Evolution, 2025, **79(3)**, 411–422
https://doi.org/10.1093/evolut/qpae174
Advance access publication 6 December 2024
Original Article

OXFORD

Contact zones reveal restricted introgression despite frequent hybridization across a recent lizard radiation

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Abstract

Introgression—the exchange of genetic material through hybridization—is now recognized as common among animal species. The extent of introgression, however, can vary considerably even when it occurs: for example, introgression can be geographically restricted or so pervasive that populations merge. Such variation highlights the importance of understanding the factors mediating introgression. Here we used genome-wide SNP data to assess hybridization and introgression at 32 contact zones, comprising 21 phylogenetic independent contrasts across a recent lizard radiation (*Heteronotia*). We then tested the relationship between the extent of introgression (average admixture at contact zones) and genomic divergence across independent contrasts. Early-generation hybrids were detected at contact zones spanning the range of genomic divergence included here. Despite this, we found that introgression is remarkably rare and, when observed, geographically restricted. Only the two most genomically similar population pairs showed introgression beyond 5 km of the contact zone. Introgression dropped precipitously at only modest levels of genomic divergence, beyond which it was absent or extremely low. Our results contrast with the growing number of studies indicating that introgression is prevalent among animals, suggesting that animal groups will vary considerably in their propensity for introgression.

Keywords: hybridization, introgression, SNP, admixture, genomic divergence, isolation-by-distance, gecko

Introduction

Hybridization is now regarded as common among sexually reproducing species. While extensive hybridization in plants has long been recognized, it is now clear that hybridization is common even across animals, with an estimate that 10%–25% of all animal species hybridize with one or more species (Mallet, 2005; Mallet et al., 2016). When hybrids are viable and fertile there is the potential for introgression—the exchange of genetic material among species—which also appears to be common among both recently diverged and more distantly related species (Edelman & Mallet, 2021; Seehausen, 2004). The prevalence of introgression in the evolution of many groups is reflected by the abundance of recent phylogenomic studies showing reticulate phylogenetic histories across taxa (e.g., Esquerré et al., 2022; Figueiró et al., 2017; Meyer et al., 2017; Selz & Seehausen, 2019; Singhal et al., 2021; Sørensen et al., 2023), but the extent and consequences of introgression can vary. In wild tomatoes, for example, introgression is frequent among species pairs while the proportion of the genome experiencing gene flow seems modest (Hamlin et al., 2020). At the opposite extreme, environmental or demographic shifts can result in such extensive introgression that species merge into one another, such as in African cichlid fishes (Seehausen et al., 1997; Shechonge & et al., 2018). This highlights the importance of understanding the factors mediating introgression and species boundaries.

Which factors influence the propensity for hybridization and subsequent introgression when divergent populations come into secondary contact? Do divergent lineages fuse, exchange alleles briefly or only in a narrow geographic area, or remain genomically isolated? The outcome will ultimately be determined by the combination of reproductive isolation (RI), dispersal, and selection (Barton & Hewitt, 1985; Dobzhansky, 1937; Endler, 1977; McEntee et al., 2020; Muller, 1942; Ramsey et al., 2003). Prezygotic RI influences the probability that hybrids are formed in the first place through factors such as mate choice, habitat and temporal separation, and mechanical isolation (Coyne & Orr, 2004). Postzygotic isolation will then determine whether hybrids can successfully develop, survive, and reproduce (Mayr, 1963), thus influencing the potential for subsequent introgression. RI generally scales with genomic divergence (Coyne & Orr, 1989; Orr, 1996; Singhal & Moritz, 2013), although the relationship can vary among prezygotic and postzygotic barriers (Coyne & Orr, 1989; Lackey & Boughman, 2017; Mendelson, 2003; Moyle et al., 2004). Postzygotic RI is tightly linked to genomic divergence through the accumulation of Dobzhansky–Muller incompatibilities over time (Dobzhansky, 1937; Muller, 1942; Orr, 1996). If RI in a group is largely mediated by genomic incompatibilities, we expect to see a tight relationship between introgression and genomic divergence (e.g., Dufresnes et al., 2021; Sasa et al., 1998; Singhal & Bi, 2017),

Received May 23, 2024; revisions received October 2, 2024; accepted December 4, 2024
Associate Editor: Scott Taylor; Handling Editor: Hélène Morlon

whereas a looser relationship might suggest that other factors—such as prezygotic RI—are also in play. Although many studies have explored the relationship between RI and genomic divergence (see Matute & Cooper, 2021), those exploring the actual relationship between introgression in the wild and genomic divergence are few (Dufresnes et al., 2021; Hamlin et al., 2020; Singhal & Moritz, 2013; Wiens et al., 2006).

What is the extent of introgression among species pairs at contact zones and how does the extent scale with a key factor—genomic divergence? We explored this question in the commonly encountered and habitat generalist lizard *Heteronotia binoei*, a taxonomically unresolved radiation of >20 largely cryptic candidate species, distinguished by genetic divergence and chromosomal rearrangements, across Australia (Figure 1). The group is likely of Plio-Pleistocene age (2–5 mya) with lineage diversity highest in the tropical north (Fujita et al., 2010; Moritz et al., 2016; Zozaya et al., 2022). Exon sequences show that genomic divergence among some of these lineages (Zozaya et al., 2023) falls across the "grey zone" of speciation as defined by (Roux et al., 2016; 0.5%–2% net synonymous divergence). Furthermore, evidence of historical introgression—including multiple events of mtDNA capture (Moritz et al., 2016), some historical evidence of genomic introgression (Zozaya et al., 2023), and the formation of all-female populations of hybrid origin (Moritz, 1993; Moritz & Heideman, 1993; Strasburg & Kearney,

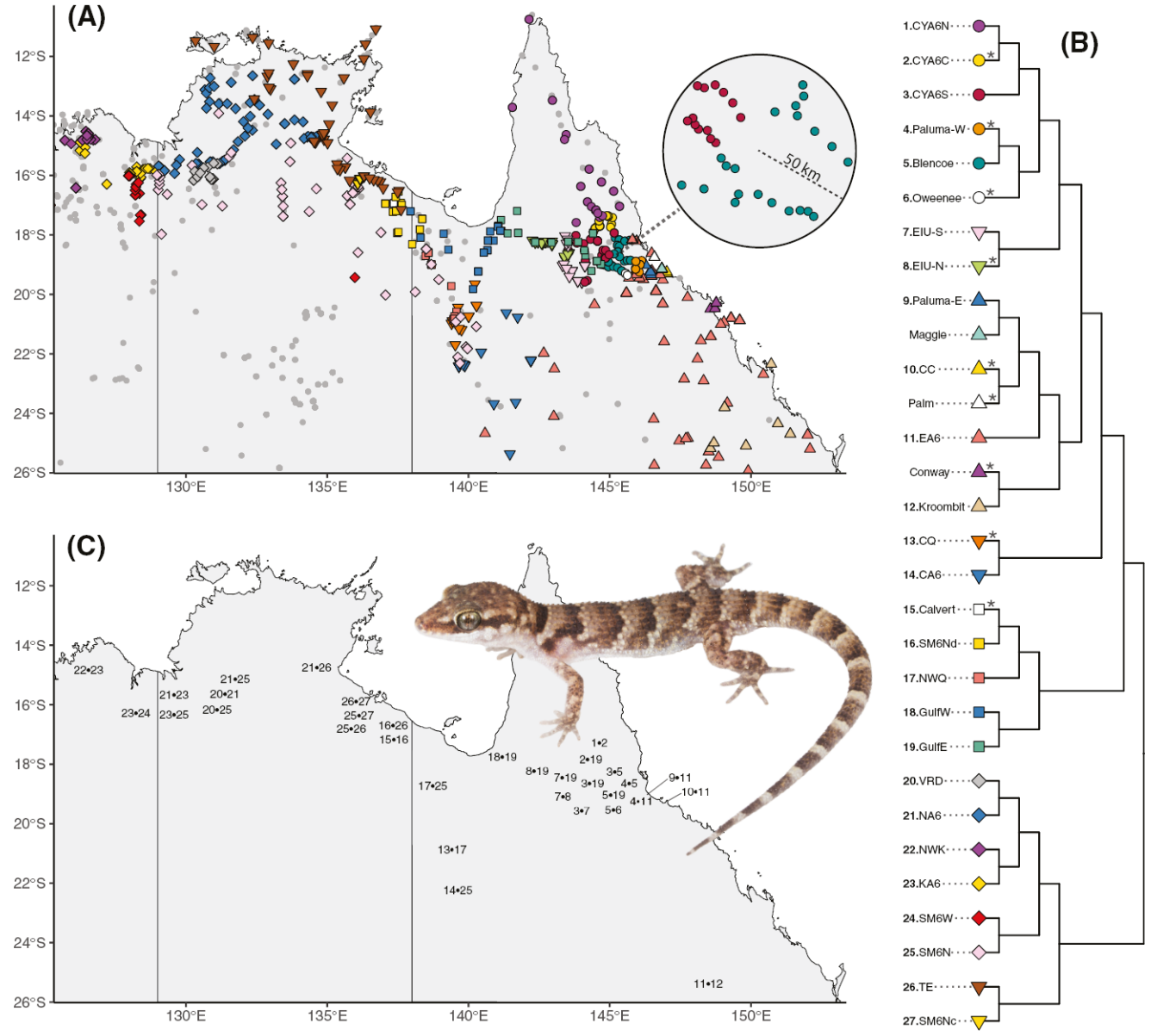

Figure 1. Geographic sampling and phylogenetic relationships of the *Heteronotia binoei* complex across northern Australia. Map (A) shows samples used for genome-wide SNP screening (colored shapes) and those with only mtDNA data (grey dots), with the inset showing an example contact zone. Most of the rest of continental Australia is occupied by just three sexual lineages (EA6, CA6, SM6W) and triploid asexual parthenogens (Moritz, 1993; Strasburg & Kearney, 2005). The phylogeny (B) shows relationships among candidate species following the phylogeny of Zozaya et al. (2022) with the placement of new lineages (marked *) inferred from SNP data via CASTER. Branch lengths are arbitrary. Shape-color combinations are unique to each candidate species and match the symbols shown in (A). Numbers refer to those candidate species included in contact zone sampling. The approximate location of the 32 contact zones analyzed herein is shown in (C), with candidate species combinations at each represented by their respective numbers separated by a dot. An example *H. binoei* is shown (VRD lineage; photo: Scott Macor).

2005)—suggest this is a promising group in which to explore the prevalence and predictors of introgression. We expect that hybridization and introgression in this recently radiated and ecologically generalist group will be common while varying in its geographic extent. Given that RI typically scales with genomic divergence, it is also expected that the degree of introgression among species pairs, which is mediated by RI, will also scale with genomic divergence. Here we assess the extent of introgressive hybridization across 32 contact zones among 27 candidate species in the *H. binoei* complex, comprising 21 phylogenetic independent contrasts. We then test the relationship between the extent of introgression and the degree of genomic divergence.

Methods

Approach and field sampling

Our goal was to estimate the extent of introgression at contact zones in a comparable way to then test the relationship between these estimates and genomic divergence. To accomplish this, we first sampled as many contact zones as possible among different pairwise combinations of candidate species (i.e., deeply divergent genetic lineages; see below) in the *H. binoei* complex. We then did the following for each contact zone (Figure 2): (i) Retained samples only within a 50–60 km radius of the contact zone (dependent on sampling) to reduce effects of within-lineage geographic variation and so that estimates from contact zones were comparable; (ii) identified F1, F2, and first-generation backcross (BC1) hybrids (hereafter referred to as "early generation hybrids") to inform other analyses and to determine whether hybrids could be produced across the scale of divergence included here; (iii) estimated admixture proportions for all individuals at each respective contact zone; (iv) assessed the presence of mitochondrial introgression, inferred by comparing mtDNA ancestry to genomic ancestry; (v) removed early-generation hybrids prior to steps (vi)–(viii) because such hybrids can be sterile or inviable, and thus might not contribute to subsequent introgression even if hybridization is common; (vi) used isolation-by-distance (IBD) plots and accompanying mixture models to verify the results from admixture analyses; (vii) estimated the extent of introgression by calculating the average admixture proportion at each contact zone; (viii) bootstrapping sensitivity analysis to assess if and how estimates of average admixture proportions change depending on variation in geographic sampling. Assuming sampling is relatively even, the average admixture proportion at each contact zone should provide a robust proxy for the geographic extent of introgression. Detailed methods for all these steps are given in the relevant sections below. We did not employ cline analysis (Endler,

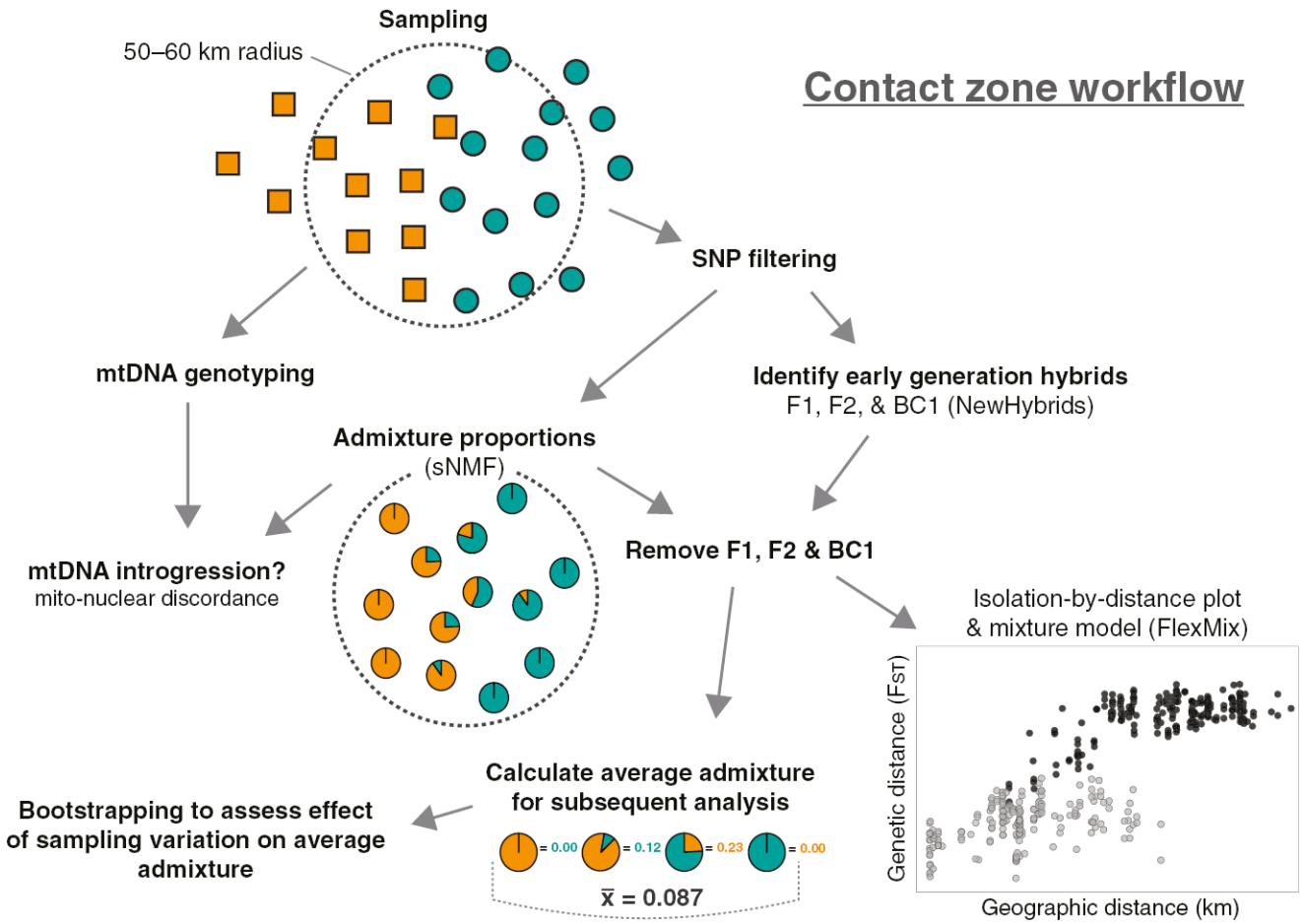

Figure 2. Analytical workflow for contact zone analysis. Orange and blue colors represent different candidate species. Pie charts under "Plot admixture map" reflect admixture proportions of individuals with respect to the two candidate species at the contact zone. The isolation-by-distance (IBD) scatterplot shows pairwise F_{ST} (*y*-axis) between individuals of the same (grey) or different (black) candidate species plotted against the geographic distances (*x*-axis) between them.

1977), which requires one to sample a transect perpendicular to the contact zone between the two respective populations. This can be difficult in cases where poor accessibility restricts sampling, the exact location and shape of the contact zone is unknown, or the contact zone is a mosaic (Rand & Harrison, 1989). All these factors apply to our study.

We first identified potential contact zones among candidate species based on previously published studies that used mtDNA, multilocus nDNA, and phenotypic data (Fenker et al., 2021; Moritz et al., 2016; Riedel et al., 2021; Zozaya et al., 2019, 2022, 2023). We then conducted fieldwork in August–December 2021 to densely sample tissues at as many of these as possible. Sampling involved active searching of sites every 1–10 km (depending on access and conditions) across the general area of each putative contact zone. This was followed by genome-wide SNP screening and mtDNA sequencing of these and more geographically dispersed samples to test previous candidate species designations and to better identify the location of contact zones among them. Informed by these preliminary results, further field sampling was undertaken in September–December 2022 to better sample contact zones, followed again by SNP screening and mtDNA sequencing. Using these data, we then performed the final analyses outlined below. Pseudonyms for previously identified genetic lineages follow Moritz et al. (2016) and Zozaya et al. (2022). Analyses were done with R v4.3.1 in Rstudio v2023.06.2 (R Core Team, 2023; R Studio Team, 2020) unless otherwise specified.

Genomic sequencing and filtering

We conducted genome-wide reduced-representation sequencing using the DArTseq platform, developed by Diversity Arrays Technology (DArT; Canberra, Australia), which involves genome fragmentation with two restriction enzymes, filtering based on fragment size, and subsequent short-read Illumina sequencing (Georges et al., 2018; Kilian et al., 2012; Sansaloni et al., 2010). Additional filtering and SNP calling was done via DArT's proprietary pipeline, with SNPs called across all individuals in the dataset. The resulting data comprised thousands of ca. 60–80 bp fragments. DArTseq data generated in this study (938 samples) were combined with data from Fenker et al. (2021) for a total of 1,032 *Heteronotia* individuals (Table S1). These previously published DArT data were generated using an older methodology that, when co-analyzed with our newly generated data, resulted in fewer variant sites. To maximize the number of variant sites for each analysis, we therefore used the SNP dataset that included these older data only for those contact zones where they contributed significantly to sampling coverage (five contact zones that included either the SM6N or NA6 lineages). Filtering of SNP data was done separately for each analysis and each contact zone to maximize the number of retained sites given the samples included. We used *dartR* v2.9.7 (Gruber et al., 2018) to filter data in the following order: minimum read depth≥5; reproducibility ≥0.99; call rate by locus ≥0.8; call rate by individual ≥0.5; minor allele count ≥3 (to ensure a given allele is present in at least two individuals); remove monomorphic loci; retain only one SNP per locus (using method = "best" to retain the site with the highest PIC score). The SNP dataset used for candidate species delimitation across the *H. binoei* complex (excluding data from Fenker et al., 2021) contained 6,070 variant sites after filtering, while contact zone datasets ranged from 373–2,652 variant sites (mean = 1,523).

Full DArTseq sequence data, including invariable sites, were used to estimate levels of genomic divergence among candidate species (see below). DArTseq reads in raw FASTQ format were trimmed of barcode sequences using *process_radtags* in STACKS v2.65 (Catchen et al., 2011, 2013). We used Trimmomatic v0.39 (Bolger et al., 2014) to trim adapters (clipping bases with alignment scores ≥10 with respect to adapters) and poly-G tails by truncating remaining sequences to 60 bp, discarding any shorter trimmed reads. Reads were mapped to the reference genome for the SM6W candidate species (NCBI accession GCA_032191835.1) using *bwa aln* and *bwa samse* in the Burrows-Wheeler Aligner v0.7.17 (Li, 2013; Li & Durbin; 2009). Locus assembly and SNP calling were performed in *gstacks* in STACKS following the *ref_map* pipeline, excluding loci missing from >10% of individuals. For subsequent analyses, we generated concatenated consensus sequences in FASTA format for each individual, with locus alleles randomly assigned to one of two haplotypes. This yielded a 319,865 bp alignment across 5,195 concatenated loci.

Mitochondrial DNA sequencing

Given a known history of mitochondrial capture in this genus, we also sequenced mtDNA to assess whether there is evidence of mitochondrial introgression at contact zones and to help identify the location of contact zones for field sampling. We sequenced 1,041 bp of the mitochondrial *NADH dehydrogenase subunit 2* (*ND2*) locus using the MinION barcoding method presented in Srivathsan et al. (2021). Where possible, this was done for samples included in SNP screening that did not already have published *ND2* sequence data. We created 20 forward and 20 reverse PCR primers, each with a unique 5' index attached to previously published *ND2* primers for *Heteronotia* ("tRNAI" & 'tRNAA'; Strasburg & Kearney, 2005), which allowed us to pool ≤400 uniquely indexed samples at a time for sequencing. The *ND2* locus was amplified by PCR, and PCR products were visualized on a 2% agarose gel and pooled based on gel band intensity. The pooled libraries were cleaned with 2X SeraMag beads followed by end-prep with NEBNext UltraII End-Repair/dA-Tailing Module and library preparation with the ONT Ligation Sequencing Kit (either LSK109 or LSK110). Libraries were sequenced on an ONT MinION R9.4.1 flowcell for 3–4 hr, with raw data processed with the High Accuracy Basecalling algorithm. We demultiplexed raw reads and generated consensus barcodes in ONTBarcoder (Srivathsan et al., 2021), using the "consensus by length" and "consensus by similarity" modules. We confirmed the reliability of this method by including seven samples in the MinION barcode sequencing with existing barcodes generated by Sanger sequencing. We found that MinION *ND2* sequences had, on average, 99.7% sequence similarity with the Sanger *ND2* sequences. Newly generated *ND2* sequences were combined with sequences from previous studies (Moritz et al., 2016; Zozaya et al., 2019, 2022) that matched individuals used in SNP screening here. Sequences were aligned using MAFFT in Geneious Prime v2021.2.2 followed by manual inspection to check for gaps, unexpected stop codons, or frame shifts. This yielded a final *ND2* alignment of 1,041 bp across 935 samples.

Candidate species delineation and phylogenetics

While the initial designation of candidate species was based on previously published studies (see above), we nevertheless

conducted simple candidate species delimitation to test these and to identify any previously unrecognized candidate species. We first performed Principal Coordinates Analysis (PCoA) to visualize and identify distinct clusters. This was done using the "gl.pcoa" function in *dartR*, first across all samples and then separately for each of the main groups of clusters (Figure S1). We then created a simple neighbor-joining tree to assess whether clusters also formed reciprocally monophyletic and deeply divergent groups (taking early-generation hybrids into account). This was done by first calculating a pairwise dissimilarity matrix with the 'dist.dna' function in *ape* v5.7-1 (Paradis et al., 2004) using the full concatenated sequence alignment of 319,865 bp across 5,195 RAD loci, including invariant sites. This matrix was then used to create a neighbor-joining tree with the "nj" function. Finally, we assigned each individual to a candidate species and then used the "gl.fixed.diff" function in *dartR* to generate a matrix of fixed allelic differences among candidate species (threshold = 0) and then used "gl.collapse" to iteratively amalgamate candidate species that had no fixed differences. Early-generation hybrids (see NewHybrids analysis below) were removed prior to this analysis. Ultimately, the contact zone analyses themselves served as post hoc candidate species delimitation.

A species tree phylogeny was needed to determine phylogenetic independent contrasts. The phylogeny of Zozaya et al. (2022)—a multispecies coalescent (MSC) tree inferred via StarBEAST2 (Ogilvie et al., 2017) using 100 exonic loci—was used as the backbone on which to infer the phylogenetic relationships of candidate species not included therein. We inferred the placement of 10 lineages onto the Zozaya et al. (2022) phylogeny using Coalescence-aware Alignment-based Species Tree EstimatoR (CASTER; Zhang et al., 2023). This was done using an alignment of 5,231 biallelic SNPs (relevant ambiguity codes used for heterozygous states) with 4 samples per candidate species, which were chosen so that none represented admixed individuals (see NewHybrids and sNMF analyses below). Relationships among relevant lineages were constrained to the topology of Zozaya et al. (2022), with *Heteronotia fasciolatus* included as the outgroup. Although we expect the exon-based phylogeny of Zozaya et al. (2022) to be more accurate than what could be inferred with SNP data here, we nevertheless repeated phylogenetic analyses without any topological constraints to assess if this changed the results of subsequent pairwise contrasts. We did this using both CASTER (as above) and SVDquartets (Chifman & Kubatko, 2014) in PAUP* v4.0 (Swofford, 2003).

We performed a phylogenetic analysis of *ND2* sequences using IQ-TREE 2.2.0 (Minh et al., 2020). The 1,041 bp alignment was partitioned by codon position, with substitution models chosen using ModelFinder (Kalyaanamoorthy et al., 2017). Statistical support was determined with 1000 ultrafast bootstrap replicates (implemented via ufboot2; Hoang et al., 2018).

Assessing hybridization and introgression at contact zones

We first chose an approximate geographic center point for each contact zone that maximized sampling evenness and extent. Samples of the respective populations within a 50 or 60 km radius of this point were included in subsequent analyses. A radius of 50 km was chosen except in six cases (Table S2) where 60 km was chosen because sampling was more spread out because of land access or the nature of the distributions themselves. Sample sizes for each population at each contact zone varied from 9 to 51 (mean = 26.3; Table S2) due to sampling access and variation in the size and shape of distributions. Samples at each contact zone were first assessed using NewHybrids (Anderson & Thompson, 2002) to detect early-generation hybrids. This was done using the 'gl.nhybrids' function in *dartR* with the "AvgPIC" method, 100 sweeps, and a burn-in of 100. We then estimated admixture proportions across individuals (including early-generation hybrids) at each contact zone using sNMF (Frichot et al., 2014) implemented in *LEA* v3.14.0 (Frichot & François, 2015). We specified $K = 2$ because each analysis included two candidate species with samples confined to a relatively small geographic area. Each analysis was run using the "snmf" function with alpha = 100, 100 repetitions, and masking = 0.05. To assess mito-nuclear congruence, we checked whether an individual's mtDNA lineage matched the population for which it had the highest ancestry proportion.

Admixture analyses such as sNMF are susceptible to biases that can over or underestimate apparent admixture (Lawson et al., 2018; Puechmaille, 2016; Wang, 2022). IBD plots and accompanying mixture models were therefore used to verify sNMF results (Prates et al., 2023). We did this by plotting pairwise geographic distance against pairwise F_{ST} (Weir & Cockerham, 1984; Weir & Hill, 2002; Wright, 1943) among individuals for the respective contact zone, excluding early-generation hybrids. Pairwise geographic distances were calculated using the "earth.dist" function in *fossil* v0.4.0 (Vavrek, 2011). Pairwise F_{ST} was calculated using the "calculate.all.pairwise.Fst" function in *BEDASSLE* v1.6.1 (Bradburd et al., 2013). We then used finite mixture modeling with latent class regression via *flexmix* v2.3-19 (Grün & Leisch, 2007; Leisch, 2004) to test whether the relationship between genetic and geographic distance is better explained by one regression line ($k = 1$) or two ($k = 2$). If the $k = 1$ model is supported over $k = 2$, we interpreted that to mean that within- and between-lineage comparisons are indistinguishable, indicating that the two "candidate species" represent a single population with genetic differentiation following what might be expected under IBD.

The relationship between introgression and genomic divergence

We sought to understand the relationship between genomic divergence and the extent of introgression across contact zones. To do this, we first estimated the average admixture proportion at each contact zone. We define admixture in this analysis as the lesser ancestry proportion inferred for an individual. For example, two samples, one with 70% ancestry from candidate species A and 30% ancestry from candidate species B, and the other with 30% ancestry from candidate species A and 70% ancestry from candidate species B, would both be assigned an admixture proportion of 30%. Average admixture was calculated by taking the mean admixture proportion at each site yielded from the respective sNMF analysis—including all samples except those identified as early-generation hybrids—followed by averaging across sampling sites for the respective contact zone. This serves as a proxy for the geographic extent of introgression, although it is agnostic with respect to the direction or symmetry of introgression. Averaging by sampling site before calculating the contact zone average is important because sites vary in the number of sampled individuals, meaning that a single densely

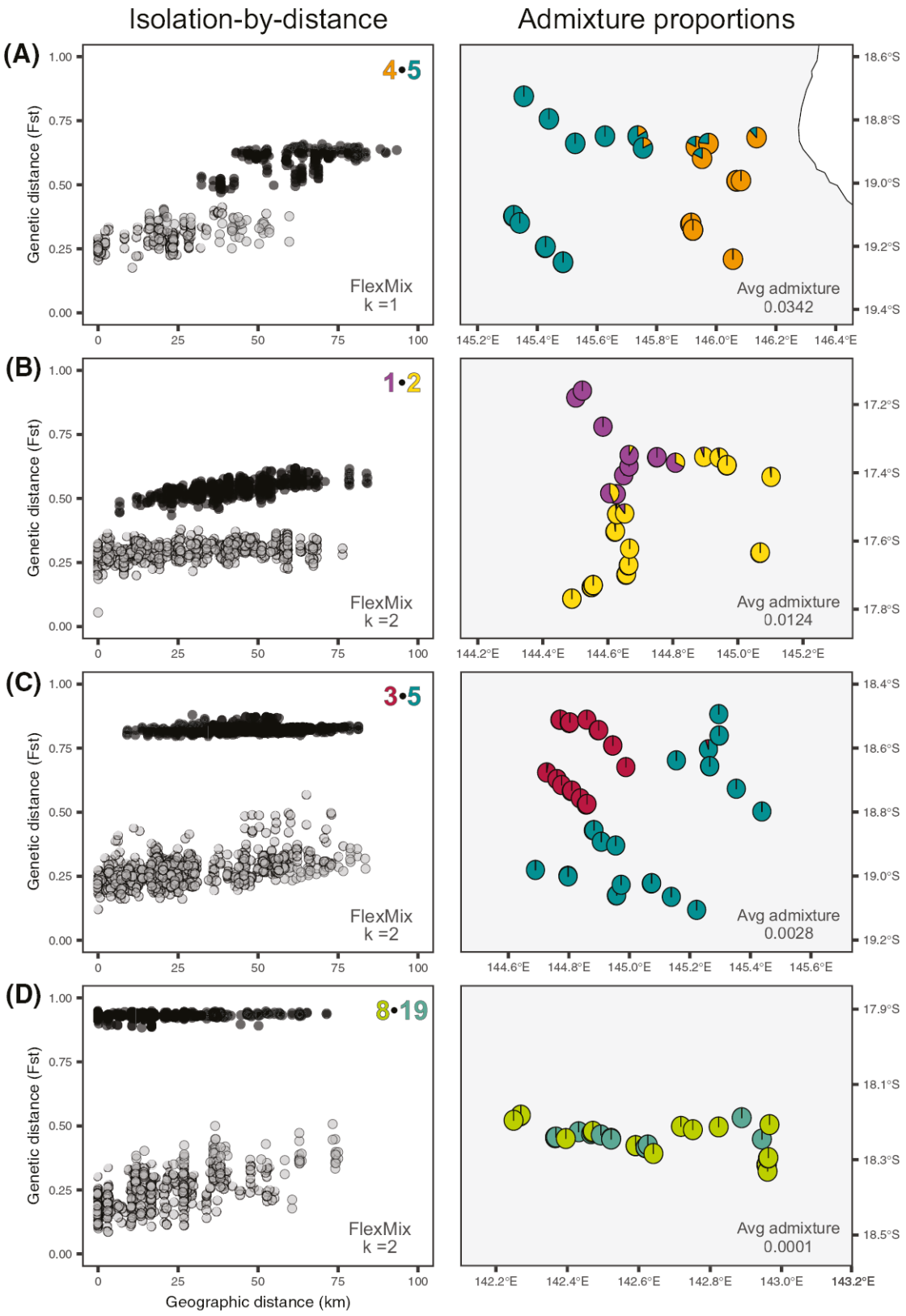

Figure 3. Four examples illustrate variation in the extent of introgression at contact zones. Each row shows a different contact zone. Isolation-by-distance (IBD) plots (left column) illustrate within-lineage (grey) and between-lineage (black) IBD, with the optimal *k*-value determined by FlexMix shown on the bottom-right. Maps (right column) show individual admixture proportions illustrated by pie charts (some charts are slightly offset for visibility) with the average admixture proportion for that contact zone shown on the bottom-right. Colored numbers in the top-right corners of each IBD plot display the combination of candidate species (designated in Figure 1B), with colors matching those in the adjacent map and those shown in Figure 1.

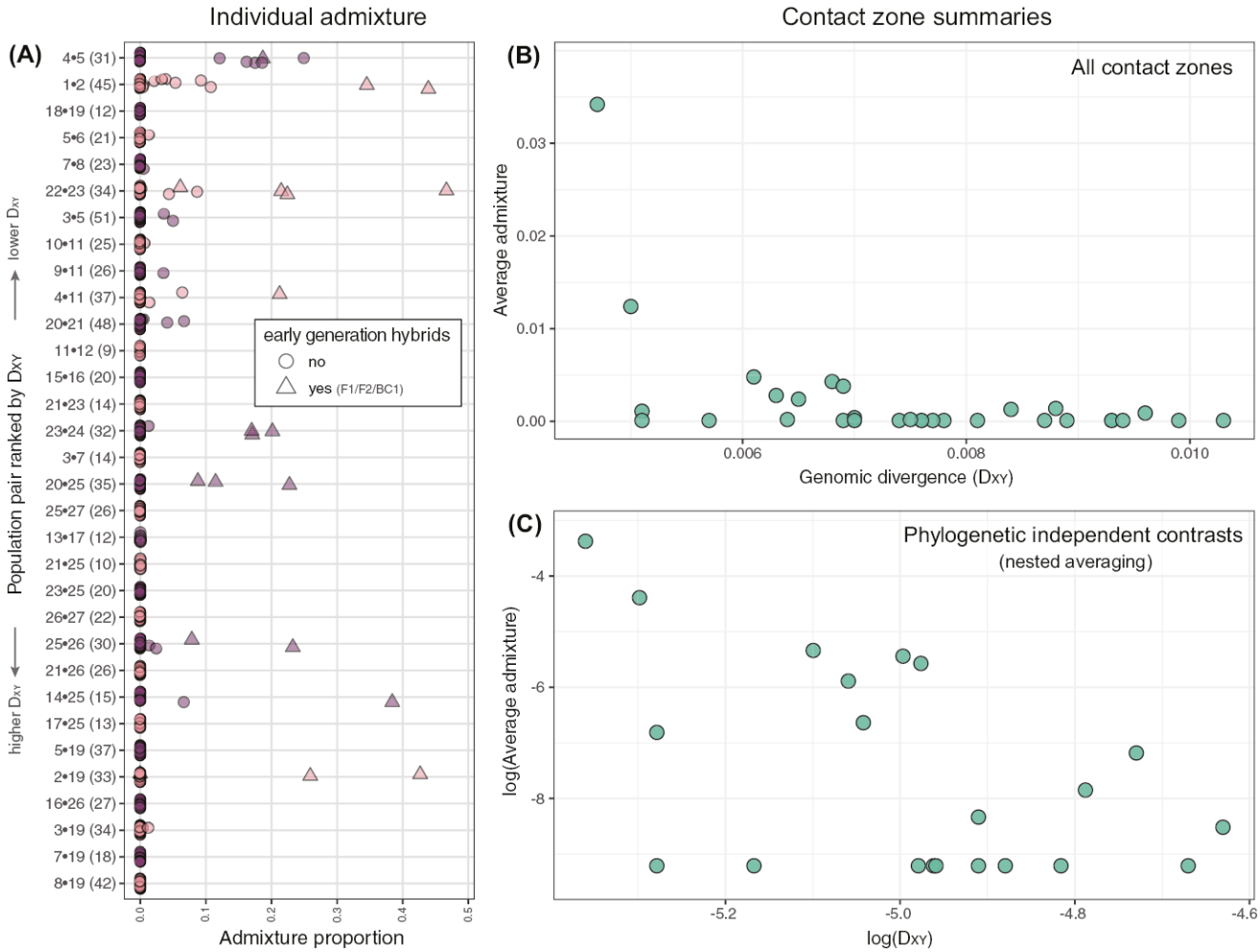

Figure 4. The relationship between genomic divergence (D_{XY}) and admixture across 32 contact zones. (A) Individual admixture proportions at each contact zone, with contact zones ordered by increasing D_{XY}. Numbers on the *y*-axis show the candidate species combination as designated in Figure 1 followed by the number of individuals in parentheses (e.g., "4•5 (31)" is the contact zone between lineage 4 and 5, which included 31 samples). Shapes indicate whether a sample was an early-generation hybrid; colors are simply aesthetic. Average admixture plotted against D_{XY} for (B) all contact zones and (C) phylogenetic independent contrasts (reflecting values determined via nested averaging; see Methods). Both average admixture and D_{XY} are log-transformed in panel (C). Early-generation hybrids were removed before calculating average admixture and D_{XY}.

sampled site would be weighted more heavily than a site with only a single sample. Samples within 500 m of each other were considered to be from the same site. Given that geography, weather conditions while field sampling, and various idiosyncrasies (such as size and shape of distributions) prevented sampling each contact zone in a perfectly comparable way, we ran a simple sensitivity analysis via bootstrapping. We randomly selected 80% of samples from each candidate species at each contact zone, repeated 1,000 times, each time calculating mean admixture. This produced a distribution of mean admixture which was then compared to our "raw" mean estimate. The effect of sampling biases with respect to our study is considered in the Results and Discussion. Because the number of SNPs varied depending on whether the samples from Fenker et al. (2021) were included in SNP calling, we also tested the sensitivity of these results with respect to the differing number of retained SNPs.

We then estimated pairwise genomic divergence between candidate species pairs by calculating D_{XY}, the number of sites that differ between two haplotypes divided by the total number of sites (Nei & Jin, 1989). This was done using the 319,865 bp concatenated sequence alignment, including invariant sites. Only samples inferred to have admixture proportions ≤1% were included in D_{XY} calculations to avoid biasing divergence estimates in the face of introgression. This resulted in the exclusion of 0–11 samples (mean = 1.8 samples) per candidate species, with the most extreme case being the removal of 11 out of 34 samples from the CYA6C lineage (a lineage that shows moderate admixture with CYA6N; see Results). Only one haplotype per individual was used. Calculations were done using the "dist.dna" function in *ape* v. 5.6-2 (Paradis et al., 2004) with "pairwise.deletion" set to "TRUE" to exclude sites with missing data for the respective pairwise calculation. Average D_{XY} was then calculated for each candidate species pair.

Finally, we tested the relationship between genomic divergence and average admixture proportion across contact zones while accounting for phylogenetic non-independence. We produced phylogenetic independent contrasts with the nested averaging (node-weighted averaging) approach used by Coyne and Orr (1989) and Fitzpatrick (2002) for subsequent analysis. Finally, using these phylogenetic independent

contrasts, we performed a general linear regression via the "glm" function with average admixture as the response variable and average pairwise D_{XY} as the explanatory variable. Both average admixture and D_{XY} were log-transformed prior to linear regression.

Results

Lineage delimitation and phylogenetics

Initial lineage delineation identified 31 putative candidate species that formed distinct genotypic clusters (Figure S1) and divergent monophyletic groups (Figure S2). Most of these were identified in previous studies (Moritz et al., 2016; Zozaya et al., 2022), while six are newly identified here (Figure 1; Figures S1–S4). Fixed differences analysis did not merge any candidate species pairs, with ≥7 fixed allelic difference (Table S3) among pairs, including those candidate species pairs with considerable introgression (admixed individuals that were not early-generation hybrids were retained in analysis). Phylogenetic relationships inferred among these candidate species using CASTER, with a fixed topology for relevant candidate species included in Zozaya et al. (2022), are shown in Figure 1c. Some deeper relationships vary considerably between this tree and the unconstrained CASTER and SVDquartets trees (Figure S4); however, these differences affect only two phylogenetic independent contrast.

Hybridization and introgression at contact zones

We assessed 32 pairwise contact zones among 27 candidate species of the *H. binoei* complex (Table S2). Figure 3 shows select examples of contact zones with varying levels of admixture. We detected a total of 20 early-generation hybrids across nine contact zones, ranging from 1 to 4 individuals at each of these contact zones, with hybrids observed from pairs of candidate species spanning the entire phylogeny and nearly the entire range of D_{XY} (Figure 4A; Table S2). While our sampling is insufficient to detect early-generation hybrids at all contact zones, or to compare the frequency of different hybrid classes, our results demonstrate that even our most divergent pairs can produce early-generation hybrids. Beyond early-generation hybrids, admixed individuals were generally rare and with low admixture proportions. What could be described as substantial intergradation—a gradient of admixture proportions indicating that populations are linked by gene flow—was observed at only two contact zones representing the least divergent candidate species pairs considered here (Paluma-W & Blencoe [4•5]; CYA6N & CYA6C [1•2]; Figures 3A and B, 4B). Only two population pairs (Paluma-W & Blencoe [4•5]; KA6 & NWK [22•23]) showed evidence of mito-nuclear incongruence at the contact zone (excluding early-generation hybrids) that suggests recent or ongoing mtDNA introgression (Figure S3; Table S1). Admixture was otherwise geographically restricted, if it was observed at all, with "pure" individuals often present within 0–5 km of the contact zone, and no later generation admixed individuals observed more than 10 km from this zone (Figure 3; Figures S5–S11). These results are robust to the number of variant sites included in the analysis (Figure S12).

Many contact zones show a complete lack of later generation hybrids, i.e., admixed individuals that are not F1, F2, or BC1 hybrids (Figure 4A). IBD analyses corroborate these results, showing that F_{ST} between individuals of different candidate species decreases with geographic proximity only in the two contact zones that show substantial introgression (Figures 3A,B). Pairwise F_{ST} between candidate species appears unrelated to geographic proximity across all other contact zones (Figures 3C,D; S5–S11), even when geographically restricted admixture was observed (e.g., Figure S5). Mixture models supported a K = 2 scenario at all but three contact zones (Table S2): the 4•5 pair mentioned above, and two relatively poorly sampled contact zones (14•25 and 17•25).

Relationship between genomic divergence and introgression

There is a significant inverse relationship between log-transformed admixture and log-transformed D_{XY} across the 21 phylogenetic independent contrasts ($t = -2.595$, $df = 18$, $p = 0.0177$; Figure 4c), with average admixture decreasing by a factor of 4.7% for every 1% factor increase in D_{XY}, although the model has relatively low explanatory power (adjusted $R^2 = 0.223$). Average admixture estimates produced from the bootstrapping sensitivity analysis were very similar, and usually slightly lower, than the raw admixture proportion averages (Figure S12).

Discussion

We sought to assess the extent of introgressive hybridization at contact zones across a recent lizard radiation and to understand how variation in extent is predicted by genomic divergence. We did this across 32 pairwise contact zones comprising 21 phylogenetic independent contrasts—a scale rare in contact zone studies. We found that introgression is remarkably rare and, when observed, geographically restricted across this recent lizard radiation. Only 2 of the 32 contact zones we assessed, involving the two least divergent candidate species pairs, exhibited introgression beyond 5 km of the contact zone. This is despite the detection of early-generation hybrids between pairs representing the entire spectrum of genomic and phylogenetic divergence in our study. Furthermore, we found evidence of recent or ongoing mtDNA introgression at only two contact zones, despite evidence of historical mtDNA introgression among several lineages (Moritz et al., 2016). This points to rapid establishment of RI in this recent and ecologically generalized radiation. While sporadic hybridization is common in this group, it is remarkable how rare introgression is at existing contact zones. These results are contrary to much of the growing body of literature demonstrating extensive introgression across many animal groups (Edelman & Mallet, 2021). More narrowly, even several lizard groups of similar ages to *Heteronotia* show considerable levels of recent introgression, such as *Sceloporus* (Bouzid et al., 2022; Grummer et al., 2015), *Podarcis* (Caeiro-Dias et al., 2021; While et al., 2015; Yang et al., 2018), *Liolaemus* (Grummer et al., 2021), and *Ctenotus* (Prates et al., 2023). Our findings are instead similar to studies of contact zones among more deeply divergent lineage pairs that show rare or geographically restricted introgression (e.g., McGuire et al., 2023; Singhal & Moritz, 2013). These findings indicate that animal groups will vary considerably in their propensity for introgression, and that introgression is not always the norm, even among closely related lineages.

Our study is one of few to assess the relationship between the extent of gene flow and genomic divergence among natural populations (Dufresnes et al., 2021; Hamlin et al., 2020;

Singhal & Moritz, 2013; Wiens et al., 2006). We found that average admixture decreases as genomic divergence increases. Postzygotic RI is expected to increase exponentially with increasing genomic divergence through the accumulation of Dobzhansky–Muller incompatibilities (Dobzhansky, 1937; Muller, 1942), that is, the "snowball effect" (Orr, 1996; Orr & Turelli, 2001). This prediction is consistent with our results, with a precipitous drop from modest introgression to no or extremely limited introgression with increasing genomic divergence. The rarity and geographically restricted extent of introgression in our study, however, suggests we have largely missed the "left side" of the relationship between genomic divergence and the extent of introgression (i.e., younger lineage pairs in the "grey zone" of speciation; Roux et al., 2016). Even relatively low levels of gene flow can act as a homogenizing force (Hartl & Clark, 1997; Wright, 1931), particularly when aided by selection (Rieseberg & Burke, 2001; Slatkin, 1987). This raises the question of whether less differentiated pairs, which generally have weaker RI, have simply merged into each other upon secondary contact (Dobzhansky, 1958; Seehausen, 2004; Taylor et al., 2006). This would result in survivorship bias in our observed contact zones: we can only see contact zones between lineage pairs that have not collapsed. The *H. binoei* complex, therefore, might be an interesting system in which to explore the prevalence of lineage fusion (e.g., Garrick et al., 2019, 2020; Kearns et al., 2018).

While the extent of admixture at contact zones was generally very low and geographically restricted, there is, nevertheless, substantial unexplained variation in the relationship between mean admixture and genomic divergence (see Figure 4C). This variation could be noise from sampling or estimation error (discussed below), or it could reflect variation in dispersal ability or the presence and strength of different RI barriers. The frequent observation of early-generation hybrids indicates that premating isolation is not complete; however, premating barriers mediated by mate choice and habitat isolation could nevertheless influence the frequency with which hybrids are produced. *Heteronotia* geckos, while generally regarded as morphologically similar, are known to exhibit variation in male chemical signals (Zozaya et al., 2019), a putative mating trait (Martín & López, 2006, 2014), and morphological adaptations to different habitats (Riedel et al., 2021). The two contact zones that showed substantial introgression (1•2 and 4•5) coincide with shifts from flat savanna woodland to rocky hills. But this description also characterizes contact zones with no observed introgression (e.g., 5•6), and several contact zones with no observed introgression are characterized by continuous savanna (e.g., 18•19). As such, there is no immediately clear link between habitat shifts and the propensity for introgression. Another possibility is that variation in selection pressures or substitution rates among different populations have caused Dobzhansky–Muller incompatibilities to accumulate at varying rates (e.g., Fitzpatrick, 2002). Disentangling mechanisms of RI in this group will require further genomic and experimental work.

Our approach to analyzing and comparing contact zones has both strengths and weaknesses. Using the average admixture proportion across a given radius centered on a contact zone should be a comparable proxy for the geographic extent of introgression if sampling is even. However, in practice, this will rarely be the case, as variation in land access, species abundances, the shape of species distributions, and the landscape itself will influence geographic sampling density. Such biases can influence estimates of mean admixture. Given our findings, which show extremely limited introgression at all but two contact zones, we assume our overall results are robust to such biases—especially given that they are supported by both admixture-based (sNMF) and IBD analyses, as well as bootstrapping sensitivity analyses. The strength of our approach is that it allows for the comparison of large numbers of contact zones where cline sampling is either difficult or impossible because of, for example, variable land access and mosaic contact zones. Ultimately, this approach sacrifices some accuracy and precision in favor of volume.

Finally, how should we interpret the scarcity of introgression in this study given the growing number of studies showing widespread historical introgression across many groups? Our results examining introgression at contact zones are counter to a known history of mtDNA capture between even distantly related *Heteronotia* lineages (Figure S3; Table S2; Moritz et al., 2016; Zozaya et al., 2022). Is it possible that the patterns we see here—limited introgression across all but the two most similar population pairs—are still consistent with the evolution of reticulate phylogenies? Historic patterns are the product of what were once contemporary processes. Evidence that reticulation is common might not always mean that introgressive hybridization is common at a given point in time—reticulation can be produced from *rare* events, given time, opportunity, and selection. It is difficult to accurately date historical introgression, and the degree to which populations were differentiated at the time cannot be known. What can be inferred is that RI could not have been complete at the time. Our study demonstrates that introgression at contact zones is not always common, even within a relatively young radiation. Considering these points, we conclude by asking: How do small-scale patterns such as this link to complex histories of reticulate evolution?

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Data and R code are available on Dryad: https://doi.org/10.5061/dryad.prr4xgxx6.

Author contributions

SMZ, MH, CJH, and CM conceived the study and acquired funding; SMZ, SAM, and WJR conducted fieldwork; RS, KO, and WJR conducted lab work; RS, CCL, SMZ, and KO performed bioinformatics procedures; SMZ conducted all statistical analyses, drafted the manuscript, and created figures; all authors contributed to editing the final manuscript.

Funding

This work was funded by an Australian Research Council Discovery Project (DP210102267) and an Australian Biological Resources Study NTRGP Postdoctoral Fellowship Grant to SMZ (NTRGI000036).

***Conflict of interest*:** The authors declare no conflicts of interest.

Acknowledgments

We thank Naomi Laven, Anders Zimny, Sofia Hayden, Jordan Mulder, Diane Chanut, Keith Christian, Kimberley Day, Kade

Skelton, and Justin Wright for assistance with fieldwork; Eridani Mulder and the Australian Wildlife Conservancy for access to several properties where fieldwork took place; Cecilia Myers for access to Theda and Doongan Stations; Monica Ruibal for helping to develop the MinION barcoding protocol; and Jessica Worthington Wilmer of the Queensland Museum and Sally South of the South Australia Museum for access to tissues.

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