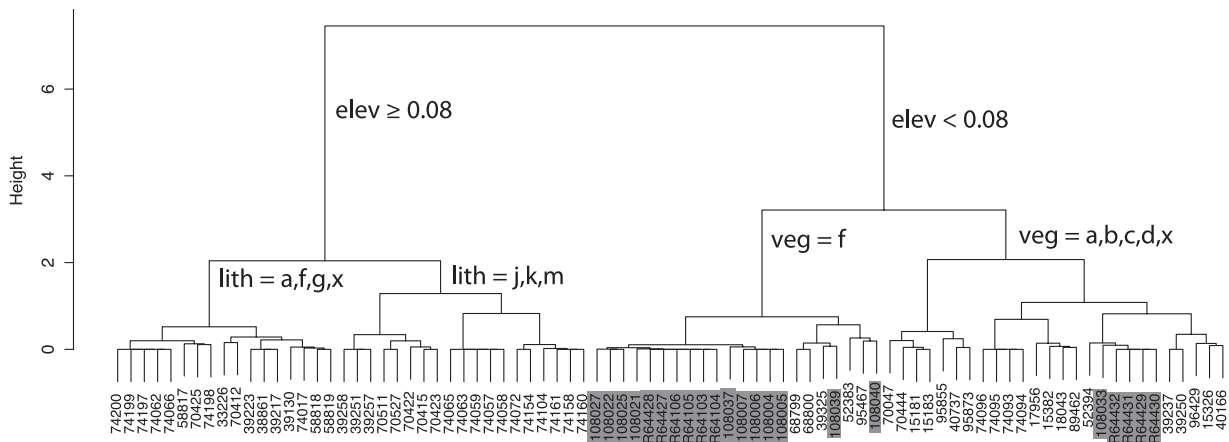
Fig. 7 Dendrogram produced by hierarchical clustering of Euclidean distances from principal component analysis scores of the environmental data. Gray highlighting designates large-bodied population specimens, with unhighlighted samples being *Gehyra lazelli* samples. The height scale represents within-dataset Euclidean distance. Notations are the results of classification tree analysis, which looks for the parameter in the dataset which groups individuals into the designated clusters most accurately, elev – elevation, lith – rock type (a – igneous felsic intrusive, f – feldspar, g – argillaceous detrital sediment, j – sedimentary carbonate, k – sedimentary siliciclastic, m – metamorphic, x - regolith), veg – vegetation type (a - Casurina, b – Eucalyptus, c – Chenopodiaceae, d – Melaleuca, f – Acacia and x – other).

Table 3 Summary of the environmental analysis.

| | Root 1 | Root 2 | Root 3 | ANCOVA | |
				F	P
Proportion of trace	0.74	0.17	0.10		
P	0.00*	0.00*	0.02*		
d.f.	79	79	79		
Climate PC1	−0.89	−1.25	0.20	19.97	0.00*
Climate PC2	−0.27	0.85	0.46	0.92	0.34
Elevation	0.07	1.18	−1.76	8.23	0.01*
Geology	1.67	−0.30	0.42	490.22	0.00*
Vegetation	−0.55	−0.43	0.65	0.89	0.35

The numbered roots are standardized coefficients of significant canonical roots resulting from discriminant function analysis. *F* and *P* values are taken from ANCOVA of variables using climate PC2 as the covariate, except in the case of climate PC2 itself for which climate PC1 was used as the covariate.
P values significant at 0.05.

Table 4 Summary of dbRDA, testing for correlation between environmental measurements and morphological distance.

| | No co-variate | Genetic distance |
	F	P
Climate PC1	18.92	0.00*
Climate PC2	1.35	0.24
Elevation	8.91	0.00*
Rock type	4.71	0.00*
Vegetation type	2.96	0.00*

F and corresponding *P* values are presented for each environmental variable when no covariate is used, when a genetic distance matrix based on F_{st} is used and when a geographical distance matrix based on longitudinal and latitudinal coordinates is used. Nineteen bioclim variables were used but condensed to two principle components to avoid autocorrelation. A significant correlation between morphology and climate PC1, elevation, rock type and vegetation type was found, which was not affected by correction for genetic or geographical distance.
dbRDA, distance-based redundancy analyses.
P values significant at 0.05.

have uncontroversially resulted in the description of LP as a separate species. In stark contrast, the mitochondrial and nuclear markers did not show any evidence of population divergence. This result is complemented by the lack of chromosomal differentiation between the two groups. In particular, the polyphyly of LP and *G. lazelli* mtDNA sequences is striking as LP sequences are distributed broadly within the two major clades (D and E) that are found only in the arid zone. This finding implies that the relationship between the two morphotypes is characterized by either widespread admixture, the retention of ancestral polymorphism over a considerable period of time, or a very recent adaptive shift in

body size associated with the occupation of differential habitats.

Morphological and ecological, but not genetic, disjunction between LP and *G. lazelli*

The presence of the distinct LP morphotype provides *prima facie* evidence for the presence of evolutionarily distinct lineages potentially representing two distinct species, as variation in phenotypes can often represent the first step in adaptive speciation (Herrel *et al.*, 2001). Morphological evidence supports the differentiation of the two groups based on phenotype, as does evidence provided by an analysis of the broad environmental conditions occupied by the morphotypes. A strong, positive correlation between morphotype and climate, elevation, vegetation and rock type is indicative of an adaptive basis to the differentiation and is a good indicator that the two morphotypes represent distinct species, as is the case for many examples of adaptive divergence in lizards (Herrel *et al.*, 2008), fishes (Nagel & Schluter, 1998; Langerhans *et al.*, 2003) and birds (McCormack & Smith, 2008). However, the conflicting evidence provided by mtDNA, microsatellite and chromosomal data indicates that this explanation is not as straightforward as might be expected. Differential morphotypes within a species are common; however, this is not a condition known from *Gehyra*, which is a genus characterized by low morphological variation within and between species, particularly in body shape and size (King, 1983).

Mechanisms resulting in differential body size

Both body size (Hibbitts *et al.*, 2005; Camargo *et al.*, 2010; Higham & Russell, 2010) and head shape (Thorpe & Baez, 1987; Vanhooydonck & Van Damme, 1999, Daza *et al.*, 2009), which are the main phenotypic traits that differentiate LP from *G. lazelli*, have been characterized as adaptive morphological traits in lizards, including geckos. This suggests that the divergence between the LP and *G. lazelli* is adaptive in nature, which is supported by the significant differences in the habitats utilized by each morphotype (Fig. 6). Theory suggests that adaptively divergent populations would be able to exploit differentiated ecological niches and thus exist in sympatry (Schluter, 2000). Such fine-scale partitioning based on body size and locomotive performance has been observed in *Anolis* lizards (Carlsbeek & Smith, 2006), benthic and limnetic partitioning of large and small stickleback fish species (Nagel & Schluter, 1998) and divergence of body size generated due to the availability of cover from predators in cichlid fishes (Takahashi *et al.*, 2009). The parapatric distribution of the two groups indicates that if adaptation is the cause of the morphological divergence, the ecological niches are geographically disjunct. Even though lithology is not identified as a major factor

separating LP from *G. lazelli* in the classification tree analysis, it is notable that LP specimens obligatorily occur on the Terrapinna granite unit, unique to the upper region of the Flinders Ranges (Neumann, 2001), and no *G. lazelli* samples have been found on this granite unit. Field observation suggests that this rock unit is characterized by very large, continuous rock faces with sparse, but deep fissures that act as refuges for the geckos. This contrasts with the surrounding rocks, which are far more fissile and provide a habitat with far more refugia and fewer open faces where extensive searching failed to yield LP specimens. This distinct geology has implications for many ecological parameters such as thermoregulatory parameters, surrounding soil type, prey availability and predation pressure and may have resulted in divergent selection for body size. Also, examination of rates of tail loss in LP specimens (70% of observed specimens) and *G. lazelli* (40.4% of observed specimens) provides a preliminary indication that predation or aggressive within-species interactions may be higher in LP specimens; however, a more thorough investigation beyond the scope of the current study would be required to make more than a speculative suggestion regarding predation rates.

Evolutionary explanations for the maintenance of body size differentiation

The lack of correspondence between morphotype and genetic structure suggests a scenario in which divergent phenotypes representing allopatric divergence and secondary introgression is unlikely. Under an allopatric scenario, divergence in microsatellite loci would be expected, and given the prevalence of differentiating chromosomal states in closely related *Gehyra* species (King, 1979, 1983; Moritz, 1986, 1987), a difference in karyotype might additionally be expected. The fact that morphotypes are distributed in adjacent but differentiated environmental conditions provides strong evidence that the nature of morphological divergence is adaptive. Further, considering the lack of support for an allopatric model of divergence, divergence has likely been ecologically driven in sympatry. While the loci used in this study have the ability to detect recent divergence in most scenarios, in some cases of very recent divergence, they have not (e.g. Elmer *et al.*, 2010) and would not be expected to under a 'genomic islands of speciation' model of divergence where differentiation only occurs in genes undergoing selection (Turner *et al.*, 2005). Lack of differentiation in the genetic data means it is not possible to distinguish between incipient speciation with recent adaptive divergence and phenotypic plasticity within a single species. Some species do show sympatric, intraspecific dimorphism of body size in relation to predation (Takahashi *et al.*, 2009) and sexual strategy (Smith & Roberts, 2003; Stuart-Smith *et al.*, 2007); however, in most cases of size dimorphism related to

sexual strategy, there is a sexual bias to size classes, which is not present in this case.

While it is unclear from our data whether or not introgression has occurred between the two groups due to the fact no population differentiation was discerned, the potential for hybridization between them exists. The maintenance of differential morphotypes through reduced hybrid fitness (Rice & Pfennig, 2010) could act to reinforce an already established morphological divergence. Conversely, introgression has the potential to be facilitating the reproductive absorption of the LP morphotype, and thus, it may disappear through the process of hybridization (Rhymer & Simberloff, 1996). As the role of interbreeding between the two morphotypes could be having significant opposite effects on the process of continued differentiation, this is an interesting and significant facet of this system to be further explored.

Acknowledgments

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Appendix S1 Information on samples and specimens used.

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