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Speciation and secondary contact in a fossorial island endemic, the São Tomé caecilian

Running Head: Diversification of the São Tomé caecilian

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ABSTRACT

A period of isolation in allopatry typically precedes local adaptation and subsequent divergence among lineages. Alternatively, locally adapted phenotypes may arise and persist in the face of gene flow, resulting in strong correlations between ecologically-relevant phenotypic variation and corresponding environmental gradients. Quantifying genetic, ecological, and phenotypic divergence in such lineages can provide insights into the abiotic and biotic mechanisms that structure populations and drive the accumulation of phenotypic and taxonomic diversity. Low-vagility organisms whose distributions span ephemeral geographic barriers present the ideal evolutionary context within which to address these questions. Here, we combine genetic (mtDNA and genome-wide SNPs) and phenotypic data to investigate the divergence history of caecilians (Amphibia: Gymnophiona) endemic to the oceanic island of São Tomé in the Gulf of Guinea archipelago. Consistent with a previous mtDNA study, we find two phenotypically and genetically distinct lineages that occur along a north-to-south axis with extensive admixture in the centre of the island. Demographic modelling supports divergence in allopatry (~300 kya) followed by secondary contact (~95 kya). Consequently, in contrast to a morphological study that interpreted latitudinal phenotypic variation in these caecilians as a cline within a single widespread species, our analyses suggest a history of allopatric lineage divergence and subsequent hybridization that may have blurred species boundaries. We propose that late Pleistocene volcanic activity favoured allopatric divergence between these lineages with local adaptation to climate maintaining a stable hybrid zone in the centre of São Tomé Island. Our study joins a growing number of systems demonstrating lineage divergence on volcanic islands with stark environmental transitions across small geographic distances.

RESUMO

Um período de isolamento em alopatria geralmente precede adaptação local e divergência subsequente entre linhagens evolutivas. Alternativamente, fenótipos adaptados localmente podem surgir e persistir apesar de fluxo gênico, resultando em fortes correlações entre variação fenotípica ecologicamente relevante e os gradientes ambientais correspondentes. Quantificar divergência genética, ecológica e fenotípica em tais linhagens pode ajudar a clarificar os mecanismos abióticos e bióticos que estruturam as populações e levam ao acúmulo de diversidade fenotípica e taxonômica. Organismos de baixa vagilidade, cujas áreas de distribuição incluem barreiras geográficas efêmeras, representam um contexto evolutivo ideal para abordar essas questões. Neste estudo, combinamos dados genéticos (mtDNA e SNPs genômicos) e fenotípicos para investigar a história de divergência de cecílias endêmicas da ilha oceânica de São Tomé, no arquipélago do Golfo da Guiné. Consistentemente com um estudo anterior de mtDNA, encontramos duas linhagens fenotipicamente e geneticamente distintas que ocorrem ao longo de um eixo norte-sul, com extensa mistura genética no centro da ilha. Modelagem demográfica suportou um cenário de divergência em alopatria (~ 300 mil anos atrás) seguida de contato secundário (~ 95 mil anos atrás). Ao contrário de um estudo morfológico que interpretou a variação fenotípica latitudinal nessas cecílias como uma clina dentro de uma única espécie amplamente difundida, nossas análises sugerem uma história de divergência de linhagens em alopatria e subsequente hibridização que pode ter confundido os limites das espécies. Propomos que atividade vulcânica durante o Pleistoceno tardio favoreceu divergência alopátrica entre essas linhagens, com adaptação local ao clima mantendo uma zona híbrida estável no centro da Ilha de São Tomé. Nosso estudo se une a um número crescente de sistemas que demonstram divergência entre linhagens em ilhas vulcânicas com transições ambientais marcantes ao longo de distâncias geográficas curtas.

Keywords:
amphibian, gene flow, hybridization, in situ diversification, island speciation, *Schistometopum ephele, Schistometopum thomense*

INTRODUCTION

A period of isolation in allopatry typically precedes local adaptation and subsequent lineage divergence that may ultimately result in speciation (Losos & Ricklefs, 2009; Mayr, 1963). Secondary contact of lineages following transient periods of allopatric divergence can result in lineage fusion or promote reproductive isolation through reinforcement (Servidio & Noor, 2003; Choi, Purugganan, & Stacy 2020). Alternatively, locally adapted phenotypes may arise and persist in the face of gene flow resulting in strong correlations between ecologically-relevant phenotypic variation and corresponding environmental gradients (Thorpe, Barlow & Surget-Groba 2015). Quantifying genetic, ecological, and phenotypic variation in these nascent lineages can provide insights into the abiotic and biotic mechanisms that structure populations and ultimately drive the accumulation of species richness and phenotypic diversity. Organisms with low dispersal potential whose distributions span ephemeral geographic barriers present the ideal evolutionary context within which to understand the relative contributions of these evolutionary processes. Here we investigate the divergence history of the enigmatic and fossorial caecilians (Amphibia: Gymnophiona) endemic to the small, volcanic island of São Tomé.

Physical barriers such as rivers (Vences, Wollenberg, Vieites, & Lees, 2009; Welton et al., 2010), sea level changes (Esselstyn, Timm, & Brown, 2009; O'Connell, Hamidy, Kurniawan, Smith, & Fujita, 2018), or volcanic lava flows (Bloor, Kemp, & Brown, 2008; Brochmann, 1984; Nater et al, 2011) often contribute to allopatric divergence. Landscapes are dynamic, however, and the elimination of such barriers can lead to population expansion, secondary contact, hybridization, and fusing of incipient species, particularly on small oceanic islands (García-Olivares et al., 2017; Garrick et al., 2014; Gow, Peichel, & Taylor, 2006; Grant & Grant, 1996; MacLeod et al., 2015; Roderick, Croucher, Vandergast & Gillespie, 2012; Sardell & Uy, 2016; Taylor et al., 2006). Spatial environmental gradients such as differences in rainfall, temperature, or soil type may further reinforce divergence in allopatry (Losos & Schluter, 2000; Rundle & Nosil, 2005). These environmental transitions can also lead to stable hybrid zones if lineages that meet secondarily are locally adapted (Barton & Hewitt, 1985).

Consequently, genetic and phenotypic differentiation along environmental gradients can be difficult to distinguish from isolation by distance (Bradburd, Coop, & Ralph, 2018; Myers et al., 2019) or allopatric divergence and secondary contact (Portik et al., 2017); however, genomic data paired with demographic modelling approaches can help differentiate among alternative historical scenarios such as divergence in allopatry versus divergence with gene flow (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013; Gutenkunst, Hernandez, Williamson, & Bustamante, 2009). Likewise, quantifying ecological divergence of lineages in the early stages of speciation can reveal the roles of environmental adaptation and geographic isolation in promoting population divergence and reproductive isolation (Marques et al., 2016; Losos & Schluter, 2000; Seehausen, Van Alphen, & Lande, 2001). Small oceanic islands are a compelling study system for addressing the role of previous isolation versus local and/or ongoing selection in shaping biodiversity because they often exhibit more transient geographic barriers to gene flow coupled with stark environmental transitions across small geographic distances (e.g., Stenson, Malhotra, & Thorpe 2002; Suárez, Pestano, & Brown 2014; Brown Paterson & Risse 2016).

Growing evidence suggests that both environmental gradients and a dynamic landscape history shaped species diversification on São Tomé, a volcanic island ~225 km off the coast of West-Central Africa in the Gulf of Guinea archipelago. The island emerged from the sea floor ~13 Mya, and despite its small size (~850 km^2), it is topographically complex, with its highest peak at 2024 m (Gillespie & Clague, 2009). Correspondingly, São Tomé exhibits environmental gradients ranging from drier and open habitat in the north to wetter and forested habitat in the south (de Lima et al., 2017; Soares, 2017). Despite the island's long geologic history, many studies of taxonomic diversification within São Tomé have inferred that divergence occurred during the Pliocene or Pleistocene (Bell et al., 2015; Daniels & Klaus, 2018; Stoelting, Measey, & Drewes, 2014), which coincides with a period of extensive volcanic activity (Barfod & Fitton, 2014). Further, a history of *in situ* divergence in allopatry followed by secondary contact and hybridization was inferred in São Tomé *Drosophila* (Coyne, Kim, Chang, Lachaise, & Elwyn, 2002; Matute & Coyne, 2010) and *Hyperolius* reed frogs (Bell, Drewes, & Zamudio, 2015; Bell & Irian, 2019). By contrast, both adaptation along

environmental gradients and allopatric divergence have been proposed to explain phenotypic (Nussbaum & Pfrender, 1998) and genetic (Stoelting et al., 2014) variation in the São Tomé Caecilian (*Schistometopum thomense*). Due to their low vagility and strong associations with particular soil types and climates (Gundappa, Balakrishna, & Shakuntala 1981; Jones, Loader & Gower 2006; Torres-Sánchez et al. 2019; Kouete & Blackburn 2019), caecilians may provide novel insights into the mechanisms that generate and maintain lineage divergence on small oceanic islands.

Globally, caecilians are distributed throughout the tropics yet are poorly known relative to most vertebrate groups due to their secretive lifestyles and because they are sometimes rare (Heyer, Donnelly, Foster, & Mcdiarmid, 2014; Measey, 2004; Measey, Gower, Oommen, & Wilkinson, 2003). However, *S. thomense* is amenable to study because it is active above and below ground and is abundant across São Tomé, where it occupies diverse habitats from 0–1440 m elevation (Measey & Van Dongen, 2006; Nussbaum & Pfrender, 1998; Stoelting et al., 2014). Morphological variation in this species roughly follows a latitudinal cline, with a yellow unflecked morph in the north and a brown, flecked morph in the south (Haft, 1992; Measey & Van Dongen, 2006; Nussbaum & Pfrender, 1998; Stoelting et al., 2014; Taylor, 1965). This morphological variation led to the description of flecked individuals as a separate species, *S. ephele* (Taylor, 1965); however, Nussbaum & Pfrender (1998) interpreted this variation as a phenotypic cline in a single widespread species and synonymized *S. ephele* with *S. thomense*. More recently, Stoelting et al. (2014) detected two distinct mitochondrial haplotype groups that roughly correspond to *"S. ephele"* and *S. thomense* with a narrow zone of putative admixture in the centre of the island, which they interpreted as evidence of allopatric divergence and secondary contact. In addition, Stoelting et al. (2014) noted that the putative admixture zone coincided with the transition between volcanic flows indicating that volcanism may have played an important role in the evolutionary history of these lineages. In the present study, we revisit these two alternative hypotheses of demographic history using phenotypic and genetic (mtDNA and genome wide SNPs) data. Specifically, we (1) leverage demographic modelling to test for historical divergence in allopatry versus divergence with continuous gene flow, (2) quantify the temporal and geographic extent of gene flow in the putative admixture zone, and (3)

contextualize the evolutionary history of the species with respect to environmental gradients and volcanic activity across its range.

MATERIALS AND METHODS

Field and museum specimen sampling and colour pattern assessment

For genetic analyses, we included 85 samples from 21 localities across the island (Table S1). Among them, 12 samples were collected by the authors between 2012 and 2016 at three localities including Obo National Park, which had not previously been sampled. Tissue samples (liver) were preserved in 95% ethanol or RNAlater for subsequent DNA extraction and genetic analyses. Additionally, we selected a subset of 73 specimens from the Stoelting et al. (2014) mtDNA study from which to collect nuDNA SNP data (see below). This sampling spans the type locality of *S. thomense* (Bocage 1873), which is only specified to the level of the entire island ("Ile Saint Thomé") but our sampling spans most accessible settlements from the colonial period, and the type locality for *"S. ephele"* Taylor 1965 ("Agua Izé, 400-700 m, Ilha São Thomé"), which is likely between Água Izé, a coastal community on the eastern side of the island, and the community of Java at ~600 m that is directly inland of Água Izé (pers comm G. Doria, Museo Civico di Storia Naturale "G. Doria", Genova; Fig. 1). For all samples, we extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen Inc.,Valencia, CA, USA) and quantified DNA yield using a QUBIT 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA). All specimens are accessioned at the California Academy of Sciences.

Following previous studies (Nussbaum & Pfrender, 1998; Stoelting et al., 2014), one author (LAS) scored the coloration of all individuals included in the nuclear dataset as flecked or unflecked. For the newly collected specimens (n=12), we compared coloration between photographs in life and the voucher specimens after > 3 years of preservation to assess consistency. The remaining individuals were only scored as museum specimens following 13–18 years of preservation. Because colours fade in preservative, we were unable to score individuals with light versus dark brown flecking

or light versus dark yellow hue as previous authors have done; the presence or absence of flecking, however, remained prominent in older specimens.

Mitochondrial DNA sequencing and haplotype network estimation

To place the 12 newly sampled specimens within the Stoelting et al. (2014) dataset, we amplified a partial fragment of the NADH dehydrogenase 4 (*ND4*) gene following their methods. We assembled both reads and edited sequences in Geneious v.11.0 (Kearse et al., 2014) and combined them with sequences (n = 137) generated by Stoelting et al. (2014) downloaded from Genbank (Table S1). We aligned sequences using ClustalW v.2.1 (Larkin et al., 2007) and estimated a haplotype network using the TCS algorithm (Clement, Posada, & Crandall, 2000) implemented in PopART (Leigh & Bryant, 2015).

SNP dataset collection

We generated double-digest restriction site associated DNA (ddRAD) libraries (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) as described in the Supplementary Methods. Briefly, extractions were digested with the restriction enzymes *SbfI* and *MspI*, and the resulting fragments were tagged with individual barcodes, multiplexed into groups of 11 uniquely barcoded individuals and size selected for fragments between 434–538 bp. Barcode groups were PCR amplified, pooled, and sequenced on an Illumina® 2500 (SE 150 bp).

Raw data were processed using ipyrad v.0.7.30 (Eaton & Overcast, 2020). After demultiplexing, we removed seven samples with < 200,000 reads and one duplicate sample that was inadvertently sequenced twice. With the remaining 77 samples, we trimmed the first six bp to remove the restriction site, allowing a maximum of five low-quality base calls per read. We followed Ilut et al. (Ilut, Nydam, & Hare, 2014) to determine an optimal clustering threshold of 0.96 and up to 16 SNPs per 150 bp locus (at which additional SNPs per locus plateaued). We allowed no barcode mismatches and used the "strict" adapter filtering option, leaving all other parameters as default values. We required each site to be present in at least 70% (55/77) of samples, and dropped three

samples missing >90% of loci. To maximize sampling of independent SNP histories, we extracted one SNP per locus, producing a final dataset of 6772 SNPs for 74 individuals.

Characterizing population structure and the extent of hybridization

Using the 6772 SNP dataset we explored genomic structure using principal component analysis (PCA) with the *dudi.pca* function implemented in 'Ade4' v.1.7.11 (Drawy & Dufour, 2007). To determine the number of genetic demes and degree of admixture among demes we implemented the maximum likelihood approach implemented in ADMIXTURE V.1.3.0 (Alexander, Novembre, & Lange, 2009) with a range of *K* values (1–10) and five iterations per *K* value. Following the recommendation of Linck and Battey (Linck & Battey, 2019), we filtered our dataset for minor allele count = 3 using VCFtools v.0.1.15 (Danecek et al., 2011) to produce a dataset for ADMIXTURE analyses that contained 3270 SNPs.

To quantify the extent of hybridization between *S. thomense* and "*S. ephele"*, we used a maximum likelihood approach implemented in the *R* package 'HIest' v.2.0 (Fitzpatrick, 2012). This method jointly infers the ancestry index (*S*; the proportion of an individual's alleles descending from alleles in one parental lineage) and interclass heterozygosity (*H*; the proportion of an individual's loci that have one allele from each ancestral lineage). *H* values close to one indicate recent hybridization (F1, F2, or backcross generations) and values closer to 0 indicate hybridization in the more distant past. Considering both values together allowed us to quantify the temporal (in generations) and geographic extent of hybridization between lineages. Following developer recommendations to retain ancestry-informative markers, we identified 10 individuals from each lineage with strong concordance between genomic, mitochondrial, and morphological data ($Q \geq 0.9$ or ≤ 0.1 in the ADMIXTURE analysis) and <10% missing data in the 3270 SNP dataset. Based on these reference "parental" samples, we estimated allele frequencies for each locus using VCFtools and only retained loci fixed between lineages (parental allele frequencies ≥ 0.95 or ≤ 0.05). We removed individuals missing > 50% of sites (13 individuals) and loci missing > 50% of individuals (one locus) resulting in a final dataset of 41 SNPs and 64 individuals. We estimated *S* and *H* using the 'SANN' method, with 1000 MCMC iterations, a starting grid = 99, and surf = TRUE. 'HIest'

assumes a continuous model of hybridization but also includes a function to compare the fit of the model when classifying each individual as one of the six standard genotype frequency classes (parental, F1, F2 and backcrosses) with that of the continuous model. To differentiate between recent and historical hybridization, we used the function 'HIclass' to estimate likelihoods for early generation hybrids (F1, F2, backcrosses), and 'HItest' to compare likelihoods to those from the continuous model.

Testing alternative demographic histories

To test alternative models of diversification history, we used the diffusion approximation method implemented in δaδi (Gutenkunst, 2009). We tested 18 historical demographic models from Portik et al., (2017) including divergence in allopatry versus divergence with continuous gene flow, secondary contact versus contemporary isolation, and instantaneous size change (full range of models shown in Portik et al., (2017); Table S2). We generated a folded two-dimensional Site Frequency Spectrum (2D-SFS) from the VCF format output from ipyrad (https://github.com/isaacovercast/easySFS). To account for missing data among individuals, we down-projected our SNP dataset to 25 diploid individuals with 3570 SNPs for *S. thomense* and 25 diploid individuals with 4377 SNPs for "*S. ephele"*. We also ran the analysis without putative hybrid individuals (>10% admixed) to ensure that these individuals did not bias model selection (*S. thomense*: 19 diploid individuals with 3109 SNPs; "*S. ephele"*: 15 diploid individuals with 3617 SNPs).

Following Portik et al. (2017) and Barratt et al. (2018), we used modified scripts from dadi_pipeline (https://github.com/dportik/dadi_pipeline) to perform five iterations of each model consisting of four rounds of optimizations with multiple replicates (see below). We used search parameter estimates from the best scoring replicate (highest log-likelihood) to seed searches in the following round. We used the following settings for each round of dadi_pipeline: grid size = 50, 60, 70; replicates = 10, 20, 30, 40; maxiter = 3, 5, 10, 15; fold = 3, 2, 2, 1. We optimized parameters using the Nelder-Mead approach (optimize_log_fmin), and used the optimized parameter sets of each replicate to simulate the 2D-SFS. The log-likelihood of each 2D-SFS was estimated for each model using a multinominal approach, we identified the best-supported model using log-likelihood and

AIC, and used the ΔAIC scores to calculate Akaike model weights (ωi). Goodness of fit tests were performed following Barratt et al., (2018) and were based on 250 simulated frequency spectra.

We estimated the divergence time between *S. thomense* and "*S. ephele"* using the Bayesian coalescent-based program G-PhoCS v.1.3 (Gronau, Hubisz, Gulko, Danko, & Siepel, 2011). By incorporating entire loci (as opposed to SNPs), G-PhoCS facilitates the conversion of posterior estimates to years using locus-based mutation rates. Due to computational constraints and developer recommendations, we used 2,000 loci and 10 individuals per lineage. To reduce potential biases introduced by admixed individuals or missing data, we sampled individuals with ancestry coefficients corresponding to > 95% of the assigned lineage and sequence data for > 90% of the loci**.** The δaδi model with the highest support indicated that divergence occurred in the absence of gene flow (see Results); thus we applied no migration bands in G-PhoCS. We followed Prates et al., (2018) to estimate prior ranges in G-PhoCS (scripts available at https://github.com/ivanprates/2018_Anolis_EcolEvol); we applied a gamma distribution to the θ (genetic diversity) and τ (root age) priors given by shape $\alpha = 1$ and rate $\beta = 275$ (mean = 0.00363). We ensured our distribution encompassed a range of θ values from 0.002 to 0.00568 based on an island-wide *Ne* estimate of 500,000 individuals (extrapolated from Measey (2006)), which we converted to θ based on the equation $4*Ne*\mu$ using upper and lower bounds for mutation rates: 1.42×10^{-9} and 2.14×10^{-9} substitutions per site per year (estimated for two frog genera by Allio, Donega, Galtier, & Nabholz, (2017)). To improve chain mixing, we applied a 500,000 generation burnin and ran the analysis for 2,000,000 generations sampling every 10,000 generations and checked Markov chain mixing in Tracer v.1.6 (Rambaut, Drummon, Xie, Baele, & Suchard, 2018). We converted our posterior estimate of the root using the mean of the two amphibian mutation rates from Allio et al., (2017), and a generation time of two years (Haft & Franzen, 1996).

Environmental variation across sampling sites

To examine whether the two lineages of São Tomé caecilians are ecologically divergent, we assessed associations for admixed and non-admixed individuals (≥90%

assignments) with landscape gradients of climate, topography, land cover, and soil type/age (both associated with periods of underlying volcanic activity). We extracted bioclimatic variables from the WorldClim database (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) that describe spatial patterns of temperature and precipitation variation. Moreover, we included geomorphological variables that likely impact fossorial organisms: elevation, land cover, and soil type/age (Caldeira & Munhá, 2002; Soares, 2017; Stoelting et al., 2014). Values were extracted from the collection sites of samples (only those for which we generated genomic data) using QGIS v.2.18.15 (available at https://github.com/qgis/QGIS). Due to the logistical difficulty of surveying the south-eastern quadrant of the island, this region remains largely uncharacterised for most variables and no caecilian specimens from this region were available for study. For continuous variables (precipitation, temperature, and elevation) we fitted ANOVAs grouping by *S. thomense*, "*S. ephele"*, and admixed individuals, and used a Tukey Honest Significant Differences test to calculate adjusted P values for group mean comparisons.

RESULTS

Phenotypic variation in São Tomé caecilians

With few exceptions, flecked and unflecked phenotypes were geographically separated across São Tomé Island, with phenotypic turnover around the latitudinal midpoint of the island (Fig. 1; S1A). Only four out of 21 localities included in this study contained both phenotypes (Bom Sucesso, Contador South, Santa Luzia, and Lemba).

Geographic structure and evidence of hybridization

The mtDNA haplotypes from the combined datasets were consistent with the clear northern and southern haplotype groups in Stoelting et al. (2014) and overlap zone in the centre of the island, where both haplotypes were present at four localities (Anselmo Andrade, Bombaim, Java, and Santa Luzia; Figs. 1B, C; S1B, C).

A genetic PCA based on the SNP dataset identified two clusters that largely corresponded to morphological and mitochondrial patterns, with the exception of individuals around the putative contact zone (Fig. 1A,B). Cross-validation of our ADMIXTURE analysis inferred roughly equal support for $K = 2$–4 (Fig. S2C), with $K = 2$

splitting individuals into northern and southern groups corresponding to the PCA group assignments and the deepest split in the mitochondrial network. We assigned individuals to *S. thomense* (north) or "*S. ephele"* (south) based on ancestry coefficients >0.90, and considered those individuals with lower coefficients as admixed for downstream analyses (restricted to Contador South, Java, Lemba, Santa Fe, and Santa Luzia; Fig. 1A). Higher *K* values (*K* = 3–4) further subdivided *S. thomense* but did not correspond to the fine-scale mtDNA structure recovered by Stoelting et al. (2014; Fig. S2).

HIest analyses based on the set of 41 ancestry-informative SNPs assigned 22 individuals to non-admixed *S. thomense* (*S* value ≤ 0.1), 14 individuals as non-admixed "*S. ephele"* (*S* value ≥ 0.9) and 28 individuals as admixed (Fig. 2A, Table S1). *H* values for admixed individuals ranged from 0 to 0.65 (Table S1), consistent with multiple generations of hybridization. Plotting *H* values relative to latitude indicated that hybridization is restricted to the centre of the island (Fig. 2B; S3). In all individuals, hybrid classifications under the continuous model were at least 2 log-likelihood units better than the best classification of early hybrid classes (F1, F2, backcrosses), thus rejecting early hybrid classes in all cases.

Demographic history of São Tomé caecilians

Demographic modelling using δaδi based on SNP data from all samples supported a model of divergence in isolation, followed by instantaneous expansion in both lineages and secondary contact with ongoing symmetric migration (Fig. 3; Table S2; ΔAIC = 83.5, ωi = 1.0). The second-best model was a three epoch model of divergence in isolation, followed by instantaneous expansion in both lineages and secondary contact with ongoing symmetric migration, followed by isolation in the recent past. When admixed individuals were excluded, this same three epoch model was best-supported (ΔAIC = 10, ωi = 1.0). Because there are no SNP-based mutation rate estimates for caecilians we refrain from converting our unscaled parameters here; however, several inferences can still be made. First, historical effective population size before and after expansion was larger for "*S. ephele"* than for *S. thomense* and *S. thomense* experienced a greater magnitude change in population size (~5x) relative to "*S. ephele"* (~2x; Table S2). Second, the relative time between secondary contact and the present (unscaled value

of 0.24) was about half that between initial divergence and secondary contact (0.52). Parameter estimates were consistent between the two and three epoch models (Table S2). Goodness of fit tests showed that our empirical values fell slightly outside simulated distributions, indicating a poor fit of the best-supported model to the data (Fig. S4). Poor model fit suggests that our cohort of models may be over-simplistic to capture the true evolutionary history of these caecilians (Excoffier et al., 2013). Future studies testing more complex models will benefit from more comprehensive sampling of genomic variation and additional sampling localities in the southern half of São Tomé.

Divergence time estimates using G-PhoCS (based on entire loci with locus-based mutation rates) indicated that initial divergence between *S. thomense* and "*S. ephele"* occurred ~303.4 kya (280.9–325.8 kya). We used this mean divergence date to convert our scaled time estimates from δaδi to infer that secondary contact occurred ~95.1 kya (88.0–101.1 kya; Fig. 3).

Environmental variation across sampling sites

We inferred that caecilians occupy a broad environmental space on São Tomé, ranging from habitats receiving between 800 and > 1400 mm/yr of precipitation (Fig. 4). Our sampling indicated that caecilians occur in most soil types/ages on the island (Fig. 4), and in all vegetation types (Fig. 4). The two lineages segregated strongly in environmental space, particularly along a gradient of precipitation (Fig. 4), with *S. thomense* occurring in drier habitats than admixed and "*S. ephele"* caecilians (adjusted $P < 0.001$). Although *S. thomense* appear to inhabit a wider range of elevations and temperatures than "*S. ephele"*, occurrence with respect to these two variables does not differ between the species (Fig. S5; adjusted $P > 0.05$). The distribution of *S. thomense* encompasses the younger basaltic lavas (<1 Mya) that dominate the central and northern half of the island and the contact zone between the lineages roughly coincides with the transition between the younger (<1 Mya) and older (3–8 Mya) basaltic lavas in the centre of the island. *Schistometopum thomense* were also associated with alluvial soils in the northern coastal plain (Fig. 4) whereas, "*S. ephele"* were associated with the older basaltic lavas (3–8 Mya) and volcanic cone formations on the southern half of the island. Caecilians occupied non-forested habitats across the island, but *S. thomense* and admixed

individuals were primarily found in shaded plantation and non-forested habitats, whereas "*S. ephele"* were found in native forest, secondary forest, and shaded plantation but not in non-forested habitat (Fig. 4).

DISCUSSION

Our genetic and phenotypic data support a history of within-island divergence in allopatry for São Tomé caecilians, followed by secondary contact and hybridization that have blurred lineage boundaries rather than a history of divergence with gene flow along an ecological gradient. The common ancestor of *S. thomense* and "*S. ephele"* arrived on the island recently, having diverged from its East African sister species *S. gregorii* ~1 Mya (Loader et al., 2007). Correspondingly, using coalescent methods we estimated that subsequent *in situ* divergence occurred ~300 kya, which is comparable to previous estimates derived from analyses of mitochondrial sequence data (Stoelting et al., 2014). Several other São Tomé organisms with contiguous contemporary distributions have a history of *in situ* diversification during the Pleistocene, including reed frogs 1.7–0.5 Mya (Bell et al., 2015), freshwater crabs 1.5–0.5 Mya (Daniels & Klaus, 2018), and fruit flies ~400 kya (Llopart, Elwyn, Lachaise, & Coyne, 2002; Llopart, Lachaise, & Coyne, 2005). These estimates broadly coincide with the most recent period of volcanic eruptive activity on São Tomé from 36–860 kya (Barfod & Fitton, 2014). Thus, lineage divergence across co-distributed groups may be associated with catastrophic late Pleistocene volcanic lava flows fragmenting species distributions and interrupting gene flow. These findings are consistent with the hypothesis that volcanic flows are an important but ephemeral mechanism for allopatric divergence in volcanic island systems (Juan, Emerson, Oromí, & Hewitt, 2000), as has been documented in a variety of taxa, including flowering plants (Brochmann, 1984), lizards (Bloor et al., 2008), birds (Milá et al., 2010) and orangutans (Nater et al, 2011).

Besides imposing transient physical barriers to gene flow, volcanic eruptions may also favour divergence through local adaptation when populations become isolated in distinct environments. This hypothesis is consistent with associations of the two caecilian lineages within distinct precipitation regimes and habitats across the island (Fig. 4), which may reflect local adaptation to specific soil microhabitats (Torres-Sánchez et al.,

2019) as demonstrated in other fossorial vertebrates (Martín, López, & García, 2013; Fouquet et al., 2021). Associations between habitat type and lineage divergence were also reported in reed frogs (Bell & Irian, 2019) and fruit flies (Coyne et al., 2002; Matute & Coyne, 2010) on São Tomé, suggesting that this pattern may be widespread across a variety of organisms on the island, although the specific mechanisms of local adaptation are likely to differ between fossorial versus surface-dwelling taxa. These observations may be somewhat confounded by the strong correlation between geography and environmental variation on São Tomé; however, similar associations have also been documented in organisms from other small volcanic islands including lizards from the Canary Islands (Brown, Woods, & Thorpe, 2017; Gübitz, Thorpe, & Malhotra, 2005; Pestano & Brown, 1999; Suárez, et al., 2014) and birds from Réunion (Gabrielli, Nabholz, Leroy, Milá, & Thébaud, 2020). Studies of climate-dependent competitive outcomes (e.g., Comeault & Matute 2021) and functional genomic variation may provide deeper insights as to the relative contributions of geographic barriers and environmental variation to lineage diversification on small (< 2500 km^2) oceanic islands (e.g., Brown et al., 2016).

Secondary contact may be pervasive when allopatric divergence results from ephemeral barriers on small oceanic islands (e.g. Brown et al., 2017). Accordingly, historical demographic analyses inferred that the São Tomé caecilian lineages came into secondary contact ~95 kya and that both lineages have experienced recent population expansion, with a greater magnitude of expansion in *S. thomense* (Fig. 3A; Table S2). This difference in expansion is consistent with more extensive and recent volcanic activity across the northern half of the island where *S. thomense* occurs (Barfod & Fitton, 2014). We hypothesize that secondary contact and population size change occurred following the expansion of suitable habitat after the erosion of lava flows, thus facilitating contact between previously separated lineages. Hybrid zones are maintained by selection against hybrid phenotypes, particularly when parental species are locally adapted (Barton & Hewitt, 1985; Mallet & Barton, 1989; Kisel & Barraclough, 2010). Consequently, small islands with distinct habitat transitions may result in particularly narrow hybrid zones (Cooper, Sedghifar, Nash, Comeault, & Matute, 2018).

In São Tomé caecilians, the contact zone appears to coincide with the transition between the younger (<1 Mya) and older (3–8 Mya) basaltic lavas in the centre of the island but we did not find a clear association between the parental species or hybrids with our broad classifications of soil type. By contrast, we found significant associations between parental species and hybrids with precipitation (Fig 4. B,C) suggesting there may be selection against hybrids in the driest parts of the island resulting in a stable hybrid zone in the centre of the island. In addition, *S. thomense* lack flecking and occur in drier habitats, while "*S. ephele"* and most hybrid individuals are flecked and occur in wetter habitats indicating there may be habitat-associated selection for divergence in coloration (Lemoine et al., 2019). Divergence in coloration between xeric and mesic habitats across small spatial scales is prevalent in other small island study systems including *Anolis* lizards in the Lesser Antilles (e.g., Lazell, 1972, Muñoz et al., 2013, Thorpe et al., 2015), lizards in the Canary Islands (Thorpe & Brown, 1989; Brown, Thorpe & Báez, 1991; Suárez et al., 2014; Brown et al., 2016), and Galapagos land snails (Kraemer, Philip, Ranken & Parent, 2018). Experimental approaches may clarify whether divergent and strong natural selection for locally adapted physiology and/or camouflage underlie correlations between coloration and environment in São Tomé Caecilians.

Differences in mate choice among incipient/recent species can also be an important mechanism for reproductive isolation (Mayr, 1963; Richie, 2007) and reinforcement can lead to greater divergence in such traits when hybridization is maladaptive (Butlin, 1987). Courtship behaviour and potential pre-zygotic or post-zygotic reproductive barriers in caecilians are very poorly understood, but molecular analyses by Torres-Sánchez et al., (2020) suggest the potential for both sexes to use species-specific peptide pheromones for species recognition and mate choice. Quantifying the peptide pheromone composition of São Tomé caecilians and their hybrids may provide further insights into this signalling modality and its role in speciation. Further, caecilians exhibit internal fertilization via an intromittent organ formed by an eversible portion of the cloaca that varies in shape and ornamentation among species (Gower & Wilkinson, 2002; Wake, 1972). Characterizing variation in phallus morphology among and within lineages of *Schistometopum* on São Tomé and in their East African relatives may clarify whether this structure plays an analogous role in

reproductive isolation to the baculum in placental mammals (Ramm, 2007) and hemipenes in squamate reptiles (Klaczko, Ingram, & Losos, 2015).

Conclusions

Our study joins a growing number of systems demonstrating speciation at small spatial scales on islands (Savolainen et al., 2006; Kisel & Barraclough, 2010; Heaney et al., 2018; Bourgeois et al., 2020; Gabrielli et al., 2020; Osborne et al., 2020). We propose that transient geographic barriers coupled with local adaption across environmental gradients can contribute to the accumulation of phenotypic and taxonomic diversity. Our integrative morphological and genetic analyses support two discrete lineages corresponding to *S. thomense* and "*S. ephele"* with a narrow zone of admixture in the centre of São Tomé Island. Demographic modelling supports a history of allopatric divergence in the late Pleistocene followed by secondary contact and hybridization, rather than a scenario of divergence with continuous gene flow. Based on this evolutionary history, we recommend recognizing these lineages as distinct species and remove *S. ephele* Taylor 1965 from synonymy with *S. thomense* (Bocage 1873).

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DATA ACCESSIBILITY

Genomic data are deposited on the SRA database (SRR12676998–SRR12677081), mtDNA deposited on Genbank (MW290290–MW290301). Input files and scripts for all analyses deposited on figshare (https://doi.org/10.25573/data.13085159.v1) and github.com/kyleaoconnell22/sao_tome_caecilians.

AUTHOR CONTRIBUTIONS

KAO, LAS and RCB conceived of the study. LAS and RCB conducted field sampling. KAO collected genetic data and LAS collected morphological data. KAO and IP implemented analyses with contributions from KPM and RCB. KAO, RCB and IP drafted the manuscript with contributions from all authors.

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Figure Legends

Figure 1: *Schistometopum* sampling on São Tomé Island. A) Map shows distribution of genomic samples with the size of circles proportional to the number of individuals at that site. Individuals with at least 90% ancestry assigned to *S. thomense* are shown in purple,

90% ancestry assigned to "*S. ephele"* in green, and admixed individuals in orange. Site abbreviations are as follows: AA = Anselmo Andrade, BO = Bombaim, BS = Bom Sucesso, CN = Contador Valley North, CS = Contador Valley South, CV = Canavial, JA = Java + Abade, LB = Lemba River, ML = Rio Maria Luisa, ON = Obo National Park, PA = Porto Alegre, QI = Quisinda, RD = Rio d'Ouro, SF = Santa Fe, SL = Santa Luzia. The type locality of " *S. ephele*" (Água Izé, 400-700m) is likely between the coastal community of Água Izé (indicated by black star) and Java. B) Plot of ancestry coefficients estimated with ADMIXTURE V.1.3.0 (Alexander et al., (2009) for $K = 2$. Circles above the plot show the haplotype of each individual from the mitochondrial *ND4* locus, and morphological assignment (yellow, unflecked = yellow; brown, flecked = gray). C) *ND4* haplotype network for new samples and previously published data (Stoelting et al., 2014) estimated in PopART (Leigh & Bryant, 2015). 26 mutations separate the haplotype groups. D) Principal component analysis of SNP data with individuals coloured according to their ancestry assignment from (B). Photo credits: A. Stanbridge.

Figure 2: Results of the 'HIest' v.2.0 (Fitzpatrick, 2012) analysis. A) Joint maximum likelihood estimates of ancestry (*S* value) and interclass heterozygosity (*H* value) for *S. thomense* and "*S. ephele"* for 41 diagnostic SNPs. A) Individuals are coloured by morphology (yellow, unflecked = yellow; brown, flecked = gray) indicating that most admixed individuals (intermediate *S* and *H* values) are flecked. B) *H* values plotted against latitude show that admixed individuals are restricted to the centre of the island at the contact zone. Individuals are coloured according to *S* values (≥ 0.9 or ≤ 0.1).

Figure 3: Results of demographic modelling (δaδi; Gutenkunst et al., 2009) and demographic parameter estimation (G-PhoCS; Gronau et al., 2011) analyses. A) Stylized representation of the best supported model from δaδi with parameters superimposed from G-PhoCS. B) The fit between the best-supported model and the data is shown using the two-dimensional site frequency spectrum (2D-SFS) and plots of the residuals.

Figure 4: Summary of environmental space occupancy analyses. A) Photos of habitat in representative dry (top) and wet (bottom) regions of São Tomé Island. B) Violin plot of precipitation values at sites for pure and admixed caecilians (top), bar plots of land cover (middle), and bar plots of soil types and ages (bottom). C) Annual precipitation (mm) across the island, with drier habitat in the north and wetter habitat in the south (top), land cover across the island, adapted from Soares (2017; middle), and soil types and ages across the island, adapted from Caldeira & Munhá (2002) and Stoelting et al. (2014; bottom). Photo credits: J. Shevock, A. Stanbridge.

Supporting Information

Figure S1: A) Distribution of phenotypes for individuals included in the genomic analyses (yellow, unflecked = yellow; brown, flecked = gray). Map shows elevation. B) Distribution of mtDNA haplotypes for 152 individuals. Adjacent contact zone localities (Bombaim, Java and Abade; Santa Luzia, Macambrara, and Radio Antenna) are grouped together for clarity. Sequence data generated for this study are designated with black arrows, all other sequences were generated by Stoelting et al. (2014). *ND4* haplotype network estimated in PopART (Leigh & Bryant, 2015). 26 mutations separate the haplotype groups.

Figure S2: A) Map and plot of ancestry coefficients estimated from 3270 SNP dataset analysed with ADMIXTURE V.1.3.0 (Alexander et al., 2009) for $K = 3$, and B) $K = 4$. Additional K values do not correspond to the additional mitochondrial lineages inferred by Stoelting et al. (2014). C) Cross validation error plots from ADMIXTURE analysis showing roughly equal support for $K = 2$–4.

Figure S3: Ancestry (S value) estimated in 'HIest' v.2.0 (Fitzpatrick, 2012) plotted against latitude showing that individuals with intermediate ancestry values (likely admixed) are restricted to the centre of the island at the contact zone. Individuals are coloured according to S values (≥ 0.9 or ≤ 0.1).

Figure S4: Visualization of goodness of fit test from demographic modelling analyses showing empirical result (blue bar) plotted on the distribution of simulated values across 100 simulations (gray bars) for log likelihood (A) and log-transformed chi-squared test statistic (B). Empirical values outside of the simulated distribution suggest poorer model fit.

Figure S5: Summary of additional environmental space occupancy analyses. A) Violin plot of elevation (m) at sites for pure (≥0.9 or ≤0.1) and admixed caecilians, and B) map of elevation. C) Violin plot of temperature at sites for pure and admixed caecilians, and D) map of temperature.

Table S1: Locality, catalogue number, and summary data for samples included in this study.

Table S2: Output summary for demographic modelling (δaδi; Gutenkunst et al., 2009) analyses. In all cases nu1 = *S. thomense* and nu2 = "*S. ephele*"