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DR. KYLE ANTHONY O'CONNELL (Orcid ID : 0000-0002-0464-9259)

MR. IVAN PRATES (Orcid ID : 0000-0001-6314-8852)

DR. KEVIN P MULDER (Orcid ID : 0000-0001-6688-8848)

DR. RAYNA CAMILLE BELL (Orcid ID : 0000-0002-0123-8833)

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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

Kyle A. O'Connell*^{1,2,3}, Ivan Prates^{1,4}, Lauren A. Scheinberg⁵, Kevin P. Mulder^{1,6,7},
Rayna C. Bell*^{1,5}

¹ *Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA.*

² *Global Genome Initiative, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA.*

³ *Department of Biological Sciences, George Washington University, Washington, DC 20052, USA.*

⁴ *Department of Ecology and Evolutionary Biology and Museum of Zoology, University of Michigan, Ann Arbor, MI 48109, USA.*

⁵ *Department of Herpetology, California Academy of Sciences, San Francisco, CA 94118, USA.*

⁶ *CIBIO/InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, 4485-661 Vairão, Portugal.*

⁷ *Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC 20008, USA.*

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32 *Corresponding authors: Rayna C. Bell, rbell@calacademy.org, [https://orcid.org/0000-](https://orcid.org/0000-0002-0123-8833)
33 [0002-0123-8833](https://orcid.org/0002-0123-8833); Kyle A. O'Connell, kyleaoconnell22@gmail.com;
34 <https://orcid.org/0000-0002-0464-9259>

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36

37 **ABSTRACT**

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

61

62 RESUMO

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

88

89

90 Keywords:

91 amphibian, gene flow, hybridization, in situ diversification, island speciation,
92 *Schistometopum ephela*, *Schistometopum thomense*

93

94

95 **INTRODUCTION**

96 A period of isolation in allopatry typically precedes local adaptation and
97 subsequent lineage divergence that may ultimately result in speciation (Losos & Ricklefs,
98 2009; Mayr, 1963). Secondary contact of lineages following transient periods of
99 allopatric divergence can result in lineage fusion or promote reproductive isolation
100 through reinforcement (Servidio & Noor, 2003; Choi, Purugganan, & Stacy 2020).
101 Alternatively, locally adapted phenotypes may arise and persist in the face of gene flow
102 resulting in strong correlations between ecologically-relevant phenotypic variation and
103 corresponding environmental gradients (Thorpe, Barlow & Surget-Groba 2015).
104 Quantifying genetic, ecological, and phenotypic variation in these nascent lineages can
105 provide insights into the abiotic and biotic mechanisms that structure populations and
106 ultimately drive the accumulation of species richness and phenotypic diversity.
107 Organisms with low dispersal potential whose distributions span ephemeral geographic
108 barriers present the ideal evolutionary context within which to understand the relative
109 contributions of these evolutionary processes. Here we investigate the divergence history
110 of the enigmatic and fossorial caecilians (Amphibia: Gymnophiona) endemic to the
111 small, volcanic island of São Tomé.

112 Physical barriers such as rivers (Vences, Wollenberg, Vieites, & Lees, 2009;
113 Welton et al., 2010), sea level changes (Esselstyn, Timm, & Brown, 2009; O'Connell,
114 Hamidy, Kurniawan, Smith, & Fujita, 2018), or volcanic lava flows (Bloor, Kemp, &
115 Brown, 2008; Brochmann, 1984; Nater et al, 2011) often contribute to allopatric
116 divergence. Landscapes are dynamic, however, and the elimination of such barriers can
117 lead to population expansion, secondary contact, hybridization, and fusing of incipient
118 species, particularly on small oceanic islands (García-Olivares et al., 2017; Garrick et al.,
119 2014; Gow, Peichel, & Taylor, 2006; Grant & Grant, 1996; MacLeod et al., 2015;
120 Roderick, Croucher, Vandergast & Gillespie, 2012; Sardell & Uy, 2016; Taylor et al.,
121 2006). Spatial environmental gradients such as differences in rainfall, temperature, or soil
122 type may further reinforce divergence in allopatry (Losos & Schluter, 2000; Rundle &
123 Nosil, 2005). These environmental transitions can also lead to stable hybrid zones if
124 lineages that meet secondarily are locally adapted (Barton & Hewitt, 1985).

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

141 Growing evidence suggests that both environmental gradients and a dynamic
142 landscape history shaped species diversification on São Tomé, a volcanic island ~225 km
143 off the coast of West-Central Africa in the Gulf of Guinea archipelago. The island
144 emerged from the sea floor ~13 Mya, and despite its small size (~850 km²), it is
145 topographically complex, with its highest peak at 2024 m (Gillespie & Clague, 2009).
146 Correspondingly, São Tomé exhibits environmental gradients ranging from drier and
147 open habitat in the north to wetter and forested habitat in the south (de Lima et al., 2017;
148 Soares, 2017). Despite the island's long geologic history, many studies of taxonomic
149 diversification within São Tomé have inferred that divergence occurred during the
150 Pliocene or Pleistocene (Bell et al., 2015; Daniels & Klaus, 2018; Stoelting, Measey, &
151 Drewes, 2014), which coincides with a period of extensive volcanic activity (Barfod &
152 Fitton, 2014). Further, a history of *in situ* divergence in allopatry followed by secondary
153 contact and hybridization was inferred in São Tomé *Drosophila* (Coyne, Kim, Chang,
154 Lachaise, & Elwyn, 2002; Matute & Coyne, 2010) and *Hyperolius* reed frogs (Bell,
155 Drewes, & Zamudio, 2015; Bell & Irian, 2019). By contrast, both adaptation along

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

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

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

189

190 **MATERIALS AND METHODS**

191

192 **Field and museum specimen sampling and colour pattern assessment**

193 ■ For genetic analyses, we included 85 samples from 21 localities across the island
194 (Table S1). Among them, 12 samples were collected by the authors between 2012 and
195 2016 at three localities including Obo National Park, which had not previously been
196 sampled. Tissue samples (liver) were preserved in 95% ethanol or RNAlater for
197 subsequent DNA extraction and genetic analyses. Additionally, we selected a subset of
198 73 specimens from the Stoelting et al. (2014) mtDNA study from which to collect
199 nuDNA SNP data (see below). This sampling spans the type locality of *S. thomense*
200 (Bocage 1873), which is only specified to the level of the entire island (“Ile Saint
201 Thomé”) but our sampling spans most accessible settlements from the colonial period,
202 and the type locality for “*S. ephèle*” Taylor 1965 (“Água Izé, 400-700 m, Ilha São
203 Thomé”), which is likely between Água Izé, a coastal community on the eastern side of
204 the island, and the community of Java at ~600 m that is directly inland of Água Izé (pers
205 comm G. Doria, Museo Civico di Storia Naturale “G. Doria”, Genova; Fig. 1). For all
206 samples, we extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia,
207 CA, USA) and quantified DNA yield using a QUBIT 2.0 Fluorometer (Life
208 Technologies, Grand Island, NY, USA). All specimens are accessioned at the California
209 Academy of Sciences.

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

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

219

220 **Mitochondrial DNA sequencing and haplotype network estimation**

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

229

230 **SNP dataset collection**

231 We generated double-digest restriction site associated DNA (ddRAD) libraries
232 (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) as described in the Supplementary
233 Methods. Briefly, extractions were digested with the restriction enzymes *SbfI* and *MspI*,
234 and the resulting fragments were tagged with individual barcodes, multiplexed into
235 groups of 11 uniquely barcoded individuals and size selected for fragments between 434–
236 538 bp. Barcode groups were PCR amplified, pooled, and sequenced on an Illumina®
237 2500 (SE 150 bp).

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

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

249

250 **Characterizing population structure and the extent of hybridization**

251 Using the 6772 SNP dataset we explored genomic structure using principal
252 component analysis (PCA) with the *dudi.pca* function implemented in ‘Ade4’ v.1.7.11
253 (Drawy & Dufour, 2007). To determine the number of genetic demes and degree of
254 admixture among demes we implemented the maximum likelihood approach
255 implemented in ADMIXTURE v.1.3.0 (Alexander, Novembre, & Lange, 2009) with a range
256 of K values (1–10) and five iterations per K value. Following the recommendation of
257 Linck & Battey (Linck & Battey, 2019), we filtered our dataset for minor allele count =
258 3 using VCFtools v.0.1.15 (Danecek et al., 2011) to produce a dataset for ADMIXTURE
259 analyses that contained 3270 SNPs.

260 To quantify the extent of hybridization between *S. thomense* and “*S. ephèle*”, we
261 used a maximum likelihood approach implemented in the *R* package ‘HIest’ v.2.0
262 (Fitzpatrick, 2012). This method jointly infers the ancestry index (S ; the proportion of an
263 individual’s alleles descending from alleles in one parental lineage) and interclass
264 heterozygosity (H ; the proportion of an individual’s loci that have one allele from each
265 ancestral lineage). H values close to one indicate recent hybridization (F1, F2, or
266 backcross generations) and values closer to 0 indicate hybridization in the more distant
267 past. Considering both values together allowed us to quantify the temporal (in
268 generations) and geographic extent of hybridization between lineages. Following
269 developer recommendations to retain ancestry-informative markers, we identified 10
270 individuals from each lineage with strong concordance between genomic, mitochondrial,
271 and morphological data ($Q \geq 0.9$ or ≤ 0.1 in the ADMIXTURE analysis) and <10% missing
272 data in the 3270 SNP dataset. Based on these reference “parental” samples, we estimated
273 allele frequencies for each locus using VCFtools and only retained loci fixed between
274 lineages (parental allele frequencies ≥ 0.95 or ≤ 0.05). We removed individuals missing >
275 50% of sites (13 individuals) and loci missing > 50% of individuals (one locus) resulting
276 in a final dataset of 41 SNPs and 64 individuals. We estimated S and H using the ‘SANN’
277 method, with 1000 MCMC iterations, a starting grid = 99, and surf = TRUE. ‘HIest’

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

284

285 **Testing alternative demographic histories**

286 To test alternative models of diversification history, we used the diffusion
287 approximation method implemented in $\delta\text{a}\delta\text{i}$ (Gutenkunst, 2009). We tested 18 historical
288 demographic models from Portik et al., (2017) including divergence in allopatry versus
289 divergence with continuous gene flow, secondary contact versus contemporary isolation,
290 and instantaneous size change (full range of models shown in Portik et al., (2017); Table
291 S2). We generated a folded two-dimensional Site Frequency Spectrum (2D-SFS) from
292 the VCF format output from ipyrad (<https://github.com/isaacovercast/easySFS>). To
293 account for missing data among individuals, we down-projected our SNP dataset to 25
294 diploid individuals with 3570 SNPs for *S. thomense* and 25 diploid individuals with 4377
295 SNPs for “*S. ephèle*”. We also ran the analysis without putative hybrid individuals
296 (>10% admixed) to ensure that these individuals did not bias model selection (*S.*
297 *thomense*: 19 diploid individuals with 3109 SNPs; “*S. ephèle*”: 15 diploid individuals
298 with 3617 SNPs).

299 Following Portik et al. (2017) and Barratt et al. (2018), we used modified scripts
300 from `dadi_pipeline` (https://github.com/dportik/dadi_pipeline) to perform five iterations
301 of each model consisting of four rounds of optimizations with multiple replicates (see
302 below). We used search parameter estimates from the best scoring replicate (highest log-
303 likelihood) to seed searches in the following round. We used the following settings for
304 each round of `dadi_pipeline`: grid size = 50, 60, 70; replicates = 10, 20, 30, 40; maxiter =
305 3, 5, 10, 15; fold = 3, 2, 2, 1. We optimized parameters using the Nelder-Mead approach
306 (`optimize_log_fmin`), and used the optimized parameter sets of each replicate to simulate
307 the 2D-SFS. The log-likelihood of each 2D-SFS was estimated for each model using a
308 multinomial approach, we identified the best-supported model using log-likelihood and

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

312 We estimated the divergence time between *S. thomense* and “*S. ephèle*” using the
313 Bayesian coalescent-based program G-PhoCS v.1.3 (Gronau, Hubisz, Gulko, Danko, &
314 Siepel, 2011). By incorporating entire loci (as opposed to SNPs), G-PhoCS facilitates the
315 conversion of posterior estimates to years using locus-based mutation rates. Due to
316 computational constraints and developer recommendations, we used 2,000 loci and 10
317 individuals per lineage. To reduce potential biases introduced by admixed individuals or
318 missing data, we sampled individuals with ancestry coefficients corresponding to > 95%
319 of the assigned lineage and sequence data for > 90% of the loci. The $\delta a d i$ model with the
320 highest support indicated that divergence occurred in the absence of gene flow (see
321 Results); thus we applied no migration bands in G-PhoCS. We followed Prates et al.,
322 (2018) to estimate prior ranges in G-PhoCS (scripts available at
323 https://github.com/ivanprates/2018_Anlis_EcolEvol); we applied a gamma distribution
324 to the θ (genetic diversity) and τ (root age) priors given by shape $\alpha = 1$ and rate $\beta = 275$
325 (mean = 0.00363). We ensured our distribution encompassed a range of θ values from
326 0.002 to 0.00568 based on an island-wide N_e estimate of 500,000 individuals
327 (extrapolated from Measey (2006)), which we converted to θ based on the equation
328 $4 * N_e * \mu$ using upper and lower bounds for mutation rates: 1.42×10^{-9} and 2.14×10^{-9}
329 substitutions per site per year (estimated for two frog genera by Allio, Donega, Galtier, &
330 Nabholz, (2017)). To improve chain mixing, we applied a 500,000 generation burnin and
331 ran the analysis for 2,000,000 generations sampling every 10,000 generations and
332 checked Markov chain mixing in Tracer v.1.6 (Rambaut, Drummon, Xie, Baele, &
333 Suchard, 2018). We converted our posterior estimate of the root using the mean of the
334 two amphibian mutation rates from Allio et al., (2017), and a generation time of two
335 years (Haft & Franzen, 1996).

336

337 **Environmental variation across sampling sites**

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

340 assignments) with landscape gradients of climate, topography, land cover, and soil
341 type/age (both associated with periods of underlying volcanic activity). We extracted
342 bioclimatic variables from the WorldClim database (Hijmans, Cameron, Parra, Jones, &
343 Jarvis, 2005) that describe spatial patterns of temperature and precipitation variation.
344 Moreover, we included geomorphological variables that likely impact fossorial
345 organisms: elevation, land cover, and soil type/age (Caldeira & Munhá, 2002; Soares,
346 2017; Stoelting et al., 2014). Values were extracted from the collection sites of samples
347 (only those for which we generated genomic data) using QGIS v.2.18.15 (available at
348 <https://github.com/qgis/QGIS>). Due to the logistical difficulty of surveying the south-
349 eastern quadrant of the island, this region remains largely uncharacterised for most
350 variables and no caecilian specimens from this region were available for study. For
351 continuous variables (precipitation, temperature, and elevation) we fitted ANOVAs
352 grouping by *S. thomense*, "*S. ephèle*", and admixed individuals, and used a Tukey Honest
353 Significant Differences test to calculate adjusted P values for group mean comparisons.

354

355 RESULTS

356 Phenotypic variation in São Tomé caecilians

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

361

362 Geographic structure and evidence of hybridization

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

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

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

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

387

388 **Demographic history of São Tomé caecilians**

389 Demographic modelling using $\delta a \delta i$ based on SNP data from all samples supported
390 a model of divergence in isolation, followed by instantaneous expansion in both lineages
391 and secondary contact with ongoing symmetric migration (Fig. 3; Table S2; $\Delta AIC =$
392 83.5 , $\omega_i = 1.0$). The second-best model was a three epoch model of divergence in
393 isolation, followed by instantaneous expansion in both lineages and secondary contact
394 with ongoing symmetric migration, followed by isolation in the recent past. When
395 admixed individuals were excluded, this same three epoch model was best-supported
396 ($\Delta AIC = 10$, $\omega_i = 1.0$). Because there are no SNP-based mutation rate estimates for
397 caecilians we refrain from converting our unscaled parameters here; however, several
398 inferences can still be made. First, historical effective population size before and after
399 expansion was larger for “*S. ephèle*” than for *S. thomense* and *S. thomense* experienced a
400 greater magnitude change in population size ($\sim 5x$) relative to “*S. ephèle*” ($\sim 2x$; Table
401 S2). Second, the relative time between secondary contact and the present (unscaled value

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

410 Divergence time estimates using G-PhoCS (based on entire loci with locus-based
411 mutation rates) indicated that initial divergence between *S. thomense* and “*S. ephèle*”
412 occurred ~303.4 kya (280.9–325.8 kya). We used this mean divergence date to convert
413 our scaled time estimates from $\delta a\delta i$ to infer that secondary contact occurred ~95.1 kya
414 (88.0–101.1 kya; Fig. 3).

415

416 **Environmental variation across sampling sites**

417 We inferred that caecilians occupy a broad environmental space on São Tomé,
418 ranging from habitats receiving between 800 and > 1400 mm/yr of precipitation (Fig. 4).
419 Our sampling indicated that caecilians occur in most soil types/ages on the island (Fig. 4),
420 and in all vegetation types (Fig. 4). The two lineages segregated strongly in
421 environmental space, particularly along a gradient of precipitation (Fig. 4), with *S.*
422 *thomense* occurring in drier habitats than admixed and “*S. ephèle*” caecilians (adjusted P
423 < 0.001). Although *S. thomense* appear to inhabit a wider range of elevations and
424 temperatures than “*S. ephèle*”, occurrence with respect to these two variables does not
425 differ between the species (Fig. S5; adjusted $P > 0.05$). The distribution of *S. thomense*
426 encompasses the younger basaltic lavas (<1 Mya) that dominate the central and northern
427 half of the island and the contact zone between the lineages roughly coincides with the
428 transition between the younger (<1 Mya) and older (3–8 Mya) basaltic lavas in the centre
429 of the island. *Schistometopum thomense* were also associated with alluvial soils in the
430 northern coastal plain (Fig. 4) whereas, “*S. ephèle*” were associated with the older
431 basaltic lavas (3–8 Mya) and volcanic cone formations on the southern half of the island.
432 Caecilians occupied non-forested habitats across the island, but *S. thomense* and admixed

433 individuals were primarily found in shaded plantation and non-forested habitats, whereas
434 “*S. ephèle*” were found in native forest, secondary forest, and shaded plantation but not
435 in non-forested habitat (Fig. 4).

436

437 DISCUSSION

438 Our genetic and phenotypic data support a history of within-island divergence in
439 allopatry for São Tomé caecilians, followed by secondary contact and hybridization that
440 have blurred lineage boundaries rather than a history of divergence with gene flow along
441 an ecological gradient. The common ancestor of *S. thomense* and “*S. ephèle*” arrived on
442 the island recently, having diverged from its East African sister species *S. gregorii* ~1
443 Mya (Loader et al., 2007). Correspondingly, using coalescent methods we estimated that
444 subsequent *in situ* divergence occurred ~300 kya, which is comparable to previous
445 estimates derived from analyses of mitochondrial sequence data (Stoelting et al., 2014).
446 Several other São Tomé organisms with contiguous contemporary distributions have a
447 history of *in situ* diversification during the Pleistocene, including reed frogs 1.7–0.5 Mya
448 (Bell et al., 2015), freshwater crabs 1.5–0.5 Mya (Daniels & Klaus, 2018), and fruit flies
449 ~400 kya (Llopart, Elwyn, Lachaise, & Coyne, 2002; Llopart, Lachaise, & Coyne, 2005).
450 These estimates broadly coincide with the most recent period of volcanic eruptive activity
451 on São Tomé from 36–860 kya (Barfod & Fitton, 2014). Thus, lineage divergence across
452 co-distributed groups may be associated with catastrophic late Pleistocene volcanic lava
453 flows fragmenting species distributions and interrupting gene flow. These findings are
454 consistent with the hypothesis that volcanic flows are an important but ephemeral
455 mechanism for allopatric divergence in volcanic island systems (Juan, Emerson, Oromí,
456 & Hewitt, 2000), as has been documented in a variety of taxa, including flowering plants
457 (Brochmann, 1984), lizards (Bloor et al., 2008), birds (Milá et al., 2010) and orangutans
458 (Nater et al., 2011).

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

464 2019) as demonstrated in other fossorial vertebrates (Martín, López, & García, 2013;
465 Fouquet et al., 2021). Associations between habitat type and lineage divergence were also
466 reported in reed frogs (Bell & Irian, 2019) and fruit flies (Coyne et al., 2002; Matute &
467 Coyne, 2010) on São Tomé, suggesting that this pattern may be widespread across a
468 variety of organisms on the island, although the specific mechanisms of local adaptation
469 are likely to differ between fossorial versus surface-dwelling taxa. These observations
470 may be somewhat confounded by the strong correlation between geography and
471 environmental variation on São Tomé; however, similar associations have also been
472 documented in organisms from other small volcanic islands including lizards from the
473 Canary Islands (Brown, Woods, & Thorpe, 2017; Gübitz, Thorpe, & Malhotra, 2005;
474 Pestano & Brown, 1999; Suárez, et al., 2014) and birds from Réunion (Gabielli,
475 Nabholz, Leroy, Milá, & Thébaud, 2020). Studies of climate-dependent competitive
476 outcomes (e.g., Comeault & Matute 2021) and functional genomic variation may provide
477 deeper insights as to the relative contributions of geographic barriers and environmental
478 variation to lineage diversification on small (< 2500 km²) oceanic islands (e.g., Brown et
479 al., 2016).

480 Secondary contact may be pervasive when allopatric divergence results from
481 ephemeral barriers on small oceanic islands (e.g. Brown et al., 2017). Accordingly,
482 historical demographic analyses inferred that the São Tomé caecilian lineages came into
483 secondary contact ~95 kya and that both lineages have experienced recent population
484 expansion, with a greater magnitude of expansion in *S. thomense* (Fig. 3A; Table S2).
485 This difference in expansion is consistent with more extensive and recent volcanic
486 activity across the northern half of the island where *S. thomense* occurs (Barfod & Fitton,
487 2014). We hypothesize that secondary contact and population size change occurred
488 following the expansion of suitable habitat after the erosion of lava flows, thus
489 facilitating contact between previously separated lineages. Hybrid zones are maintained
490 by selection against hybrid phenotypes, particularly when parental species are locally
491 adapted (Barton & Hewitt, 1985; Mallet & Barton, 1989; Kisel & Barraclough, 2010).
492 Consequently, small islands with distinct habitat transitions may result in particularly
493 narrow hybrid zones (Cooper, Sedghifar, Nash, Comeault, & Matute, 2018).

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

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

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

527

528 **Conclusions**

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

541

542

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572

573 **DATA ACCESSIBILITY**

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

579

580 **AUTHOR CONTRIBUTIONS**

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

585

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947 **Figure Legends**

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

951 90% ancestry assigned to “*S. ephèle*” in green, and admixed individuals in orange. Site
 952 abbreviations are as follows: AA = Anselmo Andrade, BO = Bombaim, BS = Bom
 953 Sucesso, CN = Contador Valley North, CS = Contador Valley South, CV = Canavial, JA
 954 = Java + Abade, LB = Lemba River, ML = Rio Maria Luisa, ON = Obo National Park,
 955 PA = Porto Alegre, QI = Quisinda, RD = Rio d’Ouro, SF = Santa Fe, SL = Santa Luzia.
 956 The type locality of “*S. ephèle*” (Água Izé, 400-700m) is likely between the coastal
 957 community of Água Izé (indicated by black star) and Java. B) Plot of ancestry
 958 coefficients estimated with ADMIXTURE v.1.3.0 (Alexander et al., (2009) for $K = 2$.
 959 Circles above the plot show the haplotype of each individual from the mitochondrial *ND4*
 960 locus, and morphological assignment (yellow, unflecked = yellow; brown, flecked =
 961 gray). C) *ND4* haplotype network for new samples and previously published data
 962 (Stoelting et al., 2014) estimated in PopART (Leigh & Bryant, 2015). 26 mutations
 963 separate the haplotype groups. D) Principal component analysis of SNP data with
 964 individuals coloured according to their ancestry assignment from (B). Photo credits: A.
 965 Stanbridge.

966
 967 Figure 2: Results of the ‘Hlest’ v.2.0 (Fitzpatrick, 2012) analysis. A) Joint maximum
 968 likelihood estimates of ancestry (S value) and interclass heterozygosity (H value) for *S.*
 969 *thomense* and “*S. ephèle*” for 41 diagnostic SNPs. A) Individuals are coloured by
 970 morphology (yellow, unflecked = yellow; brown, flecked = gray) indicating that most
 971 admixed individuals (intermediate S and H values) are flecked. B) H values plotted
 972 against latitude show that admixed individuals are restricted to the centre of the island at
 973 the contact zone. Individuals are coloured according to S values (≥ 0.9 or ≤ 0.1).

974
 975 Figure 3: Results of demographic modelling ($\delta a \delta i$; Gutenkunst et al., 2009) and
 976 demographic parameter estimation (G-PhoCS; Gronau et al., 2011) analyses. A) Stylized
 977 representation of the best supported model from $\delta a \delta i$ with parameters superimposed from
 978 G-PhoCS. B) The fit between the best-supported model and the data is shown using the
 979 two-dimensional site frequency spectrum (2D-SFS) and plots of the residuals.

980

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

989

990 **Supporting Information**

991

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

1000

1001 Figure S2: A) Map and plot of ancestry coefficients estimated from 3270 SNP dataset
1002 analysed with ADMIXTURE v.1.3.0 (Alexander et al., 2009) for $K = 3$, and B) $K = 4$.
1003 Additional K values do not correspond to the additional mitochondrial lineages inferred
1004 by Stoelting et al. (2014). C) Cross validation error plots from ADMIXTURE analysis
1005 showing roughly equal support for $K = 2-4$.

1006

1007 Figure S3: Ancestry (S value) estimated in 'Hlest' v.2.0 (Fitzpatrick, 2012) plotted
1008 against latitude showing that individuals with intermediate ancestry values (likely
1009 admixed) are restricted to the centre of the island at the contact zone. Individuals are
1010 coloured according to S values (≥ 0.9 or ≤ 0.1).

1011

1012 Figure S4: Visualization of goodness of fit test from demographic modelling analyses
1013 showing empirical result (blue bar) plotted on the distribution of simulated values across
1014 100 simulations (gray bars) for log likelihood (A) and log-transformed chi-squared test
1015 statistic (B). Empirical values outside of the simulated distribution suggest poorer model
1016 fit.

1017

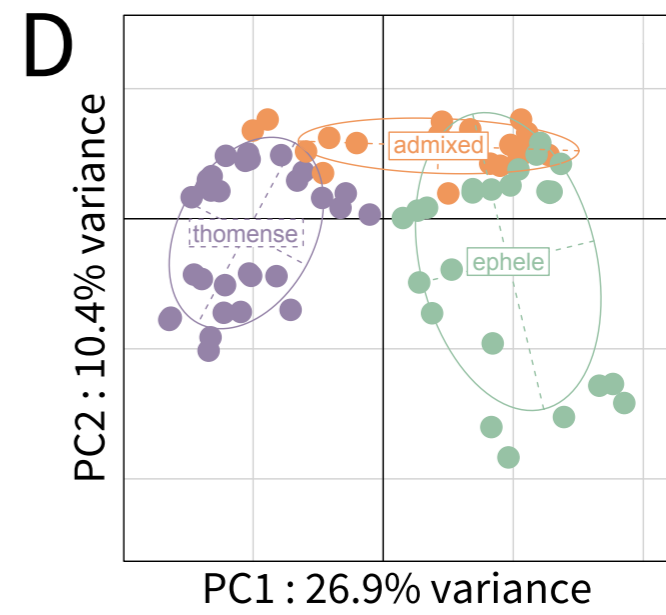
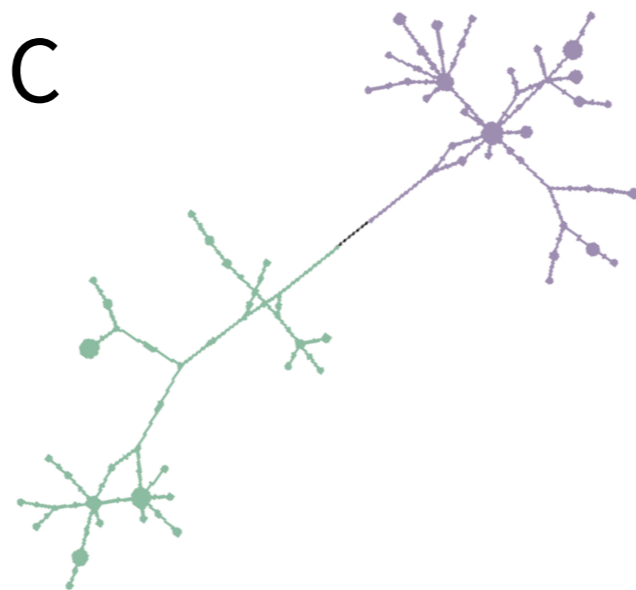
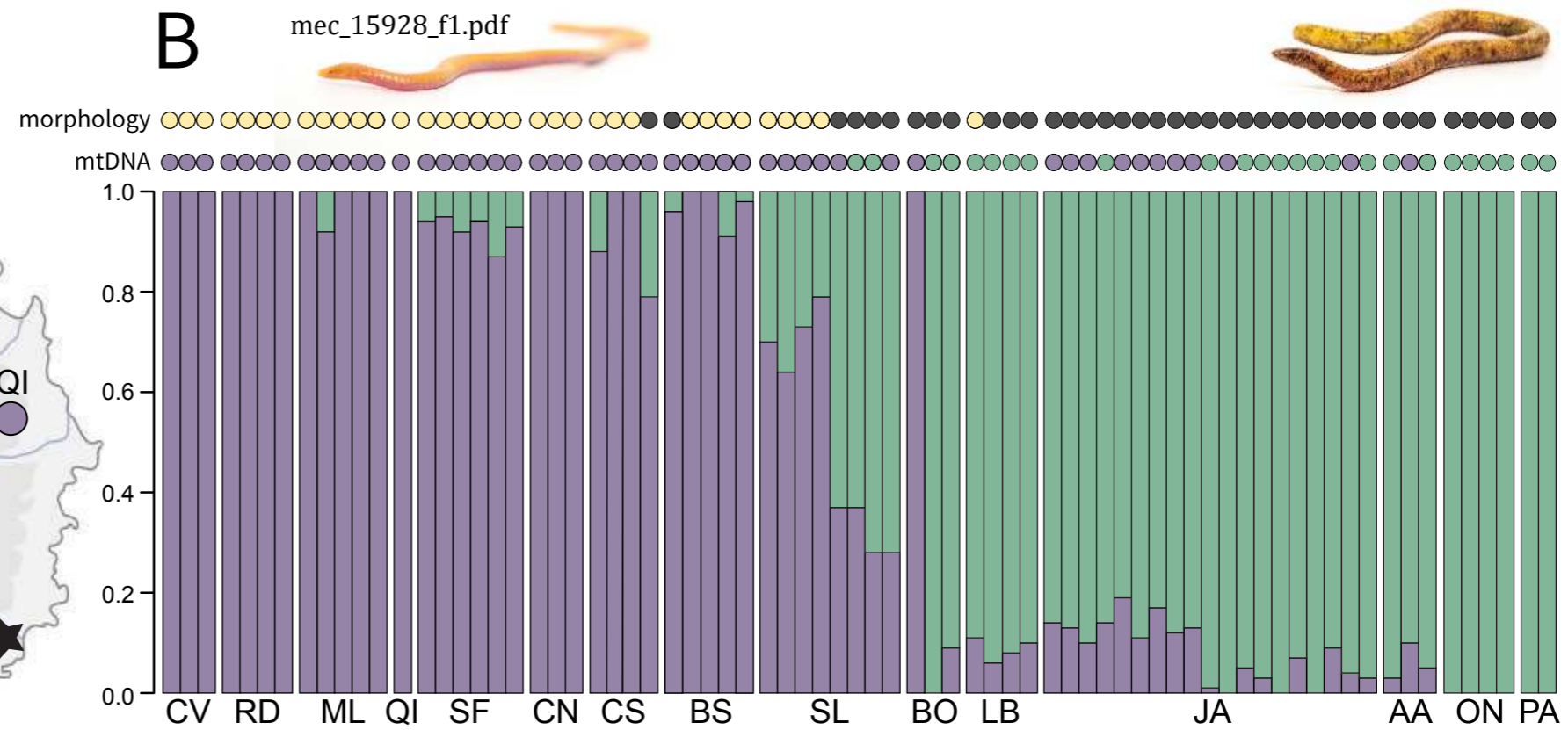
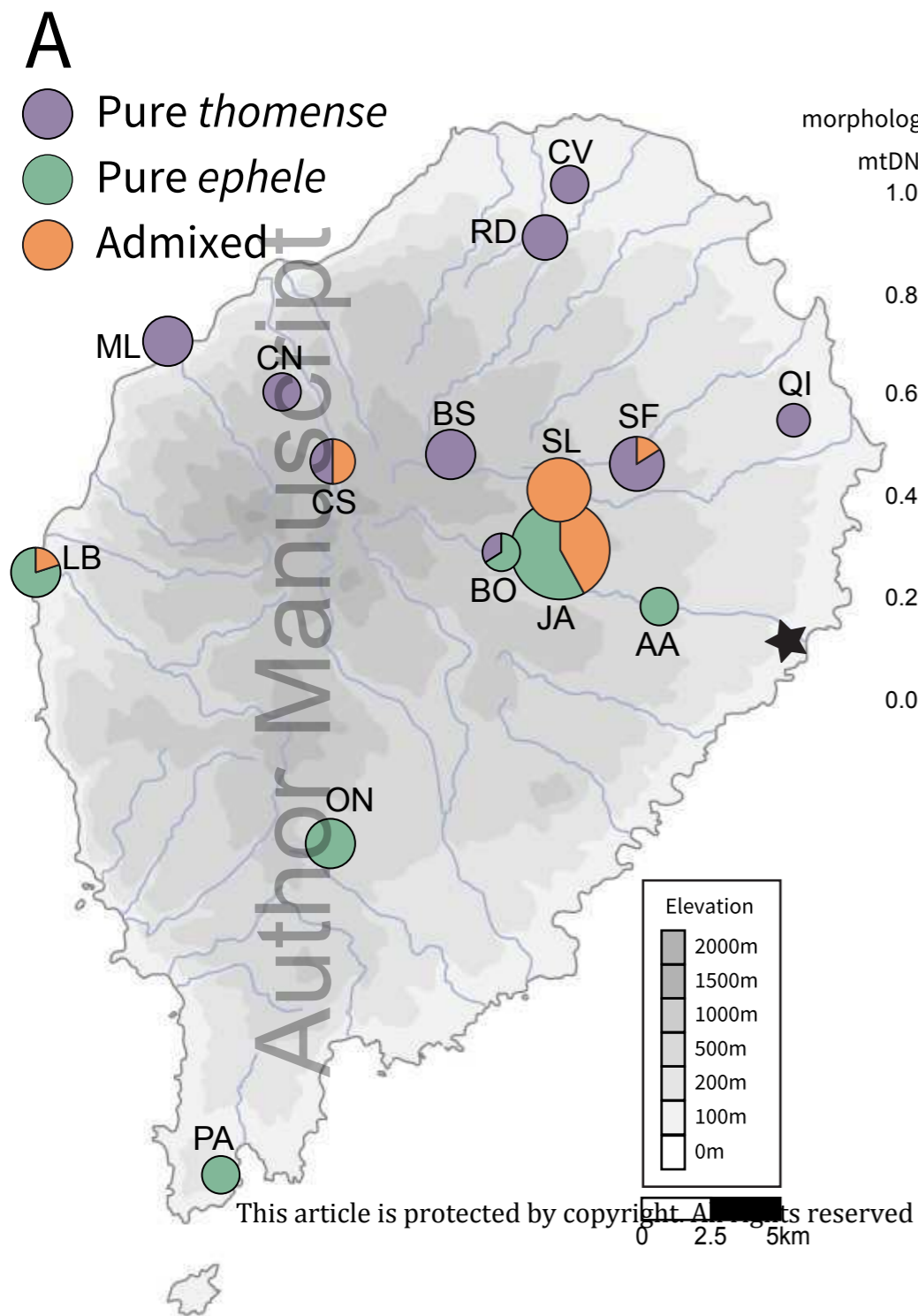
1018 Figure S5: Summary of additional environmental space occupancy analyses. A) Violin
1019 plot of elevation (m) at sites for pure (≥ 0.9 or ≤ 0.1) and admixed caecilians, and B) map
1020 of elevation. C) Violin plot of temperature at sites for pure and admixed caecilians, and
1021 D) map of temperature.

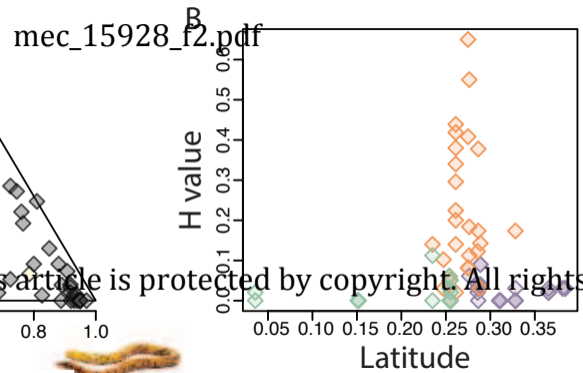
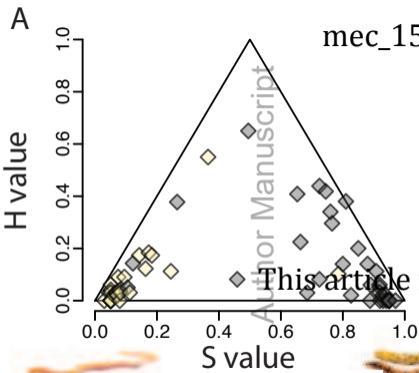
1022

1023 Table S1: Locality, catalogue number, and summary data for samples included in this
1024 study.

1025

1026 Table S2: Output summary for demographic modelling ($\delta a \delta i$; Gutenkunst et al., 2009)
1027 analyses. In all cases $nu1 = S. thomense$ and $nu2 = "S. ephela"$



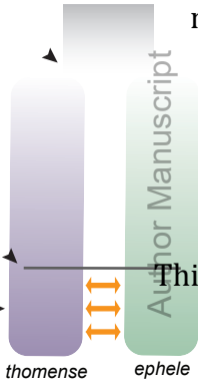


A

Divergence
281–326 kya

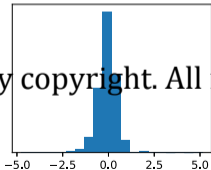
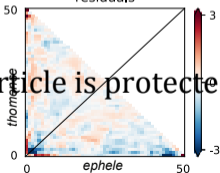
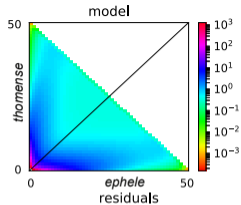
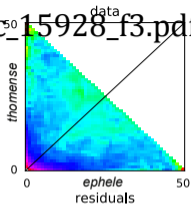
Secondary
contact
88–101 kya

Ongoing
gene flow



B

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