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Persistence in Peripheral Refugia Promotes Phenotypic Divergence and Speciation in a Rainforest Frog

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Abstract: It is well established from the fossil record and phylogeographic analyses that late Quaternary climate fluctuations led to substantial changes in species’ distribution, but whether and how these fluctuations resulted in phenotypic divergence and speciation is less clear. This question can be addressed through detailed analysis of traits relevant to ecology and mating within and among intraspecific lineages that persisted in separate refugia. In a biogeographic system (the Australian Wet Tropics [AWT]) with a well-established history of refugial isolation during Pleistocene glacial periods, we tested whether climate-mediated changes in distribution drove genetic and phenotypic divergence in the rainforest frog Cophixalus ornatus. We combined paleomodeling and multilocus genetics to demonstrate long-term persistence within, and isolation among, one central and two peripheral refugia. In contrast to other AWT vertebrates, the three major lineages differ in ecologically relevant morphology and in mating call, reflecting divergent selection and/or genetic drift in the peripheral isolates. Divergence in mating call and contact zone analyses suggest that the lineages now represent distinct species. The results show that climate shifts can promote genetic and phenotypic divergence and, potentially, speciation and direct attention toward incorporating adaptive traits into phylogeographic studies to better resolve the mechanisms of speciation.

Keywords: morphology, mating traits, Pleistocene refugia, contact zone, Wet Tropics, Cophixalus.

Introduction

The current geographic ranges of species are largely determined by climate as a consequence of physiological tolerances and habitat requirements (Grinnell 1917; Williams et al. 2007), though physical barriers and biotic interactions can also constrain distributions at local or regional scales (Soberon and Nakamura 2009). Climate change is expected to alter distributions as suitable ecological conditions for a species shift across the landscape. That this is the case is evident from fluctuation in species ranges in association with late Quaternary or more recent climate change (Jackson and Overpeck 2000; Parmesan 2006; Tingley et al. 2009), which often results in novel assemblages due to idiosyncratic responses of species (Graham et al. 1996; Williams et al. 2007; Moritz et al. 2008). Although climatic fluctuations have occurred throughout Earth’s history, particular attention has been paid to the role in shaping current diversity of the well-characterized global climatic fluctuations associated with the Pleistocene glacial cycles (e.g., Haffer 1969; Moritz et al. 2000; Hewitt 2004; Zink et al. 2004; Turgeon et al. 2005).

The net effects of past climate change on a species—range expansion, contraction, fragmentation, or consolidation—depend on how the geography of ecological conditions change across the landscape. The ensuing demographic effects should be evident in spatial patterns of current genetic diversity. Expectations include high genetic diversity in presumed refugial areas or areas of current admixture among historically isolated lineages and reduced diversity and signatures of population expansion in recently recolonized regions (Petit et al. 2003; Hewitt 2004). Accordingly, many phylogeographic studies reveal clear signatures of these past events (Hewitt 2004). More recently, such studies have been extended to include multiple loci and/or codistributed taxa and to employ increasingly sophisticated analytical methods that use coalescent simulations to test alternative hypotheses or infer parameters relating to historical demography (Turgeon et al. 2005; Knowles 2009; Hickerson et al. 2010). The field has also
advanced by using spatial modeling of potential ranges under past climates to predict geographic regions of potential persistence (“refugia”) versus instability (i.e., alternating extinction and recolonization) that can then be tested via statistical phylogeographic analysis (Hugall et al. 2002; Richards et al. 2007; Carnaval et al. 2009; Walker et al. 2009).

Less clear is how these repeated, climate-driven fluctuations in geographic range and ecological conditions translated into phenotypic divergence, and perhaps speciation, among isolates. In principle, geographic responses to climatic oscillations could alter connectivity among populations, the potential for genetic drift, and selection regimes across populations. Retraction of populations during climatically restrictive periods (e.g., at glacial maxima) could result in the following: (1) extirpation of all but one persistent lineage, which subsequently expands its range; (2) persistence of lineages in ecologically similar but isolated refugia (Haffer 1969); or (3) persistence in ecologically distinct, disjunct refugia. If the first scenario applies, we would expect genetic and phenotypic similarity over the entire range. The second scenario would also be expected to result in little phenotypic divergence. Across environmentally similar refugia, the combined effect of drift on neutral genes (Lynch 1990; Coyne and Orr 2004) and stabilizing or uniform directional selection on phenotypes (Charlesworth et al. 1982; Wiens and Graham 2005; Nosil and Flaxman 2011) is expected to produce genetically divergent but phenotypically similar lineages, that is, “cryptic” lineages (Bickford et al. 2006; Kozak et al. 2006; Bond and Stockman 2008). In contrast, in the third scenario we would expect concordant phylogeographic and phenotypic divergence among historical isolates. Differences in biotic or abiotic environment across refugia would potentially result in a heterogeneous selection regime among allopatric populations, resulting in local adaptation and potentially rapid speciation (McPeek 1996; Schluter 2001; Gavrilets 2004; McPeek and Gavrilets 2006; Hoskin and Higgs 2010). Phenotypic divergence would be expected in ecologically relevant morphology (Endler 1986; Schluter 2001; Losos 2009) and mating traits (Gerhardt and Huber 2002; Hoskin and Higgs 2010).

Rigorous phenotypic analysis integrated with thorough analyses of population history offers an approach to testing the mechanistic role of past climate shifts in diversification. Here we combine paleomodeling, genetics, and phenotypic data (ecologically relevant morphology and mating call) to assess how past climate change has shaped present genetic and phenotypic diversity across the range of a rainforest frog species. We use niche modeling to predict areas of persistence in the past, particularly at the last glacial maximum (LGM). If the range of the species was fragmented to isolated refugia in the past, and if selection pressures differed among those refugia, then we predict the following: (1) spatial correspondence between major lineages and areas of predicted persistence; (2) higher genetic diversity in regions of predicted paleorefugia, particularly where these are predicted to have been larger, and intervening areas containing a subset of the diversity contained in refugia; (3) genetic signatures of range expansion, particularly for lineages that are predicted to have contracted historically to small refugia; and (4) divergence in ecologically important phenotypic traits due to differing selection pressures among refugia. Finally, if genetic and phenotypic (particularly mating trait) divergence is substantial, reproductive isolation should be evident where the lineages are currently in contact.

The Study System

The Australian Wet Tropics (hereafter, AWT) underwent repeated fluctuations in area and connectivity during the Pleistocene, with the most severe contractions occurring at the LGM (~18 thousand years ago [kya]; Hopkins et al. 1993; Kershaw et al. 2005). During glacial periods, cool-dry conditions forced the contraction of rainforests to small, mostly montane refugia, primarily in the central and northern AWT (Webb and Tracey 1981; Nix 1991; VanDerWal et al. 2009). Under current climatic conditions, rainforest distribution is fairly continuous throughout the AWT. Genetic studies of currently widespread rainforest species have consistently revealed deep phylogeographic structure, reflecting historic isolation of populations to refugia for extended periods (Joseph et al. 1995; Schneider et al. 1998; Moussalli et al. 2009; Bell et al. 2010). However, the spatial and temporal scales of species’ responses vary; some show a relatively simple pattern of north/south lineages (Joseph et al. 1995; Schneider et al. 1998; Hoskin et al. 2005), whereas others (particularly invertebrates) have multiple, localized lineages, suggesting persistence in multiple small refugia (e.g., Hugall et al. 2002; Bell et al. 2007). Despite extended periods of isolation, little phenotypic divergence has been detected between phylogeographic lineages of AWT vertebrates (Schneider and Moritz 1999; Hoskin 2007; Moritz et al. 2009), but few have been assessed in detail (for exceptions, see Hoskin et al. 2005; Bell et al. 2010).

*Cophixalus* are small, terrestrial-breeding microhylid frogs, generally restricted to rainforest, that have low dispersal capabilities (Zweifel 1985; Hoskin 2004; Williams et al. 2010). *Cophixalus* represents the only diverse vertebrate radiation within the AWT, and the genus shows patterns of diversity and endemism that are more akin to those of the low-dispersal invertebrate groups of this region. Of the 14 Australian species, 11 form a monophyletic group that is endemic to the AWT, with most species being localized to one or a few neighboring mountaintops (Hos-
Speciation by Refugial Isolation

Figure 1: Distribution of Cophixalus ornatus in the Australian Wet Tropics as defined by 941 distribution records (black dots) and as predicted by the Maxent distribution model (yellow-to-red scale). Localities referred to in the text are marked as follows: AU, Atherton Tableland uplands; BF, Mount Bartle Frere; BK, Mount Bellenden Ker; BMC, Black Mountain Corridor; CC, Cairns-Cardwell lowlands; CU, Carbine Tableland uplands; H, Hinchinbrook Island; KU, Kirrama Range uplands; LE, Mount Lee uplands; LU, Lamb Range uplands; MT, Malbon Thompson Range; SU, Mount Spec uplands.

C. ornatus is widespread in the AWT, occurring widely in middle- and high-altitude rainforest (400–1,600 m above sea level) and some wet lowland areas (fig. 1). As with most frogs (Gerhardt and Huber 2002), the primary means of mate choice in C. ornatus is male mating call (Hoskin 2004; Felton et al. 2006). C. ornatus is a “generalist” rainforest species when compared with most other AWT rainforest vertebrates, as calculated through rainforest and ecological niche specialization scores (Williams et al. 2010). It occurs in all rainforest types within its range and extends into associated areas of sclerophyll forest and montane heath.

We chose C. ornatus, a small, low-vagility, terrestrial-breeding, generalist rainforest frog, for a study of the effects of climate shifts on genetic and phenotypic divergence for the following reasons. Small body size enhances persistence in small habitat areas, and low vagility increases the potential for isolation and subsequent divergence (Goope 2004; Turgeon et al. 2005). This is supported by the fact that low-vagility invertebrates (e.g., flightless beetles, snails, spiders) typically show fine-scale patterns of phylogeographic structuring (Hugall et al. 2002; Bell et al. 2007; Boyer et al. 2007; Starrett and Hedin 2007; Bond and Stockman 2008; Walker et al. 2009). Breeding mode is important because small montane refugial areas are more likely to have retained suitable conditions for some breeding modes (e.g., terrestrial breeding in moist leaf litter) than others (e.g., stream or pool breeders). Indeed, small-bodied, terrestrial-breeding rainforest frogs show patterns of higher persistence than aquatic breeders in the AWT (Graham et al. 2006). Regarding habitat specialization, it may be argued that specialized species are likely to be more severely isolated by habitat fragmentation (and so may be expected to show greater structuring; Knowles 2000; Turgeon et al. 2005), but these species are also more likely to become extinct when refugia become small or ecologically marginal to core habitat conditions. The relative persistence of more generalist species may therefore be reflected in more complex phylogeographic structuring. Generalist species are also more likely to persist in refugia that differ environmentally and thereby provide greater opportunity for divergent selection and consequent phenotypic change. Finally, frogs are ideal because the functionality of morphological traits is generally well known (Wells 2007) and male mating call has been well established to be the key mating trait (Gerhardt and Huber 2002).

Methods

Field Sampling

Sampling across the geographical range of C. ornatus was extensive, enabling a thorough assessment of phylogeographic and phenotypic diversity. A total of 788 individuals were sampled from 55 sites, averaging 14.3 individuals per site (app. A, available online). Intensive sampling was conducted in the regions of highest genetic diversity (on the basis of preliminary analysis), including an approximate north–south transect to the west of Mount Bartle Frere and an approximate east–west transect from the coastal lowlands over Mount Bartle Frere (fig. C1, available online). C. ornatus occurs at high density over much of its distribution; however, it is generally detected only when males call in the summer wet season. Sampling tar-
geted calling males \(N = 739; 93.8\%\) of all samples), which provided a large data set of sexually mature individuals of one sex for phenotypic analyses. All frogs were measured and tissue was sampled, and then frogs were released at the point of capture. For genetic analysis, a very small \(<2\ mm\rangle\) terminal portion of the second toe of the right foot was removed and placed in 95\% ethanol. All genetic samples and a small number of voucher specimens are currently housed at the Australian National University (C.J.H.). All collections will ultimately be deposited in the Queensland Museum. Calls were recorded from selected sites. GPS position and altitude \(\pm 5\ m\rangle\) were recorded at each site. All morphology and call data were obtained by one of us (C.J.H.). Frogs were measured using vernier calipers (to the nearest \(0.1\ mm\rangle\) and a \(5-g\) spring-loaded Pesola (to the nearest \(0.05\ g\rangle\). Morphology and call data sets are deposited at Dryad (http://dx.doi.org/10.5061/dryad.t215b).

Morphological traits encapsulating body size and shape are widely used to assess divergent selection across environments (Endler 1986; Smith et al. 1997; Schluter 2001; Hoskin et al. 2005; Losos 2009; Collar et al. 2010). We measured the following widely used frog morphology measurements (Hoskin et al. 2005; Wells 2007): snout–vent length (SVL), tarsus length (TL), head width (HW), width of the disk on the third (i.e., longest) finger (DISK), and weight (WT). Although the functionality of these traits has not been specifically established for this species, they are deemed of likely ecological relevance on the basis of patterns of variation across ecologically different Cophixalus species (C. J. Hoskin, unpublished data) and studies of other organisms (e.g., Hoskin et al. 2005; Losos 2009; Collar et al. 2010). These traits likely reflect the way a frog functions in its environment in terms of, for example, jumping and climbing abilities (size, TL, DISK), substrate use (size, DISK), and prey size selection (size, HW). Here we did not place particular relevance on variation in individual morphological traits but rather treated size and shape variation as multivariate indicators of ecological divergence.

Traits used by animals in the process of choosing their mates (mating traits), where these can be confidently identified (e.g., frog call), also offer potential insights into divergent selection across different biotic or abiotic environments (Ryan and Wilczynski 1991; McPeek 1996; Gerhardt and Huber 2002; Kirshel et al. 2009; Hoskin and Higgie 2010; Tobias et al. 2010) as well as insights into the degree of reproductive isolation between populations (Gerhardt and Huber 2002; Coyne and Orr 2004; Hoskin et al. 2005). As it is for most frogs (Gerhardt and Huber 2002; Wells 2007), mating call is the primary means of mate choice in C. ornatus (Hoskin 2004; Felton et al. 2006). Male mating calls were recorded in the field using a Sony Professional DAT recorder (TCD-D100) and a Sennheiser (K6 ME-66) directional microphone. Recording did not commence until the male had uttered an uninterrupted string of 10 calls. Following this, approximately 10 consecutive calls were recorded. Air temperature was recorded to the nearest \(0.1\ C\) and the male was measured, tissue sampled, and then released at the point of capture. Calls were sampled at 44,100 Hz on a Macintosh G4, and the software Canary, version 1.2.1, was used to measure traits. Four calls were randomly chosen for each male to give the average call characteristics of that individual. The following standard frog call traits (Gerhardt and Huber 2002; Hoskin 2004; Hoskin et al. 2005; Felton et al. 2006) were measured: dominant frequency (DF), the frequency at which the call is of greatest intensity; call duration (DUR), measured from the first pulse peak of the call to the last pulse peak of the call; and pulse rate (PULSE), in pulses per second.

### Modeling of Potential Distributions under Current and Past Climates

The maximum entropy (Maxent, ver. 3.2.1) method (Phillips et al. 2006) was applied to 941 distribution records for C. ornatus (fig. 1), using the general approach described by VanDerWal et al. (2009). The 941 records came primarily from field surveys by S.E.W. and C.J.H. and from Queensland Museum specimen data, and all are highly reliable and carefully vetted (Williams et al. 2010). The distribution records are deposited at Dryad (http://dx.doi.org/10.5061/dryad.t215b). Background points \((\sim 36,000)\) were extracted from a 1-km grid of the AWT bioregion. Seven biologically meaningful climate variables were obtained for the region from an 80-m-resolution climate surface: annual mean temperature, annual mean precipitation, minimum temperature of the coldest quarter, precipitation of the driest and wettest quarters, and temperature of the wettest and coldest quarters. Following Hilbert et al. (2007), we applied the following climate shifts (as originally estimated from local palynological records; Kershaw and Nix 1988) to three periods representative of different phases of Pleistocene climate cycles: the cool-dry LGM (18 kya), the cool-wet Pleistocene-Holocene transition (PHT; 10–8 kya), and the warm-wet Holocene Climatic Optimum (HCO; 5–3.5 kya). These three time periods are considered to best reflect major changes in rainforest distribution in the AWT through the Quaternary (Kershaw and Nix 1988; Graham et al. 2006; Hilbert et al. 2007; VanDerWal et al. 2009). The temperature variables were adjusted relative to the present climate by \(-3.5\), \(-2\), and \(+2\ C\) for the LGM, PHT, and HCO, respectively. For precipitation variables, multipliers of current conditions of 0.5, 2, and 1.25 were applied for the LGM, PHT, and HCO, respectively. The exception was for pre-
ception of the driest quarter, where the multiplier for HCO was 1.5. The paleomodeling did not account for changes in sea level because paleoecological data (Kershaw et al. 2005) and previous climatic modeling (Moussalli et al. 2009) have indicated that rainforest was not extensive on the continental shelf.

**Genetic Data and Analysis**

**Genetic Data Collection.** Total genomic DNA was extracted from toe pads, using a method similar to that outlined by Aljanabi and Martinez (1997). Using polymerase chain reaction (PCR), we amplified and sequenced one mitochondrial fragment and four nuclear fragments. The mitochondrial fragment was a section of the 16S ribosomal RNA gene (16S rDNA). Due to coamplification of a nuclear pseudogene of 16S rDNA, we designed primers specific for *C. ornatus* 16S. We also designed primers specific to the 16S pseudogene and used it as a nuclear locus for analysis. To obtain additional nuclear loci, we sequenced the flanking regions for three previously described micro-satellite markers (Bardeleben et al. 2006) from geographically diverse *C. ornatus*, using new primer sets we designed. For details of all primers and PCR and sequencing protocols, see appendix C, available online. Sequences are deposited in GenBank (accession numbers: for 16S, JF743691–JF743761; for 16S pseudogene, JN034917–JN035138; for Corn304, JN012244–JN012465) and Dryad (for Corn16, Corn53; http://dx.doi.org/10.5061/dryad.t215b).

**Mitochondrial Data Analysis.** We conducted phylogenetic analyses for the 16S mtDNA sequence data using maximum likelihood (ML) and Bayesian analysis. The 16S tree was outgroup rooted with the sister group *Cophixalus infacetus* and *Cophixalus zweifeli* (Hoskin 2004). We ran a ML search using RAxML Blackbox (Stamatakis et al. 2008). Bayesian phylogenetic analyses were conducted using MRBAYES, version 3.04b (Huelsenbeck and Ronquist 2001). We produced posterior probability distributions by allowing four incrementally heated Markov chains to proceed for 20 million generations, sampling every 1,000 generations. The Bayesian posterior probability values were estimated from the sampled trees that remained after burn-in samples were discarded. Because both the ML and the Bayesian tree topologies were the same, we have included only the ML tree with both the ML bootstraps and Bayesian posterior probabilities. Using Arlequin, version 3.1 (Excoffier et al. 2005), we estimated nucleotide diversity ($\theta_s$, $\theta_w$) and net sequence divergence among populations ($D_s$) using the Tamura-Nei model of sequence divergence. We also calculated Fu’s $F_s$ values in order to test for population expansion within lineages.

To estimate divergence times among major lineages, we used BEAST, version 1.4.8 (Drummond et al. 2005; Drummond and Rambaut 2007), with the GTR+I+Γ model of substitution, as this was the best-fit model of nucleotide substitution according to Modeltest (Posada and Crandall 1998). We assumed a substitution rate of $0.092 \pm 0.046$ substitutions per site per 100 Myr for 16S (Roelants et al. 2007). Genealogies and model parameters for each lineage were sampled every one-thousandth iteration for 50 million generations under a relaxed lognormal molecular clock with uniformly distributed priors. Effective sample size (ESS) values were >200, suggesting convergence of the sampled parameters (Weinstock et al. 2005). ESS values and demographic plots for the analyses were assessed in Tracer, version 1.4.1 (Rambaut and Drummond 2007).

**Nuclear Data Analysis.** For nuclear sequences that were heterozygous for insertions or deletions, unphased haplotypes were inferred using CodonCode Aligner. Each sample was compared with several reference sequences and manually checked to reduce error. To identify haplotypes from heterozygous individuals, we used PHASE, version 2.1 (Stephens et al. 2001). We ran each locus three times to compare the phased positions. Unique haplotype numbers were assigned to each phased nuclear locus. To infer the number ($K$) and composition of genetic populations, we used the Bayesian assignment program STRUCTURE, version 2.1 (Pritchard et al. 2000). Ten iterations were run for each value of $K$ from 1 through 10. We implemented the admixture ancestry model and assumed correlated allele frequencies among populations, with a burn-in of 50,000 and a Markov chain Monte Carlo length of 1,000,000. We used both visual inspection of changes in likelihood with increasing $K$ and the method described by Evanno et al. (2005) to identify the optimal number of clusters in our data set. We considered individuals to be admixed if the individual’s estimated membership was ≤0.8 at the “best” $K$. Admixed individuals were found in only extremely narrow zones of contact, and then in only a few populations. Because our primary interest was in identifying lineages and relationships among them, we excluded the few populations with admixed individuals in the following analyses. For each genetic population identified by STRUCTURE, we estimated diversity and divergence as described for mtDNA. To visualize overall divergence patterns, the resulting $D_s$ matrix was then used to produce an unrooted population phylogram via the neighbor-joining algorithm in PAUP*, version 4 (Swofford 2003).

**Combined Data Analysis.** To estimate the current growth rates of the lineages and subgroups, we used LAMARC, version 2.1.3 (Kuhner 2006). For each sample, we used a
combined data set of the mtDNA and the phased nDNA to estimate this parameter. Each locus was analyzed as a separate genomic region, and the results were integrated over these. We used the Bayesian analyses with 100,000 final recorded parameter sets, with a burn-in of 40,000 and sampling every 100. The GTR nucleotide evolutionary model was used for all loci, and Modeltest GTR rates and base frequencies were supplied. We adjusted the upper limit of the growth rate to 8,000 and the lower limit to \( \pm 5,000 \). Populations that were smaller in the past but that now show evidence of expansion will have positive values of the growth rate \( g \). Conversely, negative \( g \) indicates that a population was larger in the past.

**Phenotypic Analyses**

The genetic analyses (below) revealed three major monophyletic lineages: Central (C), North (N), and Hinchinbrook Island (H). We analyzed phenotypic variation among these major lineages because they are supported under all genetic analyses. Analyses also testing variation among subgroups within C (not presented here) did not alter conclusions at the major lineage level. Only data for mature males (captured calling) were analyzed (morphological analyses: \( n = 511 \) from 42 sites; call analyses: \( n = 197 \) from 26 sites). As was done for the molecular analyses, the few admixed sites were removed to avoid the inclusion of introgressed individuals in tests of among-lineage divergence. Analyses of body size and shape divergence were conducted using all five morphological traits, and call divergence was tested using all three call traits. Statistical analyses were performed in SPSS, version 18, and SAS, version 9.2. All traits were tested for normality, and transformations were performed where necessary. The phenotypic analyses aimed to determine whether differences in morphology or call could be detected among the three lineages after controlling for the effects of environmental variation within lineages. That is, how would the lineages differ in morphology and call if they existed in the same place? This enables insights into ecological divergence and the potential for premating isolation.

**Accounting for Environmental Variation in Traits.** The sources of environmental variation we accounted for were altitude and temperature variation. These have been identified in other studies (e.g., Gerhardt and Huber 2002; Hoskin et al. 2005), with the typical patterns being that frogs at higher altitudes are bigger (and bigger frogs have lower-pitched calls) and frogs calling at higher temperatures have faster pulse rates and shorter calls. Phenotypic variation due to these sources could confound the interpretation of an overall lineage effect. ANCOVAs were used to test covariance of morphological traits against altitude, and call traits against temperature and altitude (app. B, available online). All analyses were nested by site within lineage to account for among-site variation and were conducted on altitude or temperature means within groups, as this was the appropriate replicate level for effects and accounted for uneven sampling across the range of altitude or temperature data. All morphological traits and call dominant frequency and duration were dependent on altitude: at higher altitudes, frogs are bigger and therefore have lower-pitched and also longer calls (app. B). Call pulse rate and duration were dependent on temperature: as temperature increases, pulse rate increases and calls get shorter (app. B). These significant relationships were removed by taking the unstandardized residuals from a linear regression of the trait on the covariate (app. B) and using them in the following analyses (e.g., altitude-corrected SVL, temperature-corrected pulse rate).

**Divergence among the Lineages.** Multicollinearity was then tested among the covariate-corrected morphological traits and call traits. The five morphological traits had very high levels of correlation (all \( C_p > 0.8 \); table B1, available online). Because high multicollinearity may cause problems in subsequent multivariate analyses, the five morphological traits were transformed into five principal components (PCs) via a principal component analysis (PCA), using the correlations matrix (due to weight being on a different scale than the four length measurements). For highly correlated morphological measurements, PCA also has the benefit of transforming the data into biologically relevant axes of size (PC1) and shape (remaining PCs) variation (Jolliffe 2002). Loadings on each PC were reported for the trait that loaded most heavily and for any subsequent traits that loaded \( >0.7 \) times the value of the most heavily loaded trait (table B2, available online). The three call traits had low to moderate levels of correlation (highest correlation, \( C_p = 0.5 \); table B3, available online) and so were able to be used directly in the multivariate analyses.

To test the fixed effect of genetic lineage on body size, body shape, and mating call, analyses were performed using the MIXED procedure in SAS, version 9.2, with parameters estimated using maximum likelihood. All analyses were nested by site within lineage to account for among-site variation within lineages. Because of the unequal lineage sample sizes, the denominator degrees of freedom were estimated using the Kenward-Rogers method (SAS Institute 2010). To correctly perform MANOVAs to test the effect of genetic lineage on traits using the MIXED procedure, the lineage \( \times \) trait model was compared with the null model that contained only the traits (Wright 1998). The difference in \( -2 \log \) likelihoods between the two models was tested against a \( \chi^2 \)
distribution, with the degrees of freedom being the difference in number of parameters fitted between the two models.

Body size differences among the lineages were assessed by placing PC1, representing overall body size, in an ANOVA with pairwise contrasts specified to test differences between each of the lineages. Body shape differences among the lineages were assessed by placing the four remaining PCs (PC2–PC5) into a MANOVA with multivariate pair-wise contrasts specified in the lineage × trait model to test differences between each of the lineages. Univariate ANOVAs with pairwise contrasts were also conducted to test variation in each PC among lineages. Mating-call differences among the lineages were assessed by placing the three call traits into a MANOVA with multivariate pair-wise contrasts specified in the lineage × trait model to test for differences between each of the lineages. All P values reported were adjusted for multiple comparisons using the false discovery rate (FDR) method (Benjamini and Hochberg 1995). For graphical representation of the multivariate body shape and call divergence, we constructed a single canonical variate (CV1) at the lineage level for each data set from a multivariate hierarchical linear model, with site nested within lineage. The respective canonical variate equations were then applied to PC2–PC5 for body shape CV1 and to the three call traits for call CV1. These CVs represent the linear combination of traits that differ most among the lineages while accounting for among-site variation (shown in fig. 4).

Results

Predicted Distributions under Current and Past Climates

The Maxent model in the current climate was dominated by three climatic variables—positively by precipitation of the driest and wettest quarters and negatively by temperature of the wettest quarter—and produced a strong prediction relative to the known distribution (AUC = 0.94; fig. 1). Notably, *Cophixalus ornatus* is predicted to be absent from the montane rainforest in the Lee Uplands (LE), located between the southernmost-known populations in the Spec Uplands (SU) and those in the Kirrama Uplands (KU) to the north. Repeated surveys for *C. ornatus* at LE have failed to detect this species. However, the model does overpredict to multiple high-elevation rainforest areas to the north and to the south of the current distributional limits. The observed absence of *C. ornatus* from these climatically suitable areas, despite intensive surveys for microhylids, could relate to the presence of congeners (Hoskin 2004) or, more likely, limitations on dispersal across the drier forest barriers that separate these areas.

The paleomodels indicate considerable variation in the potential range of *C. ornatus* from the cool-dry LGM to the present (fig. 2), and parallel predicted responses of the rainforest itself (VanDerWal et al. 2009). At the LGM, the potential range is much reduced, especially in the southern and northern regions of the current range. Subsequently, the potentially suitable range expands during the cool-wet phase of the PHT, allowing for recolonization of northern
Mitochondrial Phylogeography

Phylogeographic structure of mtDNA was determined from 486 individuals for the 16S rDNA gene segment across 42 localities (after pooling some geographically adjacent small samples), with an average of 11.8 individuals per locality (table C1, available online). For analyses of nuclear sequences, only individuals with a complete data set of four loci were used (N = 300 individuals across 42 localities, averaging 7.3 individuals per locality; table C1). Analyses of these data revealed that C. ornatus is highly genetically structured in the manner predicted by the paleomodels above (fig. 3; tables 1, 2).

Mitochondrial Phylogeography and Diversity. The sequenced and aligned segment of the 16S rDNA gene spanned 513 nucleotide positions. The ML analysis revealed three geographically structured lineages, each with spanning 513 nucleotide positions. The ML analysis resequenced and aligned segment of the 16S rDNA gene (figs. 3 and the high areas of the coastal Malbon Thompson Range (BF) across Mount Bartle Frere, Mount Bellenden Ker, with distinct subgroups representing the Lowland (L) and northern (N), central-southern (C), and Hinchinbrook Island (H; fig. 2).

On the basis of the paleomodels, we can derive the following predictions for the genetic data: (1) the presence of multiple divergent lineages, reflecting persistence in the central AWT and in the small predicted refugia at LU and H; (2) highest genetic diversity in the central AWT, reflecting the relatively large refugial area there; and (3) signatures of population expansion in northern and southern regions, due to expansion of populations from central refugia during the early Holocene, before connections were cut in the mid-Holocene to the present.

Mitochondrial Phylogeography and Diversity. The estimated times of divergence among the three major lineages substantially predate the LGM (defined as ~18 kya; Hopkins et al. 1993; Kershaw et al. 2005), with point estimates in the Pliocene but broad confidence limits (fig. C2 in the online edition of the American Naturalist). The North and Central/Hinchinbrook Island lineages are estimated to have diverged at 4.7 million years ago (mya; 95% highest posterior densities [HPD], 2.6–8 mya) and the Central and Hinchinbrook Island lineages at 3.4 mya (95% HPD, 1.8–5.5 mya).

Nuclear Phylogeography and Diversity. Clustering of individuals’ four-locus genotypes using STRUCTURE revealed fine-scale population structure that was generally consistent with the mtDNA results and narrow zones of admixture/introgression at parapatric boundaries between the lineages (fig. 3D). One exception was the small sample (N = 4) from Hinchinbrook Island that proved to be of unstable affinity in repeated runs and was subsequently excluded from this part of the analysis (data not shown). This population is highly divergent in the other genetic analyses (see below). Using STRUCTURE, the posterior probability (ln Pr (X|K)) values increase abruptly from the K = 1 group to the K = 2 group and then more gradually to K = 5, beyond which the values plateau at K = 8 (fig. C3, available online). Using the Evanno et al. (2005) method, we found the highest ΔK value at K = 2 (fig. C4, available online), in which group 1 corresponds to populations with N mtDNA lineages and group 2 corresponds to populations with C mtDNA lineages. At higher K values, the C mtDNA lineage splits, first (K = 3) into populations with L or BF mtDNA versus those with BF or S mtDNA. Then at K = 4, the Lowland mtDNA populations (group 3) separate from the main BF mtDNA group and at K = 5 the BF/S mtDNA group splits into two geographic components, one (group 4) adjacent to Mount Bartle Frere with mostly BF but also some S mtDNA and the other (group 5) from the central Atherton Uplands to the southern limit of the range with only S mtDNA (fig. 3D). At both K = 4 (the number of lineages and subgroups detected in the mtDNA analysis, excluding H) and K = 5, there are narrow zones of admixture/introgression (fig. 3D). Geographic transition is particularly abrupt between the N and C mtDNA lineages. More introgression is evi-
Figure 3: Lineage structure in *Cophixalus ornatus*. Colors correspond to the three major lineages (Hinchinbrook Island [H], North [N], and Central [C]) and the subgroups within C (Mount Bartle Frere [BF], Lowland [L], and Southern [S]). A, Map of the Australian Wet Tropics showing localities sampled for molecular analyses. Colored dots on the map represent the lineage as determined by molecular analyses of four nuclear loci and one mitochondrial locus. Mixed-color dots represent admixed sites. The inset shows the region of secondary contact around Mount Bartle Frere. For site details, see figure C1, available online. B, 16S mtDNA maximum likelihood tree. Values above the slash are RAxML bootstrap values; values below the slash are Bayesian posterior probabilities. C, Population phylogram based on neighbor-joining of pairwise D2 values. D, Genetic structure per sampled site. Individuals are arranged according to latitude within mitochondrial lineages. Black lines denote each site, with vertical separation proportional to the number of individuals sampled; colored lines represent individuals, with the color of each representing the proportion of that individual’s membership in each cluster. Left, average mitochondrial proportion per site. Right, STRUCTURE clustering of individual four-locus nuclear genotypes for K = 4 and K = 5 groups. Potential hybrid individuals are depicted as bars with obvious contributions from two colors. The H lineage was not included in these analyses (see text).
nDNA genes were higher between the major N, C, and H lineages mtDNA.

Group 2 and group 4 populations are grouped as the BF south (group 5, S mtDNA). For subsequent analyses, mtDNA) and were distinct from the populations to the geographically proximal populations (group 2, BF (group 4, with BF, S, or both mtDNA types) clustered with populations on the western flank of Mount Bartle Frere. The Central lineage is genetically diverse, H lineages were highly distinct from each other and from the mitochondrial phylogeny (fig. 3).

Population Expansion. For mtDNA alone, all lineages (N, C, and H) and each of the subgroups within C had significantly negative $F_g$ values (table 1), indicating historical population expansions across the range of the species. The coalescent analysis of combined mtDNA and nuclear loci (LAMARC) indicated positive population growth (positive values of $g$) for all lineages and subgroups except L (table 1). However, it was only for H, the group with the highest point estimate, that the 95% confidence interval excluded 0 (table 1). Thus, for the major lineages H shows a strong population expansion signature, but for the other lineages evidence is mixed.

To test the hypothesis that N persisted in a small northern refuge (e.g. LU in fig. 2) as opposed to expanding from the periphery of the major central refuge, we assessed expansion signatures across N populations. We grouped populations north of LU (fig. CI; populations 1–3), populations around LU (fig. CI; populations 5–7), and two groups of populations south of LU (fig. CI; populations 8, 9, and 11–14). For mtDNA, only the most southerly N populations (11–14) showed range expansion ($F_g = -2.1, P < .05$). Combined LAMARC analyses on both the mitochondrial and the nuclear data indicated positive population growth in each of the two groups south of LU (populations 8 and 9, $g = 1,223 [95\% \text{ HPD}, 348–10,108]$; populations 11–14, $g = 1,018 [95\% \text{ HPD}, 315–3,385]$). By contrast, in populations around and north of LU there was no such evidence for population expansion. The results support the interpretation that the N lineage persisted in a separate refuge (LU or farther north) rather than expanding northward from the central refuge.

**Summary of Genetic Results.** The combined mtDNA and nuclear gene evidence supports the presence of three major lineages: northern (N) versus central-southern (C) populations on the mainland and the Hinchinbrook Island...
The C lineage has the highest diversity, with relatively clear demarcation between Lowland (L), Mount Bartle Frere area (BF), and Southern (S) populations. All major lineages and subgroups of C show evidence of population expansion for mtDNA, whereas coalescent estimates from combined mtDNA and nDNA demonstrate population growth for H alone. Finer-scale tests of population expansion within the N lineage suggest persistence and population growth for H alone. Finer-scale tests of population expansion within the N lineage suggest persistence and population growth for H alone.

### Summary of Phenotypic Divergence among Lineages

**Plasticity of Mating Call:** The site-nested MANOVA on the three call traits revealed highly significant call divergence among the lineages (likelihood ratio $\chi^2 = 43.48$, df = 3, $P_{\text{FDR}} < .0001$). The multivariate planned contrasts revealed that all three call traits differ significantly from each other, with N frogs being highly significantly different from both H ($F_{3,4.4} = 0.0210$) and C ($F_{3,9.9} = 0.0420$) frogs, while H and C frogs did not differ in body shape (fig. 4B; table B4). In the site-nested ANOVAs on the individual PCs, only PC2 varied significantly among the lineages ($F_{3,4.4} = 3.59, P_{\text{FDR}} = .0492$), with N frogs having relatively smaller finger disks than C frogs relative to body size (planned contrast $F_{3,4.4} = 6.89, P_{\text{FDR}} = .0209$; table B4).

**H lineage.** Population. The C lineage has the highest diversity, with relatively clear demarcation between Lowland (L), Mount Bartle Frere area (BF), and Southern (S) populations. All major lineages and subgroups of C show evidence of population expansion for mtDNA, whereas coalescent estimates from combined mtDNA and nDNA demonstrate population growth for H alone. Finer-scale tests of population expansion within the N lineage suggest persistence and population growth for H alone.

### Table 2: Corrected net sequence divergence ($D_s$) between *Cophixalus ornatus* lineages and subgroups

<table>
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<tr>
<th>Major lineage, subgroup</th>
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Note: Only lineage C has subgroups. Redundant columns and rows have been removed. n/a, not available. BF, Mount Bartle Frere; C, Central; H, Hinchinbrook Island; L, Lowland; N, Northern; S, Southern.
The mating calls of H, N, and C frogs differ significantly, with the greatest divergence being between H and N frog calls and those of C frogs being intermediate (fig. 4C). Dominant frequency differs between all three lineages, duration differs between H and N frogs, and N frogs differ from H and C frogs in terms of pulse rate.

Discussion

Our results strongly suggest that historic fragmentation of populations due to climatic shifts shaped the current genetic and phenotypic diversity in Cophixalus ornatus. Our original predictions were satisfied by (1) spatial correlation between major lineage structuring and predicted persistence: populations of C. ornatus cluster as three major lineages (C, N, and H) that correlate spatially with predicted paleorefugia; (2) higher genetic diversity in regions of persistence, particularly where these regions were larger: the C lineage, which is associated with a predicted large, spatially complex central refuge, has high genetic diversity relative to the two lineages associated with two very small peripheral paleorefugia; (3) genetic signatures of population expansion from paleorefugia, particularly where these are predicted to have been small: all populations show evidence of population expansion but it is most pronounced for the H populations and the southern populations of N; and (4) divergence in ecologically important phenotypic traits among refugia: phenotypic variation was structured concordant with major phylogeographic lineages.

The Role of Climate Shifts in Shaping Genetic Diversity

As was found for other studies (e.g., Hugall et al. 2002; Knowles et al. 2007; Walker et al. 2009), there is a strong correlation between patterns of current genetic diversity in C. ornatus and the location of predicted paleorefugia. The genetic results concord with predicted persistence of populations of C. ornatus through the LGM in a large, spatially complex central refuge and two very small peripheral refugia (LU and Hinchinbrook Island). The spatial scale of structuring in C. ornatus more closely resembles that of invertebrates (e.g., snails, beetles, spiders) in the AWT and other systems (e.g., Hugall et al. 2002; Bell et al. 2007; Boyer et al. 2007; Starrett and Hedin 2007; Bond and Stockman 2008; Walker et al. 2009) than other AWT vertebrates, which generally show fewer, broadly spread lineages correlating to core predicted northern and southern refugia (Joseph et al. 1995; Schneider et al. 1998; Moritz et al. 2009). This probably reflects the high persistence and low vagility of this small, terrestrial-breeding frog. Elsewhere in the world, other small-bodied, terrestrial-breeding rainforest amphibians provide examples of particularly pronounced fine-scale phylogeographic and species diversity (e.g., Meegaskumbura et al. 2002; Roberts et al. 2006; Köhler and Günther 2008; Vieites et al. 2009).

The central lineage (C) consists of several subgroups (BF, S, and L) that likely persisted in the large predicted areas of montane rainforest and associated lowlands in the central AWT. The genetic results and spatial modeling suggest that the N and H lineages persisted in very small refugia. In contrast to other AWT vertebrates, the paleo-
models do not suggest that *C. ornatus* persisted at CU, its northern limit, but rather that it persisted in LU (fig. 2). Genetic tests of population expansion support persistence of the N lineage in the northern half of its range with subsequent (most likely early Holocene; fig. 2) expansion southward. An alternative is persistence in a small lowland area predicted to the northeast of CU (figure 2), but this is unlikely given that it is well outside the current range. Particularly interesting is the long-term persistence of *C. ornatus* on Hinchinbrook Island. Paleomodeling suggests that rainforest persisted only in very small upland isolates on Hinchinbrook Island during climatically restrictive periods (e.g., the LGM; fig. 2; VanDerWal et al. 2009). Genetic signatures of population expansion are particularly evident in this lineage. Despite repeated land connections to the mainland over at least the last few million years, there would have been limited potential for historical contact between H and C populations because these connections were dominated by dry forest types (Nix 1991; VanDerWal et al. 2009).

The correlation between the genetic data and predicted distribution at the LGM suggests that late Pleistocene glacial cycles determined the persistence of lineages and shaped current patterns of genetic diversity within them. However, as was found in many other studies (Klicka and Zink 1997; Avise and Walker 1998; Moritz et al. 2000; Rull 2008; Moussalli et al. 2009), divergence times among the major lineages well precede the LGM, with estimates suggesting Pliocene divergences between N, C, and H frogs. This suggests that the late Pleistocene models used here also predict persistence and isolation over much longer time periods and that previous climatic oscillations resulted in spatially congruent refugia (see also Bell et al. 2010). Paleomodeling is restricted to the LGM and the Holocene because pollen-derived paleoclimate estimates for the AWT are restricted to this period (Kershaw and Nix 1988). However, detailed paleobotanical data for the region shows long-term presence of rainforest, with fluctuations in extent and composition from the mid-Miocene onward (Greenwood and Christophel 2005; Kershaw et al. 2005), overlain on geologically ancient and stable landforms (Nott 2005). Therefore, at least for the AWT, refugial areas predicted for the LGM are likely indicative of those present throughout restrictive periods of the Miocene (Greenwood and Christophel 2005; Kershaw et al. 2005) and through climatic cycles of the Plio-Pleistocene (Haffer 1997).

The big question this pre-Pleistocene model presents is how did such genetic (and phenotypic; see below) divergence accumulate in the face of regular opportunities for gene flow? This question has been raised for other regions in regard to repeated connections during the Pleistocene (Zink and Slowinski 1995; Hewitt 2000; Knowles 2000; Futuyma 2010), and it has been suggested that divergence and speciation requires rapid evolution of reproductive isolation between lineages such that mixing does not occur during subsequent interglacials (Knowles 2000; Carstens and Knowles 2006). It is clear that in our system there was ample opportunity for mixing during interglacial climates (at least between N and C frogs; figure 2). However, we suggest that the core populations in refugial areas may not have been exposed to introgression through time because the intervening mixed populations would have become extinct every time rainforest “contracted” to refugia. The core populations could therefore diverge unhindered by gene flow. This suggestion requires testing in the AWT and other such systems and will depend on a range of factors, including distance between refugia, duration and extent of connection, dispersal abilities, and selection for or against loci. Estimating gene flow between core and intervening populations across a range of taxa under current expansive conditions would be insightful.

The Role of Climate Shifts in Generating Phenotypic Diversity

We found that phenotypic variation was structured concordant with major phylogeographic lineages, which suggests that it was similarly generated by historic fragmentation of populations to refugia. This raises the question of whether phenotypic divergence was generated by drift or selection. The null approach to phylogeographic studies is that of historical isolation across environmentally similar refugia. This idea of niche conservatism clearly plays a role in driving allopatric divergence—it is due to restriction to a particular habitat (and low vagility) that gene flow is severed when habitats fragment (Wiens and Graham 2005; Kozak and Wiens 2006)—but potentially not in driving phenotypic divergence and speciation over the shorter time periods generally associated with climatic oscillations. Across environmentally similar refugia, genetic divergence will accumulate with little accompanying phenotypic divergence through nonadaptive (drift) and adaptive (stabilizing or uniform selection) processes (Charlesworth et al. 1982; Coyne and Orr 2004; Wiens and Graham 2005; Nosil and Flaxman 2011). This appears to be the norm for AWT vertebrates, with studies generally failing to find phenotypic differences between phylogeographic lineages that occupied core northern and southern refugia (Schneider and Moritz 1999; Hoskin et al. 2005; Moritz et al. 2009). This contrasts with the substantial phenotypic divergence found within some of these lineages that is associated with habitat differences (Schneider et al. 1999) or reinforcing selection in regions of secondary contact (Hoskin et al. 2005).

Environmentally heterogeneous refugia on the other
hand will more likely result in phenotypic divergence and speciation, with divergent selection among populations potentially leading to rapid divergence in ecological and/or mating traits (Rice and Hostert 1993; McPeek 1996; Schluter 2001; Gavrilets 2003, 2004; Coyne and Orr 2004; Hoskin and Higie 2010). Even weak selection (i.e., due to subtle differences in environment between refugia) can dramatically increase the rate of divergence when gene flow is absent or limited (Rice and Hostert 1993; Gavrilets 2003, 2004). We specifically chose traits relevant to ecology and mating, traits of key biological importance that are unlikely to be subject to drift (Coyne et al. 1997; Gavrilets 2003). Such traits are generally under strong stabilizing selection in an environment, so divergence in these traits is unlikely without environmental change (Charlesworth et al. 1982; Coyne et al. 1997; Gavrilets 2003). Within C. ornatus we found that N differed in body shape, H differed in being of substantially smaller body size, and all three lineages differed significantly in terms of mating call. Phenotypic divergence is particularly pronounced for H and N (figure 4). We therefore believe that this suggests divergent selection across refugia, particularly those peripheral to the large central refuge. Ultimately, however, we cannot rule out the effects of drift, particularly given the deep divergences (i.e., extended periods of isolation) between the major lineages and the evidently small population sizes in the smaller refugia.

Do the Major Lineages Represent Species?

To assess the potential for reproductive isolation among C. ornatus lineages, we consider three lines of evidence: cline divergence, genetic divergence, and preliminary analysis of secondary contact between two of the lineages (N vs. C). Calls of the three major lineages of C. ornatus differ significantly in key traits for mate choice and species recognition in frogs (Gerhardt and Huber 2002), suggesting strong potential among the lineages for premating isolation. Sequence divergence among lineages for both mtDNA and nuclear genes is comparable to that among sister species of Cophixalus (Hoskin 2004) and is sufficient to suggest the potential for at least partial postmating isolation (Sasa et al. 1998; Coyne and Orr 2004), as has been found between phylogeographic lineages in other AWT species (Phillips et al. 2004; Hoskin et al. 2005). With respect to the extent of admixture at contact zones, the parapatric boundary between N and C is abrupt (<1 km), with evidence of admixture in just one individual within the densely sampled Mount Bartle Frere transect (fig. 3). By comparison, the C subgroups show considerable admixture at some sites (L vs. BF or S) or more widespread introgression (BF vs. S; fig. 3). These multiple lines of evidence support the recognition of the three major lineages (H, N, and C) as separate species, and these will be described elsewhere.

Future Directions

This study shows divergence in key morphology and call traits among lineages, which suggests divergent selection across environmentally different refugia. However, on the basis of the data here, it is not possible to resolve the sources of selection or completely discount drift. Hypotheses can be generated, for example, that the smaller size of H frogs reflects habitat differences. The H lineage is unique in that it occupies extensive areas of montane heath, a habitat that dominates the uplands of Hinchinbrook Island but is very limited elsewhere in the species range. The shift to heath on Hinchinbrook Island may have caused selection on body size due to differences in physical structure, microenvironment, or community composition compared with rainforest. There are a number of ways in which the origins of phenotypic divergence may be further tested in this and other systems. First, the functionality of divergent traits can be tested against environmental differences (here associated with predicted refugial areas), an approach used to test for adaptation to historic and current environments (e.g., for lizards, see Losos 2009; Collar et al. 2010). Second, the array of pairwise contacts between N and C and all three subgroups of C around Mount Bartle Frere in the central AWT (fig. 3A) present an ideal setting to test for ecological and mating divergence and the importance of character displacement and reinforcement in completing speciation.

In C. ornatus, phenotypic divergence is particularly apparent for the two peripheral lineages. Similarly, in the north Queensland Cophixalus radiation more broadly, rainforest species are morphologically very similar whereas the few species occupying ecologically distinct, peripheral habitats differ obviously in body size (Hoskin 2004; C. J. Hoskin, unpublished data). This directs attention to the phenotypic analysis of currently or historically isolated peripheral populations. Phenotypic divergence (and hence speciation) may be particularly expected in refugia that are peripheral to core refugial areas because these areas are more likely to have been ecologically marginal or even atypical habitat compared with core areas (Gankin and Major 1964; Endler 1977; Lawton 1993), therefore presenting greater opportunity for divergent selection (Lesica and Allendorf 1995). Such analyses may be particularly profitable on generalist species within a broad habitat type (e.g., rainforest) because they are more likely than specialists to persist in marginal refugia and to have locally adapted phenotypic variation that could be subdivided across isolates.
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Male ornate nursery frog (Cophixalus ornatus) sitting on a leaf. Photograph by Conrad Hoskin.