

A test of alternative models of diversification in tropical rainforests: Ecological gradients vs. rainforest refugia

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Comparison of mitochondrial and morphological divergence in eight populations of a widespread leaf-litter skink is used to determine the relative importance of geographic isolation and natural selection in generating phenotypic diversity in the Wet Tropics Rainforest region of Australia. The populations occur in two geographically isolated regions, and within each region, in two different habitats (closed rainforest and tall open forest) that span a well characterized ecological gradient. Morphological differences among ancient geographic isolates (separated for several million years, judging by their mitochondrial DNA sequence divergence) were slight, but morphological and life history differences among habitats were large and occurred despite moderate to high levels of mitochondrial gene flow. A field experiment identified avian predation as one potential agent of natural selection. These results indicate that natural selection operating across ecological gradients can be more important than geographic isolation in similar habitats in generating phenotypic diversity. In addition, our results indicate that selection is sufficiently strong to overcome the homogenizing effects of gene flow, a necessary first step toward speciation in continuously distributed populations. Because ecological gradients may be a source of evolutionary novelty, and perhaps new species, their conservation warrants greater attention. This is particularly true in tropical regions, where most reserves do not include ecological gradients and transitional habitats.

Debate concerning modes of vertebrate diversification in tropical rainforests has largely focused on the role of geographic isolation, by riverine barriers (1) or in Pleistocene rainforest refugia (2, 3), vs. the action of diversifying selection across environmental gradients (4). The role of geographic isolation in speciation has become axiomatic in biology, but recent studies suggest that natural selection across habitat gradients, without significant geographic or genetic isolation, may be an important process in vertebrate diversification in tropical rainforests (5). Here we report on a simultaneous test of these competing hypotheses in the Wet Tropics Rainforest of Australia; we are able, through direct experimentation, to investigate possible mechanisms of selective divergence.

The Australian Wet Tropics offers a unique opportunity to examine the relative roles of geographic isolation and habitat gradients in diversification of rainforest vertebrates, because it is divided into two regions between which the fauna has been separated for several million years (6, 7). These conditions offer a natural experiment with which to examine the effects of historical isolation in ancient rainforest refugia on phenotypic divergence (8). Furthermore, in each of these regions, the rainforest is bordered to the west by a narrow ecotonal transition to the tall, open eucalypt forest that results from a steep gradient in rainfall and fire frequency (9). This transition occurs over short distances (hundreds of meters to a few kilometers) and provides a sharp ecological gradient across which to examine the role of selection in generating phenotypic divergence.

To determine the relative roles of long-term isolation in rainforest refugia and the action of natural selection among habitats in generating population divergence, we analyzed phenotypic and genetic variation in *Carlia rubrigularis*, a common ground-dwelling lizard that is continuously distributed from rainforest to tall open forest. Because vertebrate species in the Wet Tropics are ancient (6, 7, 10, 11), the processes that resulted in species formation are not amenable to empirical study. Instead, we focus on the processes that produce phenotypic divergence among populations of a single species. We reason that, if geographic isolation or selection across habitat gradients are general processes that lead to species formation, they should also operate to produce diversification among populations. Specifically we address the following questions: (i) Is phenotypic divergence greater among historically isolated rainforest regions (refugia), or is it greater between habitats (i.e., between rainforest and adjacent open forest); and (ii) if phenotypic divergence is found, can its likely cause (e.g., drift, selection, etc.) be identified?

Methods

We sampled adult *C. rubrigularis* from eight paired sites (rainforest, open forest) in the Wet Tropics (Fig. 1). For each individual, we measured snout-vent length (SVL), hind limb length, forelimb length, gape width, and head length. All measurements were performed by one of us (C.J.S.) on live animals in the field. SVL was measured to the nearest millimeter with a clear plastic ruler, and all other measurements were made with dial calipers to the nearest 0.1 mm. In addition, a tail tip from each individual was collected for genetic analysis. We restricted our analyses of morphology to sexually mature individuals. Male sexual maturity was judged by the presence of red on the ventral surface of the throat; females were judged to be sexually mature if they were gravid or were larger (greater SVL) than the smallest gravid female found at a site. Because significant sexual dimorphism exists, sexes were analyzed separately. Morphological data were log-transformed before statistical analysis.

From 5 to 10 individuals from each of the eight paired sites, and from a number of other sites spanning the Wet Tropics (Fig. 1), we sequenced a 400-bp portion of the mitochondrial cytochrome-*b* gene, using primers MVZ05 (12) and cytochrome-*b*2 (13) and following standard PCR and automated sequencing

Abbreviations: BMC, Black Mountain Corridor; SVL, snout-vent length; PC, principal component.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession numbers AF181037–AF181068).

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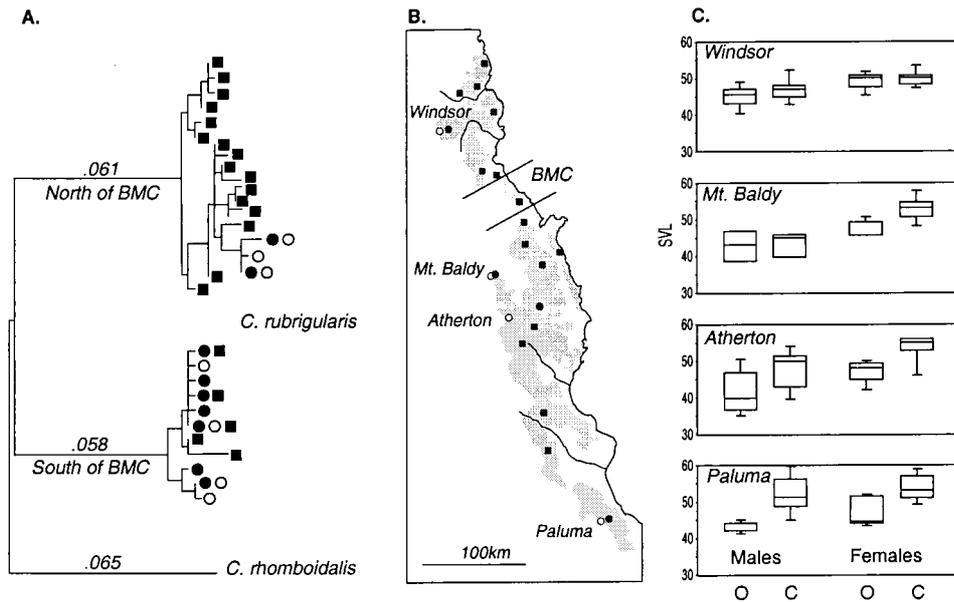


Fig. 1. (A) Neighbor-joining tree generated from Kimura two-parameter distances of cytochrome-*b* haplotypes from throughout the range of *C. rubrigularis* rooted with haplotypes from *Carlia rhomboidalis*. Branch lengths are shown above major internodes. Circles denote paired rainforest and open forest sites where both mtDNA and phenotypic variation was measured (closed circles = rainforest; open circles = open forest). Closed squares represent sites from which additional mtDNA sequences were obtained. (B) Map of the Wet Tropics showing sampling localities. Symbols are defined as above. (C) Box plots illustrating differences in snout-vent length (SVL) in *C. rubrigularis* between rainforest (c) and open forest (o) sites. BMC refers to the Black Mountain Corridor.

protocols (14). Sequences were obtained from 97 individuals and 23 localities.

After we found large phenotypic differences among populations occupying different habitats (see below), we tested the hypothesis that avian predation might be a strong selective agent that acts differently across habitats by measuring the frequency of avian attack on Plasticine lizard models in both rainforest and open forest. Four hundred eighty lizard models, in five size classes spanning the range of size variation in *C. rubrigularis*, were constructed and painted to resemble natural *C. rubrigularis*. Eighty models, drawn at random with regard to size class, were placed at 5- to 10-meter intervals along linear transects at three rainforest and three open forest sites in the southern Wet Tropics. These sites included two rainforest and two open forest sites that were sampled for phenotypic and genetic analyses before the model experiment. Models were checked for bill marks or other damage every third day. Damaged models were removed and counted as present in the experiment only up to the day when we last observed them undamaged, resulting in a conservative estimate of the number of model-days (days in which models were exposed to potential bird attack) in each habitat.

Results and Discussion

As in several other vertebrate species (6, 7) there is deep mtDNA divergence (*ca.* 12%) among populations of *C. rubrigularis* on either side of a well known biogeographic barrier, the Black Mountain Corridor (BMC), or more specifically, on either side of the Lamb Range at the southern end of the BMC (Fig. 1). Assuming a rough reptilian cytochrome-*b* substitution rate of *ca.* 1% per lineage per million years (15, 16), the level of sequence divergence indicates several million years of isolation for populations on either side of the BMC. In contrast, there is little mtDNA divergence between samples from paired rainforest and open forest sites (Table 1), and there are many shared haplotypes among populations in different habitats (Fig. 1A), indicating

high levels of recent or ongoing mtDNA gene flow between geographically proximate sites (Table 1).

Morphology is remarkably consistent among populations within habitats on either side of the BMC but differs dramatically between habitats (Table 2). Neither sex differs significantly in any single character across the BMC, but when characters are combined in a multivariate analysis of variance (MANOVA) slight differences in SVL, limb length, and head dimensions result in a significant difference in overall morphology (Wilks' $\Lambda < 0.05$; Table 2). In contrast to the relative morphological stasis exhibited by geographical population isolates, large and

Table 1. Distribution of mtDNA diversity and estimates of mitochondrial gene flow between habitats and among sites within habitats

	Φ_{st}^*	Nm [†]
Between habitats		
Paluma (P)	0.189	2.15
Atherton (A)	0.026	18.98
Mt. Baldy (MB)	0.056	8.40
Windsor (W)	0.194	2.08
Among rainforest sites		
P-A	0.857	0.08
P-MB	0.770	0.14
MB-A	0.124	3.54
Across BMC	≥ 0.974	≤ 0.01
Among open forest sites		
P-A	0.930	0.04
P-MB	0.964	0.02
MB-A	0.000	∞
Across BMC	≥ 0.968	≤ 0.02

* Φ_{st} estimates were calculated using Kimura two-parameter distances between haplotypes.

[†]Nm (the number of female migrants per generation) was estimated from Φ_{st} where $Nm = 1/2[(1/\Phi_{st}) - 1]$.

Table 2. Effects of habitat and historical isolation on ecomorphological differentiation

Variable	Females*		Males†	
	Habitat	Region	Habitat	Region
SVL	<0.001	0.421	<0.001	0.646
Hind limb length	<0.001	0.932	<0.001	0.260
Forelimb length	<0.001	0.498	<0.001	0.098
Gape width	0.002	0.556	0.002	0.837
Head length	<0.001	0.110	0.001	0.610
Wilks' Λ	<0.001	0.004	<0.001	0.032

Values are the probability of no difference in five ecomorphological variables resulting from a MANOVA using log-transformed variables with habitat and region (north or south of the BMC) as factors.

*North of the BMC, $n = 14$ (7 from open forest and 7 from closed forest) and $n = 37$ from south of the BMC (18 from open forest and 19 from closed forest).

†North of the BMC, $n = 15$ (6 from open forest and 9 from closed forest) and $n = 34$ from south of the BMC (16 from open forest and 18 from closed forest).

highly significant differences in all morphological characters occur consistently across habitats.

The most dramatic difference (in both sexes) across habitats is in size, with differences in mean SVL of up to 17% between rainforest and open forest populations. Interestingly, the minimum size at reproductive maturity is smaller in open forest than in rainforest. The smallest reproductive males in open forest ($n = 22$) were 35 mm SVL vs. 38 mm in closed forest ($n = 27$), and the smallest gravid females in open forest ($n = 25$) were 42 mm SVL vs. 46 mm in closed forest ($n = 26$). Open forest animals apparently become sexually mature at a smaller size than do closed forest animals.

Principal component (PC) analysis reveals that differences across habitats involve shape as well as size. The first PC explains 88.2% and 92.8% of the variation for females and males, respectively, and is largely a size axis, with all characters exhibiting high positive loadings (Table 3). The significant difference in size between habitats is evident in the distribution of factor scores along PC I, where open forest animals are significantly

smaller than closed forest animals (ANOVA; $F_{1,47} = 12.62$, $P < 0.001$ for females; and $F_{1,45} = 9.67$, $P = 0.003$ for males), whereas size does not differ significantly within habitats on either side of the BMC ($F_{1,47} = 0.27$, $P = 0.606$ for females; and $F_{1,45} = 0.91$, $P = 0.346$ for males). The second PC explains 5.4% and 3.6% of the variation in females and males, respectively, and is largely a shape axis, with negative loadings for limb length and positive loadings for gape width and head length. To investigate further the differences in shape among habitats while minimizing the influence of size, we regressed log-transformed variables against log SVL and used the residual values for subsequent PC analyses (17). The first size-adjusted PC explains 40.2% and 46.9% of the variance in females and males, respectively, and has high positive loadings for residual log hind-limb length and residual log forelimb length (Table 3). The distribution of factor scores along this axis differs between habitats, indicating that open forest individuals have significantly smaller limbs than closed forest individuals ($F_{1,47} = 15.11$, $P < 0.001$ for females; and $F_{1,45} = 8.55$, $P = 0.005$ for males). The second size-adjusted PC explains an additional 33.8% and 37.4% of the variation for females and males, respectively, and has high positive loadings for residual log gape width and residual log jaw length (Table 3). Inspection of factor scores revealed a trend for open forest individuals to have larger heads than closed forest individuals, but the distribution of factor scores along this axis does not differ significantly between habitats ($F_{1,47} = 2.45$, $P = 0.10$ for females; and $F_{1,45} = 0.77$, $P = 0.39$ for males). In summary, individuals from different habitats differ in both size and shape, with open forest individuals of both sexes being smaller, having relatively shorter limbs, and tending to have larger heads for their size.

The large differences in morphology and life history between habitats, relative to that between historical isolates, suggest that selection is more important than geographic isolation in promoting phenotypic divergence in *C. rubrigularis*. A previous study also found a lack of ecomorphological differentiation among similarly isolated populations of other lizard species in the Wet Tropics (8). Although morphological and life history differences between habitats may reflect phenotypic plasticity in response to environmental differences, which cannot be ruled out, two lines of evidence suggest that phenotypic plasticity may not explain all of the observed variation. First, between-habitat shifts in shape as well as in size suggest that natural selection acts

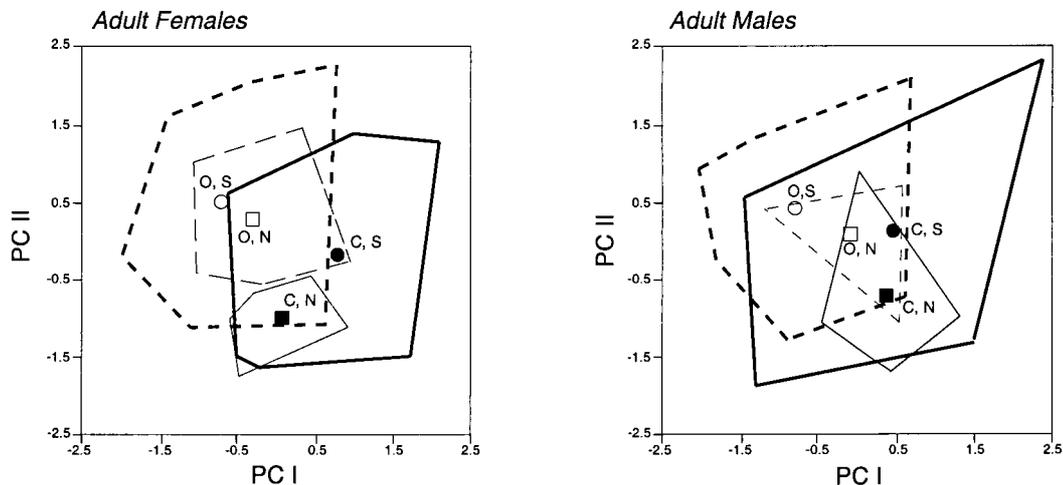


Fig. 2. Plot of the first two PC axes resulting from analysis of log-transformed morphological variables in *C. rubrigularis*. Sexes are plotted separately. Points from a single region and habitat are enclosed by minimum convex polygons (heavy lines, area south of the BMC; light lines, area north of the BMC; dashed lines, open forest; solid lines, closed forest). Group centroids are shown (C, closed forest; O, open forest; N, north of the Black Mountain Corridor (BMC); S, south of the BMC).

Table 3. Factor scores from PC analysis of log-transformed morphological variables; and the same variables adjusted for size

Variable	Log-transformed*				Size-adjusted†			
	Females		Males		Females		Males	
	PC I	PC II	PC I	PC II	PC I	PC II	PC I	PC II
SVL	0.964	0.042	0.966	0.053	—	—	—	—
Hind limb length	0.948	−0.246	0.972	−0.194	0.886	0.098	0.929	0.219
Forelimb length	0.944	−0.197	0.964	−0.229	0.896	0.099	0.941	0.141
Gape width	0.898	0.394	0.946	0.275	0.126	0.805	0.048	0.910
Jaw length	0.940	0.113	0.968	0.101	0.056	0.828	0.356	0.775
Eigenvalue	4.41	0.27	4.64	0.18	1.61	1.35	1.88	1.50
% of variance	88.2	5.4	92.8	3.6	40.2	33.8	46.9	37.4

*Unrotated axes.

†Varimax rotation.

on particular morphological characters, particularly relative limb length, and, to a lesser degree, head size. Both relative limb length and head size have direct ecological performance consequence for lizards (18, 19), and are therefore likely to be subject to natural selection. Second, if the risk of mortality in open forest is higher than that in rainforest, the shift toward smaller size at first reproduction in open forest is in the direction predicted by life history theory (20, 21).

We suspect that predation risk is greater in the open forest because of a larger number of lizard-eating avian predators. To test this hypothesis, we deployed Plasticine lizard models in both habitats to determine whether avian attacks were more frequent in the open forest than in the rainforest. In a total of 3,105 model-days in the open forest and 3,181 model-days in the closed forest, we found unambiguous bird-bill marks on 25 models, with significantly more models attacked in the open forest sites than in the closed forest sites (21 vs. 4; Fisher's exact test, $P < 0.001$). This result is consistent with our prediction that bird predation is greater in the open forest, and it supports the notion that higher risk of mortality in open forest may be driving the difference in size at sexual maturity between rainforest and open forest populations. Our results are similar to those of Reznick *et al.* (20), who found significant shifts in life history toward earlier reproduction and smaller size in a Trinidad guppy in high-predation streams.

Our primary objective in this study was to determine whether historical isolation was more or less important than selection across a narrow ecological gradient in producing phenotypic divergence among lizard populations in a tropical rainforest. We have demonstrated that phenotypic differences across narrow habitat gradients are much greater than those between historically isolated populations in similar habitats. In addition, we have identified a possible selective agent that may be driving the differences in body size among habitats. The changes in morphology and life history across habitats, despite current or recent mtDNA gene flow, suggest rapid adaptive evolution in response to natural selection. In stark contrast, differences among ancient isolates are comparatively minor, suggesting that natural selection across habitat gradients is more important than geographic isolation alone in producing phenotypic divergence among populations of *Carlia*.

The link between population divergence and species formation in *Carlia* is not yet clear. However, it has been shown that reproductive isolation can evolve quickly in response to strong, multifarious, divergent selection, even with moderate levels of gene flow (22). The fact that significant phenotypic differences among *Carlia* populations occur despite recent or ongoing

mitochondrial gene flow suggests the action of strong disruptive selection. If characters that vary among habitats (e.g., body size) lead to positive assortative mating, as has been demonstrated in other systems (23), then reproductive isolation could evolve quickly (22). Additional analyses of nuclear gene flow and reproductive compatibility among ecotypes and among historically isolated populations of *Carlia* are needed to determine whether selection across habitat gradients or long-term isolation has resulted in reproductive divergence.

The results presented above add to the mounting body of evidence indicating that isolation through vicariance in rainforests may be insufficient to produce phenotypic divergence among populations (5, 8), and therefore may be less important in species formation than previously thought. These results further indicate that microevolutionary processes leading to population divergence, and perhaps ultimately, species formation in rainforest vertebrates may be primarily the result of selection rather than the result of geographic isolation per se. Geographic isolation will facilitate genetic and/or reproductive divergence (24), but diversifying selection, whether across environmental gradients (4, 5, 25) or among historical isolates with differing selective regimes, appears to be a key factor in phenotypic diversification and speciation (22, 26). Although this view is not new (22, 27), the importance of ecology and selection in species formation has been eclipsed by debates centering instead on the geographic context in which reproductive isolation evolves and on the role of founder events and genetic drift (24, 27). We believe that a renewed emphasis on the importance of selection in species formation is warranted. This view has important implications for conserving evolutionary processes that generate biodiversity in tropical rainforests (and elsewhere), placing added emphasis on the conservation of transitional and topographically complex areas (28), which provide the differing selective regimes that may ultimately generate biodiversity.

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