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STRONG SELECTION AGAINST HYBRIDS MAINTAINS A NARROW CONTACT ZONE BETWEEN MORPHOLOGICALLY CRYPTIC LINEAGES IN A RAINFOREST LIZARD

Sonal Singhal[1,2,3] and Craig Moritz[1,3]

[1]*Museum of Vertebrate Zoology, University of California, Berkeley, 3101 Valley Life Sciences Building, Berkeley, California 94720-3160*

[2]*E-mail: singhal@berkeley.edu*

[3]*Department of Integrative Biology, University of California, Berkeley, 1005 Valley Life Sciences Building, Berkeley, California 94720-3140*

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Phenotypically cryptic lineages comprise an important yet understudied part of biodiversity; in particular, we have much to learn about how these lineages are formed and maintained. To better understand the evolutionary significance of such lineages, we studied a hybrid zone between two morphologically cryptic phylogeographic lineages in the rainforest lizard, *Lampropholis coggeri*. Analyzing a multilocus genetic dataset through cline inference, individual-based methods and population measures of disequilibrium and using simulations to explore our genetic results in context of theoretical expectations, we inferred the processes maintaining this hybrid zone. We find that these lineages meet in a hybrid zone that is narrow (≈400 m) relative to inferred dispersal rate. Further, the hybrid zone exhibits substantial genetic disequilibrium and sharply coincident and largely concordant clines. Based on our knowledge about the region's biogeography, the species' natural history, and our simulation results, we suggest that strong selection against hybrids structures this system. As all clines show a relatively narrow range of introgression, we posit that this hybrid zone might not yet be in equilibrium. Nonetheless, our results clearly show that phylogeographic lineages can evolve substantial reproductive isolation without concomitant morphological diversification, suggesting that such lineages can constitute a significant component of evolutionary diversity.

KEY WORDS: **hybrid zones, phylogeography, speciation, *Lampropholis coggeri*, simulations.**

The growth of phylogeography, or the study of geographic variation in genetic diversity within a species, has shown that many species consist of multiple, highly divergent genetic lineages. These lineages often exhibit levels of genetic divergence equal to or greater than those between morphologically defined species (Avise 2000) yet have no or limited phenotypic differentiation. These morphologically cryptic lineages are common yet understudied; in particular, understanding how these lineages form and what maintains boundaries between these lineages in the absence of overt phenotypic differentiation are open questions (Bickford et al. 2006). Contact zones, or geographic regions in which previously isolated lineages meet and interact, are an excellent tool for exploring and understanding the forces that maintain lineages' identities (Harrison 1993). Although such studies traditionally focus on contacts between morphologically differentiated species, contact zones can be particularly useful in evaluating the

significance of morphologically cryptic phylogeographic lineages as a component of biodiversity. After all, contact zones exhibit a continuum of outcomes that reflect how the lineages meeting diverged; lineages can exchange genes freely in the absence of reproductive isolation (Sequeira et al. 2005), gene flow can be spatially limited if barriers to gene flow (i.e., assortative mating or selection against hybrids) have evolved (Harrison 1986), or lineages can show complete reproductive isolation and remain phenotypically and genetically distinct at contact (Weir and Price 2011). Looking at contact zones between cryptic lineages can help us start to answer these questions; already, studies have shown that such lineages can evolve substantial barriers to gene flow (Phillips et al. 2004; Hoskin et al. 2005; Kawakami et al. 2009). This initial work suggests that cryptic lineages might not just be evolutionary ephemera; rather, they can be regarded as nascent species (Avise and Wollenberg 1997). However, these studies are still in their infancy, and we have much more to learn about cryptic biodiversity.

The Australian Wet Tropics (AWT) suture zone offers a set of replicated natural experiments with which we can address these questions. This suture zone reflects effects of late Quaternary climate change on rainforest and the species endemic to it (Moritz et al. 2009; Hewitt 2011). Palynological data and ecoclimatic models show that the forest contracted into two major refugia during the glacial cycles of the Late Quaternary (Nix 1991; Graham et al. 2006; VanDerWal et al. 2009). From 3 to 8 Kya, the rainforest expanded rapidly from these refugia, allowing diverse rainforest-specialist fauna to form spatially clustered contact zones (Moritz et al. 2009). Genetic divergence at these contacts varies widely, yet lineage pairs are morphologically similar and occur in the same ecological settings. Previous analyses of contacts in frogs, lizards, and mammals have shown several evolutionary outcomes: speciation by reinforcement, postzygotic isolation without assortative mating, and no reproductive isolation (Pope et al. 2000; Phillips et al. 2004; Hoskin et al. 2005; Dolman 2009). These results show that lineages can maintain their genetic integrity at secondary contact, even without concomitant morphological diversification. However, studies from this suture zone are still few, and analysis of additional contacts in lineage pairs with different levels of divergence is needed to test predictions such as the correlation between genetic divergence and extent of reproductive isolation (Weir and Price 2011).

We add to this emerging portrait of secondary contact outcomes in a suture zone by characterizing a contact in the AWT-endemic rainforest skink, *Lampropholis coggeri*. *Lampropholis coggeri* (family: *Scincidae*) is a small, semifossorial lizard (SVL $\approx$ 45 mm) that is often found in sun patches within or at the edge of forests (Williams et al. 2010). Previous sampling and genetic analysis of *L. coggeri* identified two major lineages in this species, which we name Northern and Southern (Fig. 1A; Bell et al. 2010). These lineages are 9.4% divergent at mitochondrial DNA and 1.1% divergent at nuclear introns, yet do not differ in any morphological traits (i.e., size, shape, scale counts, coloration) (Bell et al. 2010). In this study, we locate and characterize the contact zone between the Northern and Southern lineages by collecting a multilocus genetic dataset and conducting individual-based, population-level, and cline-based analyses. Then, motivated by results from the genetic analyses, we use simulations to explore alternative hypotheses about the forces governing this hybrid zone, and to extend the relevance of our results, we compare our findings to those found for other hybrid zone systems. Given previous results from similar studies in this system (Phillips et al. 2004) and the expectation that postzygotic isolation scales with genetic divergence (Coyne and Orr 1997), we predict substantial levels of genetic disequilibrium and narrow genetic clines in the present system.

Methods

SAMPLING

Based on the initial characterization (Bell et al. 2010) and subsequent sampling, we identified the contact zone between the Northern and Southern lineages. From 2008 to 2010, we sampled the contact extensively along a linear transect running through the contact zone (the Gillies Transect) and, to provide some level of replication, opportunistically around a nearby lake (Lake Barrine) bisected by the contact zone (Fig. 1; Table S1). Although each of these regions are forested, the area between them was cleared in the early twentieth century, preventing further sampling. Animals were captured by hand, sampled for tail tissue, measured, and sexed, geo-referenced, and then released at the site of capture. For the linear transect, we sampled 17 localities (average of 17 individuals per locality) over 2.5 km. At Lake Barrine, we sampled 58 animals at 28 unique locations. Additionally, we sampled two localities (17–18 individuals per locality) at 2.5 km on either side of the hybrid zone center to determine whether any of the alleles showed introgression outside of the central hybrid zone. Finally, from each lineage, we sampled one locality (12 individuals) about 40 km away from the hybrid zone center. These latter individuals were sampled for liver tissue and were collected as voucher specimens to be accessioned at the Museum of Vertebrate Zoology, Berkeley, California. As these localities are geographically isolated (hereafter, "allopatric") from the hybrid zone center, they are unlikely to contain alleles introgressed from the hybrid zone center, and thus, we used them for marker development.

MARKER DEVELOPMENT

To assay hybridization and introgression in the contact zone, we used one mitochondrial locus and 10 nuclear loci. We used

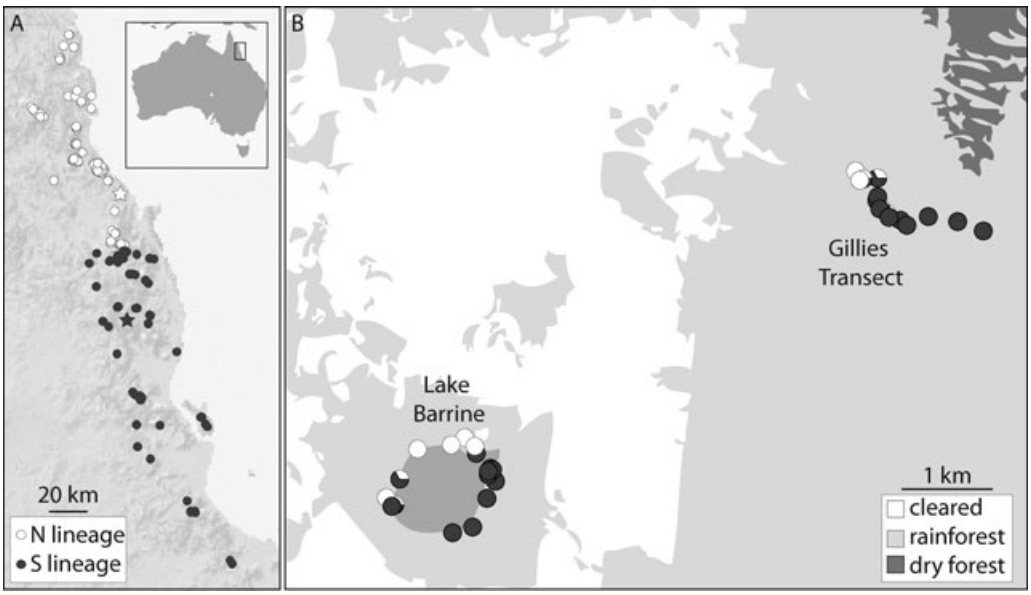

Figure 1. A. Range of *L. coggeri* with major lineages identified; localities used for marker development (≈40 km away from the hybrid zone center) are shown by stars. B. Close-up of contact zone, showing sampling points and present land cover.

previously published primers to amplify the mitochondrial locus ND4 (Arevalo et al. 1994) and three nuclear loci: β-globin (Dolman and Phillips 2004), LC5, and LC17 (Bell et al. 2010). To design additional markers, we developed markers based on data from a high-throughput sequencing run. Briefly, we extracted total RNA from five individuals from each of our Northern and Southern allopatric localities, pooled equimolar amounts of individual RNA for each locality, isolated mRNA, and prepared a sequencing library as directed by Illumina (Bentley et al. 2006). Each of the two resulting libraries was sequenced at one lane on an Illumina Genome Analyser II (Bentley et al. 2006). Resulting reads were trimmed for quality and for adapter sequence and assembled using the de novo assembler ABySS; contigs were annotated using a custom vertebrate gene database compiled from Ensembl (Birney et al. 2003; Biron et al. 2009). To find fixed differences between the two lineages, we mapped the trimmed reads from the Northern and Southern localities to the Northern reference assembly using *bwa* (Li and Durbin 2009). Resulting single nucleotide polymorphism (SNP) calls were parsed using *samtools* (Li et al. 2009), and we identified SNPs that were fixed in the lineages for different alleles. We then identified a subset of SNPs that were in annotated genes, that were either noncoding (i.e., located in the 5′ or 3′ untranslated region) or resulted in synonymous amino acid changes, and that could be resolved using commonly available and robust restriction enzymes. We used Primer3 (Rozen and Skaletsky 2000) to design primers for 12 of these SNPs and Sanger-sequenced these alleles in a larger sample from the Northern and Southern allopatric localities (12 individuals each) to confirm these SNPs were fixed or nearly fixed between the localities. We successfully sequenced 11 loci and selected for further analysis the seven most robust loci (*ABHD5*, *AUTO*, *NDST2*, *LEMD2*, *PCBD1*, *RTN3*, and *SAR1*). The bioinformatics pipeline to develop these markers was written in Perl, available at https://sites.google.com/site/mvzseq/.

COLLECTION OF GENETIC DATA

We extracted genomic DNA from tail tissue using a high-salt method (Aljanabi and Martinez 1997) and confirmed DNA quality and quantity using a Nanodrop. To amplify DNA, we used standard polymerase chain reaction (PCR) conditions in a 15 μl reaction. Each of the 11 markers contained a diagnostic SNP that was fixed or nearly fixed between the two lineages and that could be genotyped using PCR-RFLP (restriction fragment length polymorphism). To genotype loci, we digested 10 μl of the amplified product in a 25 μl reaction with one unit of the appropriate enzyme, following manufacturer's suggestions for use (NEB). We visualized the digested products on a 1.5% agarose gel stained with ethidium bromide and scored genotypes manually. Details on primer sequences, annealing temperatures, enzymes used, and restriction patterns can be found in Table S2.

ANALYSES

We conducted four types of analysis. First, we determined hybrid composition using individual-based methods, as the genetic makeup of hybrids can reflect the nature of selection against different hybrid classes (Wiley et al. 2009). Second, we fit clines to our

data to measure introgression extent and to determine whether clines were concordant and coincident, as we expect greater introgression extent and lack of coincidence and concordance if there are no barriers to gene flow. Third, we estimated population parameters of disequilibrium, as disequilibrium only persists after secondary contact if there is selection against hybrids or strong assortative mating (Barton and Gale 1993). Fourth, we combined estimates of cline width and linkage disequilibrium (LD) to infer dispersal and selection in the hybrid zone given a tension zone model. We were unable to obtain sufficient samples at Lake Barrine to enable cline-based analyses; thus, these samples were only included in the individual-based analyses and disequilibrium measures.

First, to determine the composition and type of hybrids in the contact zone, we used two programs, Structure (Pritchard et al. 2000) and NewHybrids (Anderson and Thompson 2002). For both programs, we used genotypic data from all 10 nuclear loci for all 406 individuals sampled. Using a Bayesian approach, Structure estimates the probability that an individual belongs to a genetic cluster by minimizing linkage and Hardy–Weinberg (HW) disequilibrium within a cluster (K). We ran Structure 10 times under the "admixture" model for each of nine K values (ranging from two to 10), recording posterior probability distributions for admixture proportion. We then determined the best-supported K value following Evanno et al. (2005) as implemented in StructureHarvester (Earl 2011), summarized results across that K value using Clumpp (Jakobsson and Rosenberg 2007), and plotted results using Distruct (Rosenberg 2004). NewHybrids also implements a Bayesian approach to determine the probability that an individual belongs to one of the six genotypic classes that results from the first two generations of crossing (i.e., either parental form, F1 hybrid, F2 hybrid, or first-generation backcross). We ran NewHybrids five times and summarized runs by averaging probabilities across runs.

To fit clines to our data, we first pooled our unique sampling points along the transect into localities. On average, the area around pooled points (as measured by minimum convex polygons) was 297 m^2, with areas ranging from 3 to 1498 m^2. We then calculated the location of the pooled points along the transect by collapsing the points to a one-dimensional (1D) transect; because our sampling regime followed a linear transect, the results were nearly identical to the original sampling points. We then fit three models of clines to our data via the maximum-likelihood framework implemented in Analyse (Barton and Baird 1998). First, we fit a basic two-parameter sigmoidal model (Sig) which describes the transition in allele frequency through space (p) with respect to cline center (c) and width (w) as:

$$p = \frac{1 + \tanh\left[\frac{2(x-c)}{w}\right]}{2}. \quad (1)$$

Here, cline width is the inverse of the maximum slope of the curve. Sigmoidal clines do not necessarily evoke a specific selection model, and thus, can be used to describe a frequency change in any trait. Second, we fit a four-parameter stepped model (Step) in which the center of the cline is described by the sigmoidal model and the tails of the cline are described by the parameter B and the exponential decay function (Szymura and Barton 1986):

$$p \propto \exp\left(\frac{-4x\theta^{\frac{1}{2}}}{w}\right). \quad (2)$$

The stepped model is appropriate for multitrait data; it allows for a sharp change in frequency at the center of cline, as might be seen due to epistatic interactions. In the tails of the cline, where recombination has broken down epistasis and selection is accordingly weaker, introgression occurs more quickly and clines are shallower (Barton 1983). The parameter θ reflects the strength of selection against the character outside of the cline center, and B describes the size of the tails, or the proportion of alleles that are introgressing. Finally, we fit a special case of the stepped model, the six-parameter asymmetric stepped model (Astep), in which either side of the cline has different introgression extent (i.e., θ and B are fit separately to either side of the cline). This model describes scenarios in which introgression is greater into one lineage than the other. In each of these three models, we allowed $p_{\min}$ and $p_{\max}$ to vary at either end of the cline. Each of these models is nested within each other; thus, to determine whether more complex models fit the data better, we calculated twice the difference of log likelihoods and found significance by comparing to the critical value. Clines were fit to each of the 11 loci and a composite multilocus hybrid index obtained from the Structure results. After fitting the clines, we determined whether clines were coincident (i.e., sharing the same center) and concordant (i.e., sharing the same width). We would expect coincidence if the hybrid zone is recent or if selection is strong and concordance if selection strength is uniform across loci. Following Phillips et al. (2004), we constructed log-likelihood profiles for each locus over a range of center (c) and width (w) values. We then calculated two likelihood values: (1) for the noncoincident model, we summed the locus-specific maximum-likelihood values and (2) for the coincident model, we summed the c log-likelihood profiles over all loci to find the maximum-likelihood value for the loci's shared center. To determine whether noncoincidence fit the data better than coincidence, we determined if the difference in likelihood between the two models was significant using a χ^2 distribution (df = 10, or one less than the number of loci). The same approach with cline widths was used to determine cline concordance.

To calculate within-locus and between-locus disequilibrium, we also used Analyse. Within-locus disequilibrium, or

HW disequilibrium, results when the proportion of heterozygotes at a locus deviates from the expected proportion under random mating. We calculated HW disequilibrium by estimating maximum-likelihood values for F_{IS} across all nuclear loci and across all sites. Analyse calculates between-locus disequilibria, or LD, as:

$$R_{ij} = \frac{D_{ij}}{\sqrt{p_i q_i p_j q_j}}. \quad (3)$$

Because the magnitude of LD relies, in part, on the allele frequencies at the loci under consideration, this method reduces this dependency by dividing the estimate of LD by the square root of the product of the allele frequencies at the loci (Barton and Gale 1993). To calculate LD, two challenges arise: first, how to account for within-locus disequilibrium as it can affect measures of LD, and second, how to estimate multilocus disequilibrium properly when pairwise measures of disequilibria are not independent. Analyse addresses the first issue by accounting for within-locus disequilibria by downsizing the effective sample size of a population, as each allele sampled does not reflect a unique datapoint when there is within-locus disequilibrium. This method does not, however, address the second issue; it assumes that pairwise LD estimates are independent. Other multilocus LD methods, which allow dependency, are not appropriate here. Barton's (2000) method for estimating multilocus disequilibria cannot handle a dataset of this size, and Barton and Gale's (1993) method for estimating disequilibria by hybrid index assumes no within-locus disequilibrium.

Assuming a tension zone model (i.e., that all clines are in migration–selection equilibrium), estimates of cline width and LD can be used to estimate dispersal in the hybrid zone as represented by σ, the variance in position between offspring and parents (Barton and Gale 1993). If selection in the hybrid zone is weak, σ is given by:

$$R_{ij} = \frac{4\sigma^2}{w^2 r}, \quad (4)$$

where r is the recombination rate between loci; we assume no linkage between markers and thus take r to be 0.5. Here, R_{ij} is calculated at the center of the zone, where it is predicted to be the largest. We calculated average LD at each of the four center localities in the hybrid zone and averaged these estimates to derive a value for R_{ij}. As our clines were not concordant, we calculated σ for the range of observed widths we saw, the mean width, and the width of the composite cline based on hybrid index. Once σ has been estimated, we can estimate selection against heterozygotes by using the equation:

$$s^* = 8\left(\frac{\sigma}{w}\right)^2, \quad (5)$$

where s^* is a measure of effective selection on a locus, which reflects both selection acting directly on the locus and on loci in disequilibrium with the locus (Barton and Gale 1993).

For these analyses, we used R and the package "ggplot2" to conduct all mathematical and statistical operations and to make all graphs (Wickham 2009; R Development Core Team 2011).

SIMULATIONS

Similar patterns of cline width and genetic disequilibrium in hybrid zones can emerge from very different biological realities; in fact, it can be notoriously challenging to distinguish between competing hypotheses for hybrid zone maintenance (Harrison 1986). Thus, to place our genetic results in context of hybrid zone models, we simulated secondary contact between two isolated lineages using the forward-time simulation program simuPOP (Peng and Kimmal 2005). Although models for hybrid zones are abundant in the literature and have contributed greatly to our understanding of hybrid zone dynamics (Baird 1995; Kruuk et al. 1999; Durrett et al. 2000), few incorporate assortative mating and selection against hybrids in a multilocus framework as we do here. For this model, we implement a 1D chain of 60 populations; the two lineages occupy either side of the chain at time zero (Fig. S4). At time zero, the populations begin exchanging migrants under a stepping-stone model of migration. Under a 1D stepping stone model, the proportion of migrants is related to σ by $\sigma^2 = m\epsilon^2$, where ϵ is the distance between demes. Cline width is thus a dimensionless value measured in deme number; converting to distance to compare to empirical systems necessitates an estimate of ϵ. Selection against hybrids was defined by a multiplicative selection model, where fitness was dependent on the number of loci at which an individual was heterozygous. We allowed strength of assortative mating to vary from random mating to nearly complete associative mating. In the model of assortative mating used here (a multilocus model based on Felsenstein's [1981] "group-based model") , some proportion of individuals (α) mate preferentially with any individuals who share at least 90% or more of their ancestry, and the remaining individuals (1−α) mate randomly. We simulated clines under a range of scenarios, including varying the number of loci under selection (2, 5, 10), the strength of selection (0, 0.2, 0.4, 0.6, 0.8, 0.9, 0.95, 0.99), the strength of assortative mating (0, 0.2, 0.4, 0.6, 0.8), and migration rates (0.1, 0.3, 0.5). Population sizes ($N = 1000$) and recombination rates ($r = 0.5$) were kept constant across simulations, and in each simulation, we modeled 10 neutral loci to look at introgression patterns under neutrality. Simulations were run for 1000 generations as preliminary runs suggested that, where relevant, this was sufficient for runs to reach equilibrium. We recorded data from the simulations every 100 generations, which included estimates of cline width and center (as calculated by Kruuk et al. 1999), per locus HW disequilibrium, LD, and hybrid indices. We

simulated 1000 datasets for each parameter combination. Supporting information contains more information about model choice and parameterization (Table S4 and Fig. S4).

Results

IDENTIFICATION OF THE CONTACT ZONE AND DEVELOPMENT OF MARKER LOCI

We located the zone of secondary contact at two places, along a road running through both lineages along the Gillies Range (Gillies Transect) and around Lake Barrine. The habitat is continuous through the contact zone, with no major transitions in habitat types, and *L. coggeri* are abundant throughout. This contact zone is near several other contact zones in the AWT suture zone; in particular, Lake Barrine is home to an admixed population of two frog species *Austrochaperina robusta/fryi*, and the previously described contact in the skink *Carlia rubrigularis* is located 15 km northward (Phillips et al. 2004; Moritz et al. 2009).

High-throughput sequencing of the pooled libraries from the allopatric localities resulted in the identification of over 20,000 putatively fixed and annotated SNPs between the two lineages. Sanger-sequencing data of a subset of these SNPs and their surrounding regions from allopatric (c. 40 km distant samples; $N = 12$ each) localities confirmed that all were fixed or nearly fixed, although other variants close to the target SNPs ($\pm$200 bp) showed incomplete lineage sorting.

Our mitochondrial locus and seven of the 10 nuclear SNPs were completely fixed between allopatric localities of the two lineages. SNPs located in three loci (*PCBD1*, *LC17*, and *ABHD5*) showed incomplete fixation when comparing the Northern and Southern allopatric localities, with F_{ST} estimates of 0.92, 0.75, and 0.96, respectively. Although this incomplete fixation could be due to introgression from the contact zone, this is unlikely as these localities are geographically disjunct from the hybrid zone.

In total for the contact zone, we genotyped all 10 nuclear and one mitochondrial markers in 406 lizards, 348 from the Gillies transect and 58 from Lake Barrine. These data are available at Dryad at doi:10.5061/dryad.4gh6hf5g

ESTIMATES OF HYBRID FREQUENCY AND COMPOSITION

Results from the individual-based Structure analyses showed that the individuals at the contact zone best fit a two-population model, where each parental lineage is a genetic cluster and hybrids are the result of admixture between these clusters. At the Gillies Transect, population assignment tests showed that the majority of hybrids (45 out of 63; 71%) were limited mostly to just four geographically close localities, spanning from 1041 to 1151 m along the transect. Here, hybrids are defined as any individual that has an admixture proportion of ≥ 0.10, and for whom the posterior probability of admixture proportion does not include 0 or 1. Of the 81 individuals in these four central localities, 45 (or 55%) were hybrids, of which 65% and 35% had Northern and Southern mitochondrial types, respectively. As shown in Figure 2B, the admixture proportion (i.e., hybrid index) in the hybrid zone center (localities at 1041 to 1151 m) spans a wide range, a pattern that has been described as flatly uniform (Jiggins and Mallet 2000), as opposed to unimodal or bimodal patterns. Outside of these four localities, only 18 additional lizards (5% of genotyped lizards) were identified as hybrids, and the two localities located 2.5 km from the hybrid zone center showed no sign of introgression (Fig. 2A). Thus, introgression beyond the hybrid zone center, as estimated from these markers, appears very rare.

Using NewHybrids to assign individuals to hybrid class showed that the hybrid zone center contained no F1s, 16 parents from the Northern lineage (20%), and 12 parents from the Southern lineage (15%) (Fig. S1). Delineating between F2s, first-generation backcrosses, and older backcrosses confidently is challenging (Pereira and Wake 2009); thus, we do not categorize the 53 hybrids further.

The pattern seen along the Gillies transect was matched at Lake Barrine (Fig. 1B). Of the 58 individuals genotyped there, 16 (27%) were hybrids, and none could be confidently classified as F1s. These hybrids were all found in two narrow bands on either side of the lake, each measuring about 300 m in width (Fig. S2).

ESTIMATES OF CLINE SHAPE

All clines were best fit by the sigmoidal model; whereas some markers showed a marginally better fit under the stepped or asymmetric stepped model, the improvement over the sigmoidal model was insignificant. We report and discuss the results from the sigmoidal clines only. Clines were exceptionally narrow—average cline width was 403 m, and widths ranged from 280 m (*LC5*) to 695 m (*LEMD2*) (Fig. 3; Table 1). The mitochondrial cline and the composite hybrid index cline were both narrower than average; they had widths of 300 m and 370 m, respectively. For most clines, $p_{\min}$ and $p_{\max}$ were 0 and 1, respectively; however, the three loci for which fixation between allopatric samples was incomplete had, as expected, nonzero $p_{\min}$ (Table 1).

Visual inspection of the clines showed that they had coincident centers; all centers were within 100 m of each other. A formal test of cline coincidence confirmed this observation ($\chi^2 = 7.26$, df $= 10$, $P = 0.70$); the best-fitting center was located 1.18 km from the start of the transect, at the southern edge of the four localities at the core of the hybrid zone. Yet, the clines were not concordant; allowing cline width to vary across loci fit the data significantly better than constraining clines to the same width ($\chi^2 = 184.8$, df $= 10$, $P < 0.05$). Although the clines are not concordant, their widths fall within a narrow range. Most cline widths are

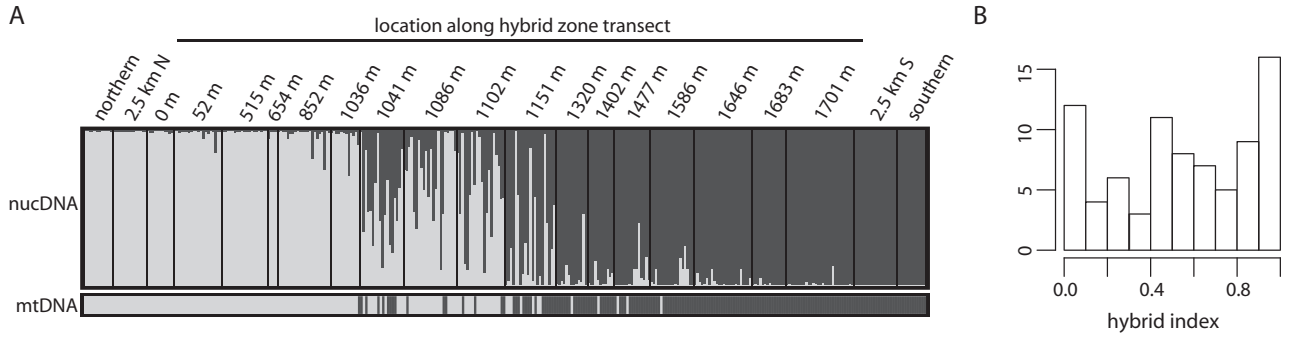

Figure 2. **A. Structure results for all individuals in the Gillies transect, showing the point estimate for admixture coefficient. Individuals are arranged north to south. B. Histogram of point estimates of hybrid index for individuals at the center of the hybrid zone.**

within 400 ±100 m, which, given that $\sigma \approx 80\frac{m}{\sqrt{gen}}$ (see below), is a relatively small deviation from concordance.

ESTIMATES OF DISEQUILIBRIUM

Estimates of F_{IS} and R_{ij} showed that both within-locus and between-loci disequilibrium is substantial in the hybrid zone. In most localities, power to measure within-locus disequilibrium was limited as many localities only had one allele at each locus. However, six localities showed significant departures from Hardy–Weinberg equilibrium (HWE) at one or more loci. The four localities at the center of the hybrid zone had across-loci F_{IS} values ranging from 0.239 to 0.453; per locus measures showed that almost all loci had significantly nonzero F_{IS} values (Fig. 4 A). Two localities away from the center of the hybrid zone showed deviations from HWE; per locus results indicated that departures

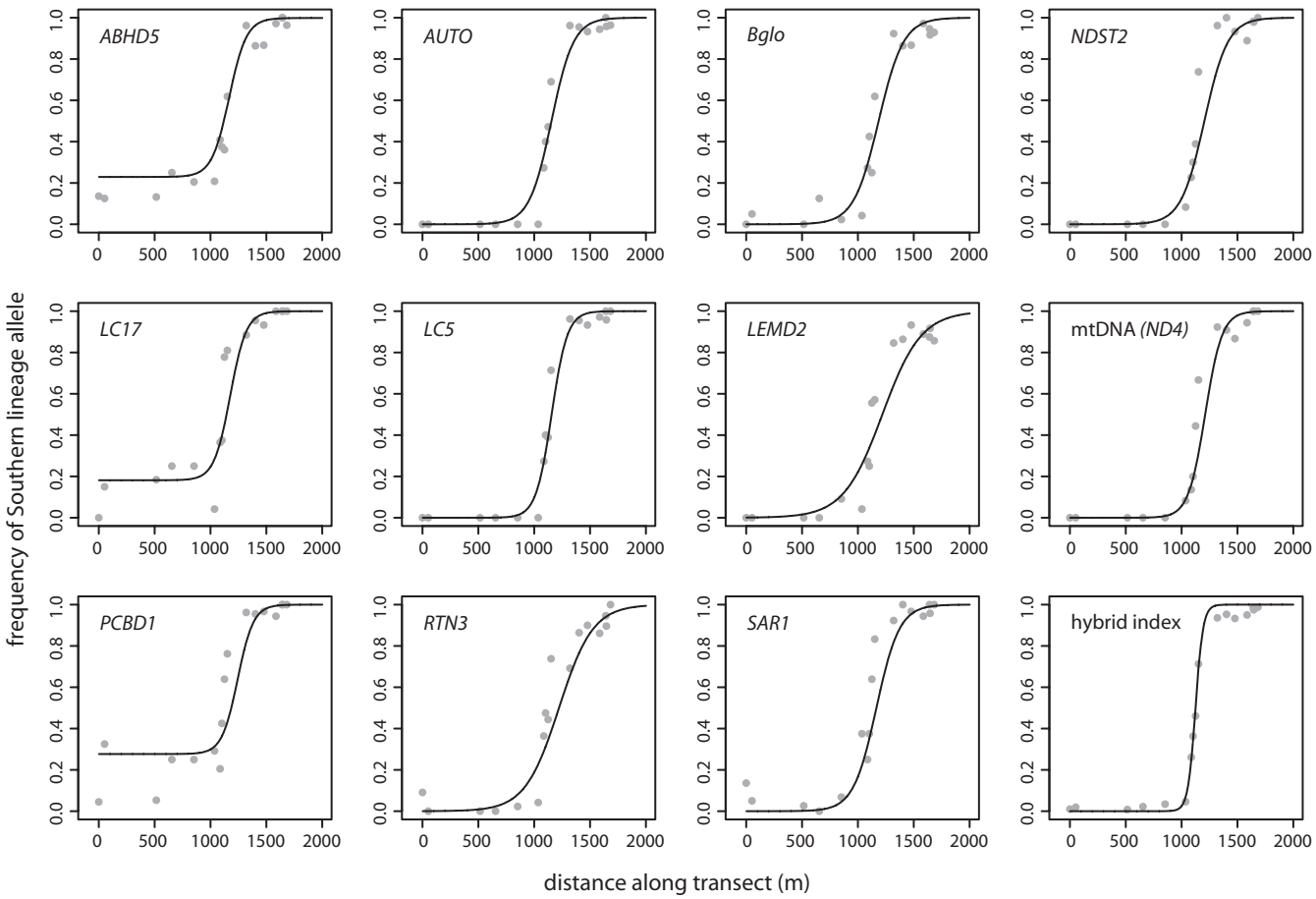

Figure 3. **Maximum-likelihood estimates for cline shape and location for 11 markers studied in this hybrid zone and the cline for our composite measure, hybrid index.**

Table 1. Summary of estimates of cline parameters with two-unit support limits shown in parentheses.

Locus	LnL	p_{min}	p_{max}	Width (w) in Meters	Center (c) in Meters
mtDNA (*ND*4)	−4.469	0	1	300 (223–416)	1211 (1165–1266)
Hybrid index	−3.21	0	1	370 (310–456)	1150 (1104–1216)
*ABHD*5	−1.952	0.23	1	311 (3–621)	1167 (1036–1313)
AUTO	−8.842	0	1	395 (234–464)	1155 (1118–1227)
Bglo	−4.07	0	1	444 (334–610)	1185.5 (1114–1249)
*LC*17	−0.786	0.18	1	288 (14–488)	1176.5 (1091–1312)
*LC*5	−2.764	0	1	280 (175–436)	1156 (1104–1215)
*LEMD*2	−3.624	0	1	696 (519–980)	1219 (1123–1290)
*NDST*2	−8.799	0	1	419 (312–562)	1204 (1143–1262)
*PCBD*1	−7.705	0.28	1	282 (2–372)	1243 (1094–1317)
*RTN*3	−2.619	0	1	607 (460–826)	1222 (1144–1293)
*SAR*1	−3.471	0	1	411 (316–573)	1165.5 (1106–1229)

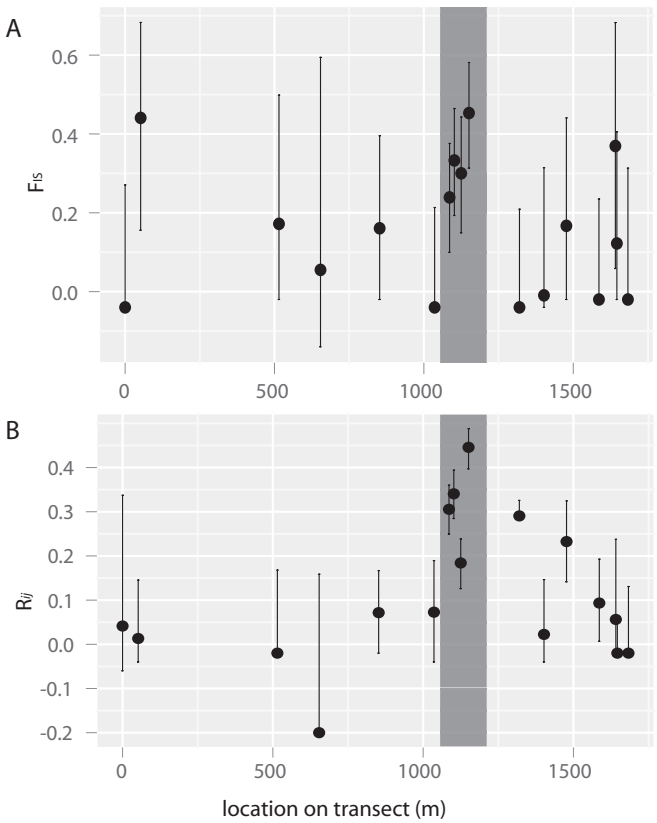

Figure 4. (A) Hardy–Weinberg disequilibrium F_{IS} measures and (B) linkage disequilibrium R_{ij} in localities along the linear transect. Darker gray box outlines the localities in the hybrid zone center.

from HWE at two of the incompletely sorted loci, *PCBD*1 and *LC*17, drove this pattern. Disequilibrium where the two lineages meet at Lake Barrine was similarly substantial; across-locus F_{IS} was estimated to be 0.408 (0.266–0.540).

Similarly, power to estimate LD was limited in many localities as most localities consisted of individuals homozygous at all loci. However, six localities had significant R_{ij}, all in or near the center of the hybrid zone (Fig. 4B). The four localities at the center of the hybrid zone all had significant, positive LD at nearly all between-locus comparisons; average LD across all loci at these localities ranged from 0.184 to 0.446. Again, disequilibrium at Lake Barrine was strong; almost all locus pairs had significant, positive LD and the multilocus estimate of LD was 0.490 (0.440–0.523).

ESTIMATES OF DISPERSAL RATE AND SELECTION

Assuming a tension zone model and that the hybrid zone is at migration–selection equilibrium, measures of cline width and LD can be used to estimate dispersal rate in the hybrid zone and selection against hybrids. These measures do not explicitly account for variation in cline width among characters; thus, we report here point estimates based on the width of our multilocus cline, as well as the range of values corresponding to the range of cline widths. Dispersal rate (here measured as σ) was estimated as 80 $\frac{m}{\sqrt{gen}}$ (40 − 160 $\frac{m}{\sqrt{gen}}$). Although estimating dispersal rate is fraught with assumptions, in particular that the system conforms to a tension zone model, our estimates correspond well to those from two closely related species. In the rainforest skink *C. rubrigularis* (Phillips et al. 2004), σ was estimated to range from 90 to 133 $\frac{m}{\sqrt{gen}}$ by using both the method described here and an F_{ST} based measure (Rousset 1997), and in the rainforest skink *Gnypetoscincus queenslandiae*, σ, as estimated via the Rousset (1997) method, was 29 $\frac{m}{\sqrt{gen}}$ (Sumner et al. 2001). Using our estimate of σ for *L. coggeri*, we estimate average effective selection (s^*) at a locus as 0.403 (0.106–0.653). The wide range of possible values for selection strength reflects both the challenge in measuring the various parameters of this composite measure and the variation of selection strength across loci. However, our estimates suggest that, if the equilibrium tension model is appropriate for this system, then selection against hybrids is substantial. If our system does not fit a tension zone model, then we are likely

over-estimating σ, in which case average effective selection would be less than we suggest here. However, we think that our estimate of σ is reasonable, as it overlaps with that for *C. rubrigularis*, a species with similar natural history and habitat use as *L. coggeri* (Williams et al. 2010).

RESULTS FROM HYBRID ZONE SIMULATIONS

Motivated by the finding of strong and ubiquitous genetic disequilibrium and narrow clines in this hybrid zone, we used simulations to explore how reproductive isolation (both prezygotic and postzygotic) versus neutral diffusion affects hybrid zone dynamics. Under models that ranged from random mating to nearly complete assortative mating and from neutral diffusion to nearly complete selection against hybrids, we simulated expected patterns of (1) hybrid composition, (2) cline width and concordance, and (3) disequilibrium. For ease of presentation, we discuss and show results from just one parameter set (migration rate = 0.3 and 10 loci under selection); results from other parameter combinations are qualitatively similar (Fig. S3). Several key conclusions emerge from these simulations. First, strength of assortative mating has no significant effect on any of the measured parameters; whereas assortative mating slows initial introgression and the decay of disequilibrium, the equilibrium outcomes are the same under strong assortative mating and random mating (Fig. S4). Second, under neutral diffusion (e.g., no selection against hybrids) and very soon after secondary contact (<50 generations under a range of demographic parameters), the system exhibits many of the same patterns one would see in a tension zone model—that is, narrow clines, high disequilibrium, and flatly uniform distribution of hybrid indices (Fig. 5). Beyond this point, disequilibrium is low and clines increasingly broad with gradually increasing variance in cline width. Third, patterns of disequilibrium and cline shape diverge between markers under selection and unlinked neutral markers rapidly after secondary contact (<50 generations) and only remain concordant when total selection against hybrids is strong (>90%; Fig. 5). In particular, assuming independent assortment between neutral and selected loci, maintaining narrow clines at neutral loci requires total selection against hybrids to be nearly complete (>95%). Fourth, continuing this theme, variance in cline width among neutral, unlinked loci increases rapidly after secondary contact, even under strong selection against hybrids. Although average cline width remains narrow at neutral loci when selection against hybrids is strong, some loci show stochastic behavior and wide clines, increasing variance. Seeing this variance, however, requires a large number of loci to be sampled. Finally, hybrid index tends toward unimodality, unless contact is recent or there is strong selection against hybrids (>90%), in which case it is bimodal to flatly uniform. Further, in all these cases, finding F1s in the hybrid zone is rare. These results suggest that our pattern of narrow, fairly concordant clines, and high disequilibrium is likely the result of very recent secondary contact (e.g., <50 generations) or strong selection against hybrids.

Discussion

In this article, we show that the major lineages of *L. coggeri* meet in an extremely narrow hybrid zone, which we can describe further as:

(1) evincing clines of average width 403 m in a species with dispersal rate of approximately 80 $\frac{m}{\sqrt{gen}}$,
(2) consisting of coincident clines, which while not concordant, show low variance in cline width,
(3) showing substantial LD and HW disequilibrium at the center of the zone at nearly every marker,
(4) having a flatly uniform distribution of hybrid index with no F1 hybrids, and
(5) exhibiting the same general patterns of limited introgression and extensive disequilibrium at a second independent sampling site, Lake Barrine.

Narrow hybrid zones can emerge due to several processes (Moore 1977). Here, we describe what we consider the four dominant causes, noting that these are not mutually exclusive. First, as a null hypothesis, a narrow hybrid zone can result from neutral diffusion after secondary contact between previously isolated lineages (Endler 1977). If there are no barriers to gene flow between the lineages, the two lineages will eventually become genetically and phenotypically indistinguishable. Prior to this equilibrium, however, the system will exhibit clines, the width of which are a function of dispersal length and time since contact (Barton and Gale 1993). Second, narrow clines can result from selection against heterozygotes or, more generally, hybrids under the environmentally independent "tension zone" model (Bazykin 1969; Szymura and Barton 1986). Here, clines are stable at the equilibrium between parental dispersal into the hybrid zone and selection against resulting hybrids, and selection is independent of the environment though the clines often cluster in areas of low environment suitability (Szymura and Barton 1986). Third, narrow clines can result due to assortative mating between parental forms, whether due to active mate choice (e.g., *Heliconius* butterflies [Mallet et al. 1990]) or habitat selection (e.g., in *Bombina* toads [Maccallum et al. 1995]). Finally, narrow clines can form at the edge of an ecotone between two distinct environments, when populations on either side of the ecotone are differentially adapted to these conditions (Endler 1977). Here, we explore these possible explanations for the *L. coggeri* hybrid zone by considering their predictions in light of our data and simulation results.

Although environment-dependent selection is certainly important in shaping numerous hybrid zones (e.g., in *Iris* flowers [Cruzan and Arnold 1994] and in *Colaptes* birds

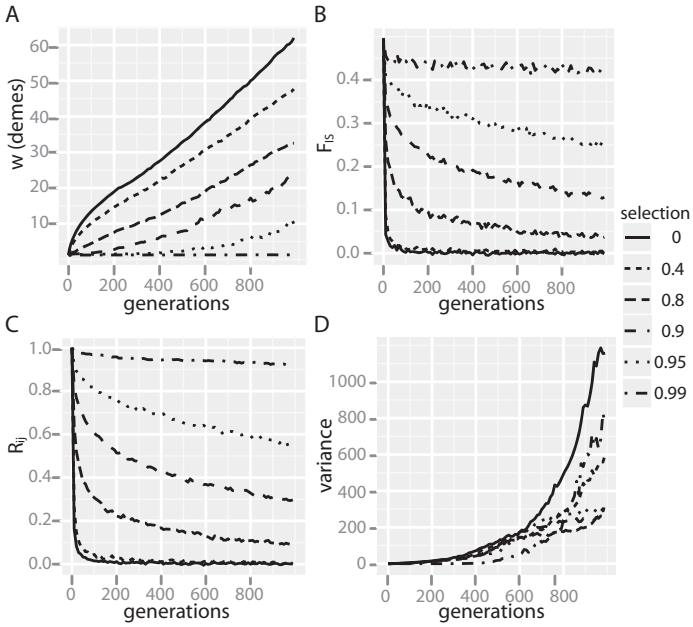

Figure 5. Results from the simulations of secondary contact, (A) Cline width (in demes), (B) F_{IS}, and (C) R_{ij} at neutral loci for a range of values for selection against hybrids. Shown for migration rate of 0.3, with 10 loci under selection, and random mating.

[Moore and Price 1993]), it likely not a factor contributing to this hybrid zone. Bioclimatic analysis of the suture zone relative to adjacent refugial (source) areas indicated the former has relatively low suitability, but also that the parental lineages are from analogous rainforest habitats (Moritz et al. 2009). As there is no noticeable difference between parental habitats or in ecophenotypes (Bell et al. 2010) and this region of low suitability is much broader and more subtle than one would expect for such a narrow hybrid zone, we think environmental selection is unlikely to contribute significantly here. Distinguishing between the remaining three explanations is notoriously difficult (Arnold 1997), but it is key if we want to use hybrid zones to understand speciation better. After all, by understanding what maintains a hybrid zone, we can understand what barriers have evolved to gene flow, and thus, what factors are contributing to lineage maintenance.

If the system is described by neutral diffusion, then cline width is given by $w = \sqrt{2\pi}\sigma\sqrt{t}$, where w is cline width and t is the time (in generations) since secondary contact (Endler 1977). We do not have direct estimates of t for this system, but we use what we know about the system to evaluate the plausibility of this nonequilibrium model. First, paleomodels suggest the rainforest expanded from glacial refugia sometime 3–8 Kya (VanDerWal et al. 2009), and as *L. coggeri* is found at rainforest edges and gaps, it likely tracked this expansion closely. Assuming we estimated σ correctly and assuming a conservative three years/gen, then under neutral diffusion, we would expect clines to be (at minimum) 6.3-km wide, nearly 15 times wider than our average cline width. To get clines as narrow as those measured here given this estimate of time since secondary contact, σ would need to be just $14\ \frac{m}{\sqrt{gen}}$. This estimate of dispersal rate is nearly an order of magnitude lower than that estimated in the closely related and ecologically similar species, *C. rubrigularis* (Phillips et al. 2004). Further, *L.coggeri* is a gap-edge species such that population densities are naturally dynamic in space, and thus based on natural history, this estimate is too low to be realistic (Williams et al. 2010). Moreover, under this scenario, our simulations show that we would not expect to see extensive disequilibrium and cline coincidence as we do. However, it is possible that we have wrongly inferred time since secondary contact, and secondary contact is more recent. Given the width of our multilocus and mitochondrial clines and using a range of dispersal rates for a closely related lizard species (*C. rubrigularis*; $90 - 133\ \frac{m}{\sqrt{gen}}$), we estimate time since secondary contact as 0.81–2.70 generations, or two to eight years given a conservative generation time of three years. Our simulations suggest that in the early stages of neutral diffusion (<50 generations), we can expect to recover all the patterns of hybridization seen in this contact zone (i.e., high disequilibrium, sharply narrow and concordant clines, a flatly uniform hybrid distribution; Fig. 5). However, it seems unlikely that the lineages met for the first time in just the last 10 years. It is more likely that the lineages met in the past, and environmental change, whether natural or human-induced, have affected the hybrid zone such that the clock for time since secondary contact has been reset (Patton 1993). However, even in this scenario, the lineages would have been previously in secondary contact, during which there

would have been ample time for broad-scale introgression, which is noticeably absent. In conclusion, although neutral diffusion can result in the patterns of introgression and disequilibrium we see here, the timing of secondary contact and dispersal rate necessary to generate such patterns are unrealistic based on our knowledge about this system.

Tension zones can produce the same patterns as well (Fig. 5), and most systems that exhibit similar patterns as the *L. coggeri* zone tend to be defined as tension zones (e.g., the *Vandiemenella* grasshoppers [Kawakami et al. 2009] and the *Carlia* lizards [Phillips et al. 2004]). However, these and our systems do not fit the tension zone model in one important way; even when hybrids are under strong selection, introgression patterns are expected to be uneven across loci. Introgression extent is inversely proportional to effective selection strength, which is both a function of direct selection on a locus and selection on any loci in LD with the focal locus (Barton 1983). For neutral loci, which experience no direct selection, theory suggests that indirect selection can retard introgression (Barton 1983). But, as theory further suggests and as our simulations show, recombination breaks down LD between neutral loci and loci under selection, over time increasing extent of introgression and variance in cline width (Barton 1979, Fig. 5) . The speed at which this occurs depends on the strength of selection relative to the rate of recombination between the neutral and selected loci (Baird 1995). Under equilibrium, we would thus expect to see a wide range of cline widths; however, in our study, much like most other studies that have measured cline width, the range of cline widths is fairly limited (Table 2).

In the context of the tension zone model, this pattern of limited introgression has three possible roots. First, it is possible all our markers are under strong direct selection. Others have argued this is likely when researchers chose to assay introgression extent using diagnostic markers, which have possibly become fixed due to divergent natural selection (Yuri et al. 2009). However, in this system, we see that even our markers with incomplete lineage sorting have narrow clines. Second, as theory and our simulations suggest (Gavrilets 1997), if total selection against hybrids is great ($>95\%$), then introgression at neutral alleles, even if unlinked to loci under selection, will be greatly reduced. Although we measured strong effective selection at individual loci (average $>40\%$) in this system, we cannot easily convert this to total selection strength as this measure is dependent on the number of loci under selection and the recombination rates between these loci. Finally, it is possible that selection is strong and so widely dispersed over the genome that every marker, even if neutral, is closely linked to multiple markers under selection (Barton 1983). In such systems, multilocus clines can be narrower than predicted by the direct selection each locus is experiencing, and neutral loci can be slow to introgress past the cline (Baird 1995). Testing pervasive selection as a possible mechanism for narrow clines at neutral loci would require investigating introgression at more loci, ideally in context of their genomic location.

Finally, assortative mating has been invoked in several hybrid zones as maintaining lineage boundaries (Saetre et al. 1997; Haas et al. 2010). Theoretical studies have shown that assortative mating can limit introgression at neutral loci (Gavrilets and Cruzan 1998; Moore 2008; M'Gonigle and FitzJohn 2009), but this result depends on the model used for assortative mating as all these models used a one-locus or two-locus model for mating traits and preference (for a counterexample where assortative mating has little effect on outcomes, see Sadedin and Littlejohn (2003). When using a multilocus model (appropriate for quantitative traits) and group-based mating as done here, assortative mating does little to limit introgression further. Thus, although assortative mating could potentially solidify lineage boundaries through reinforcement (e.g., the *Litoria serrata* frog [Hoskin et al. 2005]), we think assortative mating is unlikely to be an important force structuring this hybrid zone. However, to test this conclusively, we would need to do mate choice experiments for the lineages meeting in the hybrid zone.

Of all the possible forces structuring this hybrid zone, it seems most likely that there is strong selection against hybrids. It is, of course, possible both that secondary contact has been recent and that selection is strong, such that this hybrid zone is not yet at equilibrium. In the case of nonequilibrium, using cline width to infer selection will over-estimate selection strength. In particular, when selection is pervasive across a genome, approach to equilibrium can be slowed down by multilocus effects (Baird 1995). If so, we would expect to see a limited range of cline widths as we see here (Table 1). Ultimately, to disentangle how recency of contact and selection against hybrids are contributing to hybrid zone dynamics, we need to (1) collect data on hybrid viability and fitness based on experimental crosses or field-based studies and/or (2) look at distributions of the length of the introgressed blocks to estimate the age of the contact (Pool and Nielsen 2009). Upon introgressing into the foreign population, chromosomes will be whole, but with time, recombination will break up the introgressed chromosome into small blocks of introgression. As such, even with strong selection, blocks should be much smaller in older contacts than in recent contacts (Baird 1995) and, given estimates of the population recombination rate, should enable us to determine the age of these contacts.

Data from this hybrid zone suggest that selection against hybrids is key in maintaining narrow clines. Very few studies have investigated contact zones between morphologically cryptic lineages; most analyzed hybrid zones are between phenotypically distinct lineages or chromosomally distinct races (Table 2). The few other studies of contact zones between morphologically cryptic lineages have shown a range of outcomes—from neutral diffusion (*Chioglossa lusitanica* salamander; [Sequeira et al. 2005])

Table 2. A summary of minimum and maximum widths for studies measuring clines. All studies found by doing a search for ("hybrid zone" or hybridization) and clin*) in Web of Knowledge on 19 April 2011. Only studies that measured clines using a sigmoidal or stepped model and that assayed three or more loci were included. Coefficient of variation is taken for the square of the widths, as $s^* \propto \frac{1}{w^2}$.

Citation	Species	Taxa	Minimum width (km)	Maximum width (km)	Coefficient of variation	Number of loci	s^*
Dasmahapatra et al. (2002)	*Anaratia fatima* and *amathea*	Butterfly	26	28	0.06	4	–
Sites et al. (1995)	*Sceloperous grammicus* (chromosomal races)	Lizard	0.87	1.29	0.26	3	0.3
Porter et al. (1997)	*Pontia daplidice* and *edusa*	Butterfly	18.95	25.15	0.29	4	0.5
Szymura and Barton (1991)	*Bombina bombina* and *variegata*	Toad	5	7.92	0.3	6	0.22
Alexandrino et al. (2005)	*E. eschscholtzii xanthoptica* and *E. e. platensis*	Salamander	0.5	0.9	0.43	9	0.46–0.75
Brumfield et al. (2001)	*Manacus candei* and *vitellinus*	Bird	3.9	11	0.56	4	–
Buno et al. (1994)	*C.p. erythropus* and *C.p.parallelus*	Grasshopper	33.02	55.82	0.6	3	–
Yanchukov et al. (2006)	*Bombina bombina* and *variegata*	Toad	0.86	4.25	0.66	8	–
This study	*L. coggeri* (C and S lineages)	Lizard	0.28	0.696	0.73	11	0.11–0.65
Machalon et al. (2007)	*Mus musculus* and *domesticus*	Mouse	6.43	18.07	0.84	6	0.056–0.090
Carling and Brumfield (2008)	*Passerina cyanea* and *amoena*	Bird	175	523	0.87	7	–
Gay et al. (2008)	*Larus glaucescens* and *occidentalis*	Bird	400	1140	0.93	7	–
Mettler and Spellman (2009)	*Pheucticus melanocephalus* and *ludovicianus*	Bird	82	356	0.99	4	–
Carling and Brumfield (2009)	*Passerina cyanea* and *amoena*	Bird	2.8	584	1.11	10	–
Phillips et al. (2004)	*Carlia rubrigularis* (N and S lineages)	Lizard	0.45	2.24	1.15	4	0.50–0.70
Kawakami et al. (2009)	*Vandiemenella viatica* (chromosomal races)	Grasshopper	0.093	0.347	1.89	12	0.197
Teeter et al. (2008)	*Mus musculus* and *domesticus*	Mouse	6.5	341	2.41	38	–
Dufkova et al. (2011)	*Mus musculus* and *domesticu*	Mouse	0.23	16.76	2.95	13	0.25

to narrow and stable clines (*C. rubrigularis* lizard; [Phillips et al. 2004] and *Lacerta schreiberi* lizard; [Stuart-Fox et al. 2009]). Such studies are still too few to draw broad conclusions about the nature of hybrid zones between phylogeographic isolates. Our results are striking, both in comparison to these studies and other systems (Table 2), in how narrow the clines are, and certainly suggest that reproductive isolation can evolve without overt morphological differentiation. The evolution of substantial reproductive isolation is particularly fascinating as these lineages likely came contact during previous interglacials, during which they could have merged (Futuyma 2010; Hewitt 2011). Yet, these lineages have remained distinct at all assayed loci, suggesting that substantial reproductive isolation can evolve quickly despite opportunity for cyclic introgression and without detectable morphological or ecological divergence.

With this work, *L. coggeri* joins an ever-growing list of systems in which researchers have identified evidence for deep phylogeographic structure with little or no overt morphological or ecological divergence (e.g., *Heteronotia binoei* lizard [Fujita et al. 2010], *Hemidactylus fasciatus* lizard [Leache and Fujita 2010], *Aptostichus atomarius* spider [Bond and Stockman 2008], *Aneides flavipunctatus* salamander [Rissler and Apodaca 2007], *Mielichhoferia elongata* moss [Shaw 2000]). As yet, there are very few cases where the extent of reproductive isolation has been estimated from either experimental crosses or hybrid zone analyses. However, like the *L. coggeri* system, these cases often demonstrate strong barriers to gene flow (e.g., the *L. myola*/*serrata* frog [Hoskin et al. 2005], *C. rubrigularis* lizard [Phillips et al. 2004], *Astraptes fulgerator* butterfly [Hebert et al. 2004], *Brachionus plicatilis* rotifers [Gomez et al. 2002], *Draba* sp. plants [Grundt et al. 2006]). This work suggests that, in contrast to the current emphasis on divergent ecologically based selection as a driver of speciation, divergence in less obvious niche dimensions (e.g., shifts in physiology or in chemiosensory cues [Janzen 1967; Smadja and Butlin 2009], divergence due to parallel adaptation [Mani and Clarke 1990; Nosil and Flaxman 2011], or drift-driven, nonadaptive divergence [Wake 2006]) might be more important in species formation than generally recognized. Indeed, this growing body of work suggests that phylogeographic lineages are, like species, "merely one stage in progressive modification," and thus, important in their own right (Grinnell, as cited in Sunderland [in press]).

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Supporting Information

The following supporting information is available for this article:

Figure S1. NewHybrids classification of hybrid class of individuals located in the hybrid zone center.

Figure S2. A map of Lake Barrine (shown in light gray), with individual hybrid indices shown in bold.

Figure S3. Cline width (in demes) at a neutral locus, F_{IS} at a neutral locus, and R_{ij} at a neutral locus for a range of values for selection against hybrids and migration rates.

Figure S4. Cline width (in demes) at a neutral locus, F_{IS} at a neutral locus, and R_{ij} at a neutral locus for a range of values for selection against hybrids and strength of assortative mating.

Figure S5. A cartoon schematic of how simulations were conducted.

Table S1. Sampling points for this study.

Table S2. Loci used in this study, including their diagnostic SNPs and cutting patterns with listed restriction enzyme.

Table S3. Parameters for simulation for this study.

Supporting Information may be found in the online version of this article.