Demographic history explains mitochondrial genome capture and mitonuclear discordance among South American collared *Tropidurus* lizards

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Abstract

Mitonuclear discordance—evolutionary discrepancies between mitochondrial and nuclear DNA phylogenies—can arise from various factors, including introgression, incomplete lineage sorting, recent or ancient demographic fluctuations, sex-biased dispersal asymmetries, among others. Understanding this phenomenon is crucial for accurately reconstructing evolutionary histories, as failing to account for discordance can lead to misinterpretations of species boundaries, phylogenetic relationships, and historical biogeographic patterns. In this study, we investigate the evolutionary drivers of mitonuclear discordance in the *Tropidurus spinulosus* species group, which contains nine species of lizards inhabiting open tropical and subtropical environments in South America. Using a combination of population genetic and phylogenomic approaches, applied to mitochondrial and nuclear data, we identified different instances of gene flow that occurred in ancestral lineages of extant species. Our results point to a complex evolutionary history marked by prolonged isolation between species, demographic fluctuations, and potential episodes of secondary contact with genetic admixture. These conditions likely facilitated mitochondrial genome capture while diluting signals of nuclear introgression. Furthermore, we found no strong evidence supporting incomplete lineage sorting or natural selection as primary drivers of the observed mitonuclear discordance. Therefore, the unveiled patterns are most consistent with neutral demographic processes, coupled with ancient mitochondrial introgression, as the main factors underlying the mismatch between nuclear and mitochondrial phylogenies in this system. Future research could further explore the role of other demographic processes, such as asymmetric sex-biased dispersal, in shaping these complex evolutionary patterns.

Key words: Mitogenome; Molecular Evolution; Phylogenomics; Squamata; Tropiduridae.

Significance

Mitochondrial and nuclear genomes evolve independently, through distinct processes, often leading to conflicting genetic patterns—a phenomenon known as mitonuclear discordance. While widely documented, the underlying causes of such discordant patterns are not always clear. In this study, we investigated the drivers of mitonuclear discordance using a lizard group (the *Tropidurus spinulosus* species group) as a model system. Our findings suggest that changes in population size and historical isolation, possibly followed by secondary contact, were key drivers of ancient introgression among species, especially affecting the mitochondrial genome. These results provide valuable insights into how natural population dynamics shape the evolutionary processes behind mitonuclear discordance.

Introduction

Mitochondrial and nuclear genomes evolve independently, through distinct processes, often leading to conflicting genetic patterns—a phenomenon known as mitonuclear discordance. Cases of mitonuclear discordance are increasingly reported across a wide range of animal species. Recent examples can be found among mammals (Good et al. 2015; Phuong et al. 2016; Seixas et al. 2018; Fedorov et al. 2022), birds (Andersen et al. 2021; DeRaad et al. 2023), reptiles (Firneno et al. 2021), amphibians (Zieliński et al. 2013; Dufresnes et al. 2020; Rancilhac et al. 2021), and insects (Hinojosa et al. 2019; Dong et al., 2023). Generally, conflicting patterns between nuDNA and mtDNA arise from processes such as introgression, demographic fluctuations, sex-biased dispersal asymmetries, incomplete lineage sorting (ILS), human introductions, or *Wolbachia* infection in insects (see reviews by Toews and Brelsford 2012; Bonnet et al. 2017; Deprés 2019). Determining whether mitonuclear discordance is driven by any of these factors requires a clear understanding of the genealogies among

73 different loci, the demographic history of populations, and their historical
74 introgression/migration patterns and rates. Notably, these complexities often present
75 significant empirical challenges in resolving the underlying causes of discordance.

76 Most cases of discordance are attributed to mtDNA introgression (Toews and Brelsford
77 2012) and are generally associated with processes such as gene flow across hybrid zones
78 (Zieliński et al. 2013; Good et al. 2015; Dufresnes et al. 2020; Mao and Rossiter 2020) and
79 mitochondrial capture (Andersen et al. 2021). The latter refers specifically to a form of
80 complete introgression, where the mitochondrial genome of one species is entirely replaced
81 by another's (Zieliński et al. 2013). Mitochondrial capture can occur at various stages of the
82 speciation process and between distantly related species (Rancilhac et al. 2021). Additionally,
83 mitochondrial introgression can take place with minimal or not accompanying nuDNA
84 introgression (Zieliński et al. 2013; Good et al. 2015; Andersen et al. 2021; Rancilhac et al.
85 2021; Fedorov et al. 2022), making it challenging to detect introgressed nuclear and
86 mitochondrial markers simultaneously (Bonnet et al. 2017; Seixas et al. 2018). Therefore, ILS
87 is another phenomenon that cannot be overlooked in discussion of mitonuclear discordance,
88 given the challenges in distinguishing it from introgression (Andersen et al. 2021; DeRaad et
89 al. 2023). While some studies have identified ILS as a primary driver of mitonuclear
90 discordance, these remain relatively rare (e.g., Firneno et al. 2021).

91 Demographic factors, such as fluctuation in population sizes, particularly at the
92 periphery of a species' geographic range, can also contribute to mitonuclear discordance
93 (Phuong et al. 2016; Dufresnes et al. 2020; Fedorov et al. 2022). In this scenario, neutral
94 processes such as genetic drift can help in the fixation of mtDNA haplotypes at range edges of
95 expanding populations (Bonnet et al. 2017). Even in situations where introgression involves
96 both mtDNA and nuclear loci, mtDNA is expected to reach fixation faster, since it has a smaller

97 effective population size and is non-recombining (Moore 1995). Following demographic
98 expansions and secondary contact, nuclear genomes can fully recombine, thereby diluting
99 historical introgression signals, whereas divergent, non-recombining mitochondrial
100 haplotypes may persist.

101 Finally, selection can also play a role in generating mitonuclear discordance (Toews and
102 Brelsford 2012). If a beneficial mitochondrial mutation arises but is initially incompatible with
103 the nuclear genetic background of a population, selection may favor nuclear variants that
104 restore mitonuclear compatibility. This compensatory selection could lead to a scenario where
105 the advantageous mitochondrial mutation spreads widely, while the nuclear DNA follows a
106 different evolutionary trajectory. Empirical evidence robustly demonstrates that natural
107 selection can drive mtDNA introgression between populations through hybridization, even in
108 the absence of corresponding nuDNA introgression, provided there is even a minor selective
109 advantage (Excoffier et al. 2009; Zieliński et al. 2013; Good et al. 2015; Phuong et al. 2016). As
110 a result, the mitochondrial genome with the highest fitness may introgress into another
111 species, regardless of whether it originates from the resident or the invading population.

112 Here, we investigate the drivers of mitonuclear discordance in a biological system with
113 known occurrences of this phenomenon, the lizard family Tropiduridae (e.g., *Microlophus*:
114 Benavides et al. 2007; 2009; *Tropidurus*: Carvalho et al. 2016). Specifically, we explored the
115 evolutionary history of nine lineages within the *T. spinulosus* group using a multi-locus
116 genomic approach. This diverse group of lizards is distributed across open-vegetation tropical
117 and subtropical environments in South America and predominantly inhabit rocky and arboreal
118 habitats (Frost et al. 1998; Carvalho 2013, 2016; Carvalho et al. 2013). Although the taxonomy
119 of the *Tropidurus spinulosus* group has been relatively stable, new species continue to be
120 described (e.g., Carvalho 2016). Additionally, our research includes all taxa presently admitted

121 for the group plus one undescribed species, which is currently undergoing formal description
122 (Carvalho et al. in prep) and is referred to as *Tropidurus sp. nov.* in this study.

123 We integrated mitochondrial genomes, nuclear ultraconserved elements (UCEs), and
124 single nucleotide polymorphisms (SNPs) derived from UCEs to assess whether the
125 mitochondrial genealogy corresponded to reciprocally monophyletic nuclear lineages. To
126 determine the potential sources of the mitonuclear discordance, we examine the relative
127 contributions of neutral and selective processes. Specifically, we first compared mtDNA and
128 nuclear phylogenies to identify conflicts and potential introgression patterns, using methods
129 that either account for ILS or do not. In a scenario where these approaches yield largely
130 congruent results, this would suggest that ILS may not play a major role in the observed
131 mitonuclear discordance. Second, we evaluated whether mtDNA introgression was
132 accompanied by detectable levels of nuDNA introgression, as most cases of discordance are
133 attributed to processes such as gene flow across hybrid zones. We also assessed signals of
134 positive selection on mtDNA to evaluate potential adaptive processes in shaping mitonuclear
135 discordance. Finally, as demographic processes can contribute to emerging phylogenetic
136 discordant patterns as well, we examined historical changes in population size to assess their
137 role in shaping the observed discordance, as this would align with expectations under a non-
138 adaptive scenario.

139

140 **Results**

141 *UCE and mtDNA phylogenomic datasets*

142 After processing our data using the Phyluce pipeline (Faircloth 2016), we obtained a
143 final dataset including 820 UCE loci for 43 individuals of nine lineages of the *Tropidurus*
144 *spinulosus* species group. Our complete matrix comprised alignments with 725,505 bp after

145 internal-trimming. Matrices with 75% and 95% of completeness were also generated, which,
146 after internal-trimming, generated alignments with 2,269 and 1,613 loci, 1.98M and 1.40M
147 bp, respectively. Nuclear phylogenetic analyses using both a partitioned approach in the IQ-
148 TREE software (Minh et al. 2020), based on complete and incomplete matrices
149 (Supplementary Figures S1-S3), and a species tree approach based on BPP (Figure 1; Flouri et
150 al. 2018), indicated that our samples can be grouped into three main clades: (*T. sp. nov. + T.*
151 *xanthochilus), (T. lagunablanca + T. tarara), (T. guarani + T. spinulosus + T. teyumirim)*, in
152 addition to *T. callathelys and T. melanopleurus* as external lineages to the clade comprising
153 the remaining species. The trees inferred using ASTRAL (Zhang et al. 2018), which account for
154 ILS, produced similar patterns (Supplementary Figures S4-S6). Compared to the UCE dataset,
155 the mtDNA dataset (alignment with 40 samples, 15 genes, and 13,578 bp) produced markedly
156 different results regarding topology, indicating a clear case of mitonuclear discordance (Figure
157 1; Supplementary Figure S7-S8). Notably different from the nuclear trees, our mitochondrial
158 genealogy shows that while most *T. xanthochilus* samples were grouped as sister to the clade
159 containing *T. spinulosus* and *T. guarani*, two *T. xanthochilus* samples, the *T. sp. nov.* and the *T.*
160 *teyumirim* samples, were all recovered within the *T. tarara* + *T. lagunablanca* clade. Besides,
161 despite *T. xanthochilus* and *T. sp. nov.* forming a clade based on nuclear data, they do not
162 appear closely related in the mitochondrial dataset. Similarly, most groups observed in these
163 two phylogenetic trees are reflected in the haplotype network inferred for the *T. spinulosus*
164 group, showing the same pattern of discordance compared to the nuclear data
165 (Supplementary Figure S9).
166

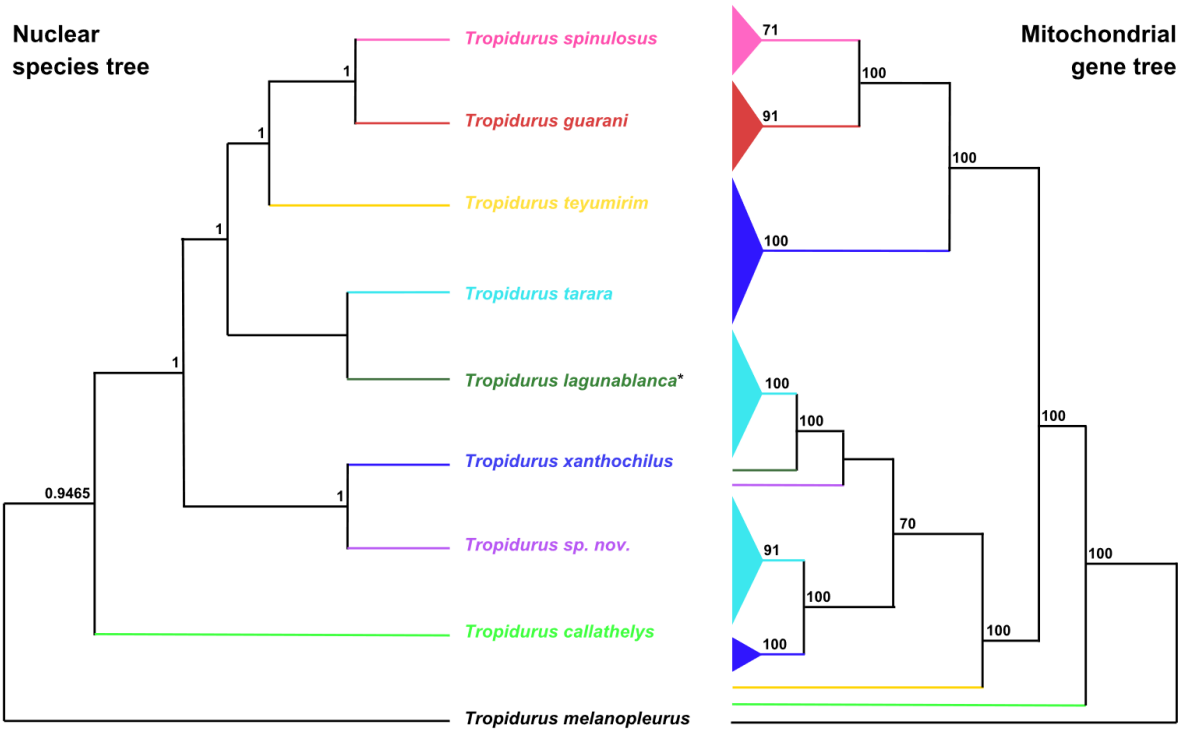


Fig. 1. Comparison between the nuclear species tree and the mitochondrial gene tree for the group *Tropidurus spinulosus*. The left panel shows the species tree inferred from multilocus nuclear data using BPP, with branch support values indicated next to nodes (posterior probabilities). The right panel displays the mitochondrial gene tree inferred through IQ-TREE, with branch support shown as bootstrap values (in this case, we only show values above 70). Colored clades correspond to the same taxa in both trees. Collapsed branches in the mitochondrial tree represent intraspecific variation (for the fully detailed tree, see the Supplementary Material). *All nuclear gene trees recovered *T. lagunablanca* nested within *T. tarara* samples, indicating that these lineages likely represent a single evolutionary unit. Accordingly, the *T. lagunablanca* sample was assigned to *T. tarara* in the BPP analysis, so that they represent a single branch in the estimated species tree. However, for a clearer comparison with the mitochondrial genealogy, in this figure we present the two lineages as separate branches within the same clade.

Divergence dating with nuclear data indicates that the initial split within the *Tropidurus spinulosus* group occurred between 20 and 15 million years ago (Ma), separating *T. melanopleurus* from the other lineages. The split involving *T. callathelys* occurred approximately 10 Ma ago, and most subsequent divergence events among the remaining species of the group took place between 7.5 and 2.5 Ma (Figure 2; Supplementary Figure S10). In most cases, the divergence times estimated using RelTime (Tamura et al. 2018) fall within the same 95% highest posterior density (HPD) intervals provided by MCMCTree (dos Reis and Yang 2019), but some differences can be noticed (Supplementary Figure S10-11).

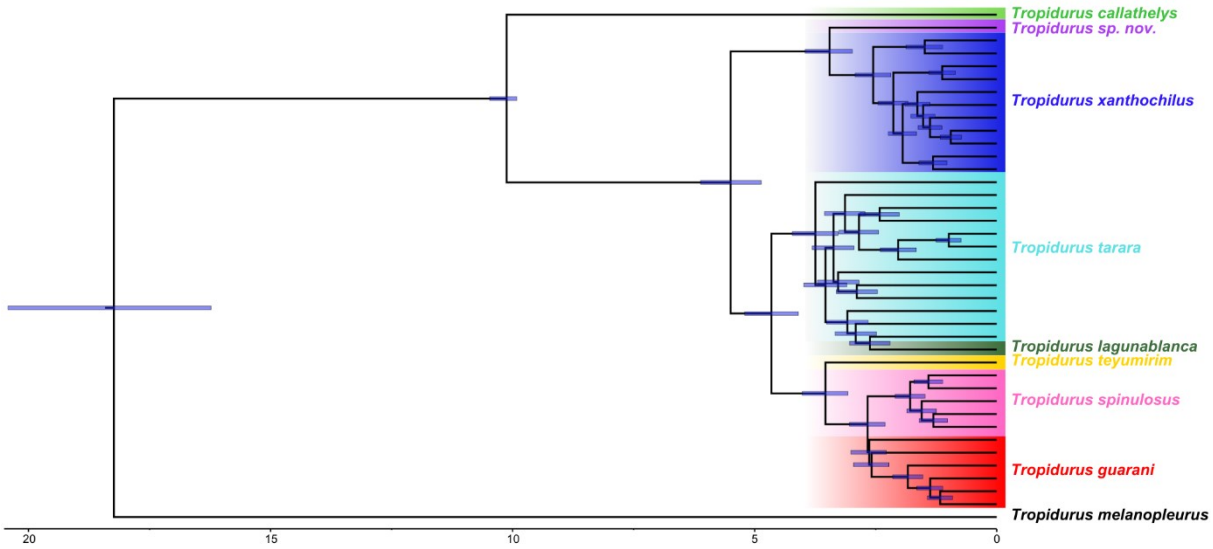


Fig. 2. Divergence times (Ma) estimated for the *Tropidurus spinulosus* species group using the UCE dataset. The 95% HPD interval of the posterior estimates (blue shaded bars), as estimated through the MCMCTree program, is shown above each node of the tree. The scale bar is in Ma. Specimen names are collapsed (for the fully detailed tree, see the Supplementary Material).

Phylogenetic networks and gene flow

We evaluated the potential for reticulate evolution in our dataset using five different approaches: (i) the phylogenetic networks applying quartets (SNaQ) method implemented in PhyloNetworks (Solís-Lemus and Ané 2016; Solís-Lemus et al. 2017); (ii) the maximum pseudolikelihood estimate (MPL) method implemented in PhyloNet (Wen et al. 2018); (iii) the extension of the typical tree-based model to general networks, assuming no ILS and independent loci, available in the NetRAX program (Lutteropp et al. 2022); (iv) ABBA-BABA tests for detecting gene flow between pairs of species, through the D-suite program (Malinsky et al., 2021); and (v) the MSC-M model implemented in BPP v.4.6 (Flouri et al. 2018; 2023) to estimate migration rate between pair of species that showed signals of mitonuclear discordance. The first four methods serve as exploratory tools to identify potential reticulations or gene flow events, while the BPP-based approach directly quantifies migration rates.

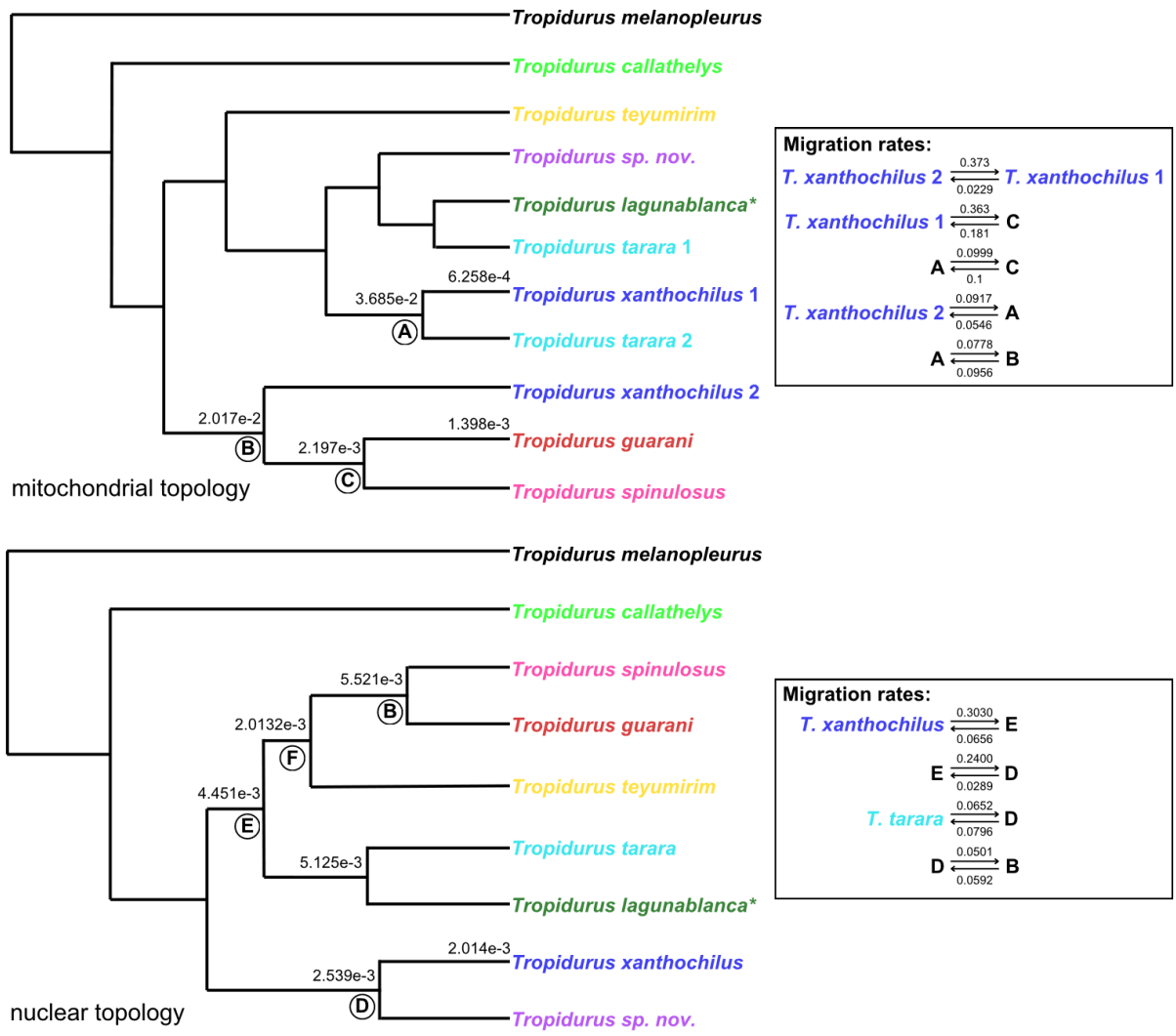
Analyses of the UCE dataset using PhyloNetworks revealed that models incorporating up to three reticulation events provided a better fit to our data compared to strictly bifurcating

210 trees, where gene tree discordance is attributed solely to ILS. However, these events are
211 mostly intraspecific and do not involve the species with discordant relationships, thus
212 Phylonetworks does not indicate that reticulation is the main source of mitonuclear
213 discordance (Supplementary Figures S12-S15; Table S1). Analyses using the other
214 phylogenetic network approaches corroborated the pattern of minimal reticulations between
215 species observed in our UCE data (Supplementary Figures S16-S18), and D-suite tests
216 (Malinsky et al., 2021) for detecting gene flow also do not indicate significant nuclear
217 admixture within the *Tropidurus spinulosus* species group (Supplementary Table S2). Please
218 refer to the Supplementary Material, section "*Details on Phylogenetic Networks methodology*
219 *and results*," for a detailed explanation on all reticulation tests.

220 Analyses in BPP under the MSC-M model with nuclear data—100 UCE loci (93,246 bp)
221 selected for clock-likeness using SortaDate (Smith et al. 2018)—indicates minimal or no gene
222 flow among extant species, but inclusion of ancestral populations yields higher migration rate
223 estimates (Figure 3; Supplementary Tables S3-S4), particularly those associated with the
224 observed phylogenetic discordance. This pattern is robust to the choice of different guide
225 trees: whether constrained by the mitochondrial topology or by the partitioned nuclear
226 topology, although the first produces relatively higher migration rate values. Notably, both
227 guide tree inferences implicate *Tropidurus xanthochilus*, or its ancestors, in these ancient gene
228 flow events, suggesting that historical mitochondrial capture involving this lineage may
229 underlie the main observed discordances between nuclear and mitochondrial phylogenies.
230 Also, when combining the nuclear dataset alongside the complete mitochondrial genome (100
231 UCE loci, 93,246 bp + 1 mitochondrial partition, 13,578 bp), higher rate estimates were
232 generally produced than those obtained using the nuclear dataset only. In this case, migration
233 rates using the combined dataset (with mitochondrial and nuclear partitions) were higher

234 than the estimates using solely the nuclear data (Table S4). In summary, across all migration
235 scenarios evaluated, including information derived from the mitochondrial data (whether
236 based on topology or mitogenome alignments) consistently resulted in higher inferred
237 migration rates compared to nuclear-only analyses, demonstrating that the inclusion of
238 mtDNA in the analysis strengthens the detection of historical gene flow in this system.

239



240
241 Fig. 3. Migration (MSC-M) model for *Tropidurus spinulosus* species, displaying only the scenarios with the highest
242 estimated migration rates. For a complete list of all tested migration scenarios and their corresponding rate
243 values, please refer to the Supplementary Material. Population size (theta, θ) values for each species or ancestral
244 node involved in gene flow events are shown above the branches, with the remaining theta values also provided
245 in the Supplementary Material. The upper panel presents the analysis using the mitochondrial topology as the
246 guide tree, whereas the lower panel shows the same analysis based on the nuclear topology, in this case using
247 both nuclear and mitochondrial data as input. In both trees, the circled letters below the nodes denote the
248 ancestral lineages included in the migration tests.
249

250 Estimates of population size (θ) for both current species and ancestral populations
251 further support the previously described scenario (Figure 3; Supplementary Tables S5-S6), for
252 instance indicating a directional gene flow event in which the larger ancestral population of
253 the *T. guarani* + *T. spinulosus* clade might have captured *T. xanthochilus* mitochondrial
254 genomes.
255
256 *Selection*
257 Regarding the mtDNA dataset, our analyses indicate minimal evidence of positive
258 selection acting on the mitogenomes of *Tropidurus spinulosus* lineages. Codon-based tests
259 detected limited signatures of episodic positive or diversifying selection: MEME (Mixed Effects
260 Model of Evolution; Murrell et al. 2012) identified nine sites (two in ATP6, one in ATP8, one in
261 COX1, two in COX2, and one in COX3) with p-values ≤ 0.05, while FUBAR (Fast, Unconstrained,
262 Bayesian AppRoximation; Murrel et al. 2013) detected two sites (one in COX1 and one in
263 COX2) with posterior probabilities ≥ 0.95. In contrast, both BUSTED (Branch-Site Unrestricted
264 Statistical Test for Episodic Diversification; Murrel et al. 2015) and aBSREL (adaptive Branch-
265 Site Random Effects Likelihood; Smith et al. 2015) analyses did not reveal significant episodic
266 selection along any branches in the mitogenomic phylogeny, even after formal testing of 36
267 branches with correction for multiple comparisons. Furthermore, we did not observe any
268 species with extreme nucleotide diversity (π) values, which could indicate selection on
269 mitochondrial lineages; instead, the diversity values were nearly identical across all
270 populations (Supplementary Table S7). Notably, the significantly negative Tajima's D (p ≤ 0.05,
271 Supplementary Table S8) indicates an excess of rare variants, a pattern that can arise from
272 both demographic expansion and selection. Subsequent analyses support a scenario of recent

273 expansion for the main populations, suggesting that this signal is evidence of historical
274 demographic changes rather than selection acting on the mitogenomes.
275
276 *Demographic history*
277 As previously demonstrated, BPP estimates of population size reveal differences
278 among ancestral populations likely involved in the ancient introgression events underlying the
279 observed discordance; these differences also support the inferred direction of gene flow
280 (please refer to the *Migration tests in BPP* section of the Supplementary Material as well).
281 Furthermore, our results indicate that demographic fluctuations (particularly rapid population
282 expansions) may have facilitated the fixation of mitochondrial haplotypes through neutral
283 processes such as genetic drift. These demographic shifts could also have diluted signatures
284 of introgression in the nuclear genome, even if such events occurred prior to the observed
285 population size changes detailed below.
286 Specifically, our Extended Bayesian Skyline Plots (EBSP; Heled and Drummond 2008)
287 revealed a pronounced increase in effective population size for all populations, particularly in
288 the last 100.000 years (Figure 4). Given that *Tropidurus tarara* was recovered in two distinct
289 clusters within the mitochondrial tree, we highlight that independent analyses were
290 conducted for each group. As results were identical across both groups, we present the EBSP
291 for only one cluster, which we consider representative of the species as a whole. For the
292 nuclear dataset, Stairway Plot analyses (Liu and Fu 2020) for *T. xanthochilus*, *T. tarara*, *T.*
293 *spinulosus*, and *T. guarani* revealed a consistent pattern across species, characterized by
294 population expansion mainly beginning 60,000 years ago, followed by relatively stable
295 population sizes thereafter (Figure 5). It is important to note that, since *T. guarani* was
296 recovered as paraphyletic in the nuclear phylogenetic analyses, we only considered samples

that consistently formed a clade, regardless of the analytical approach employed (see the Methods section for details).

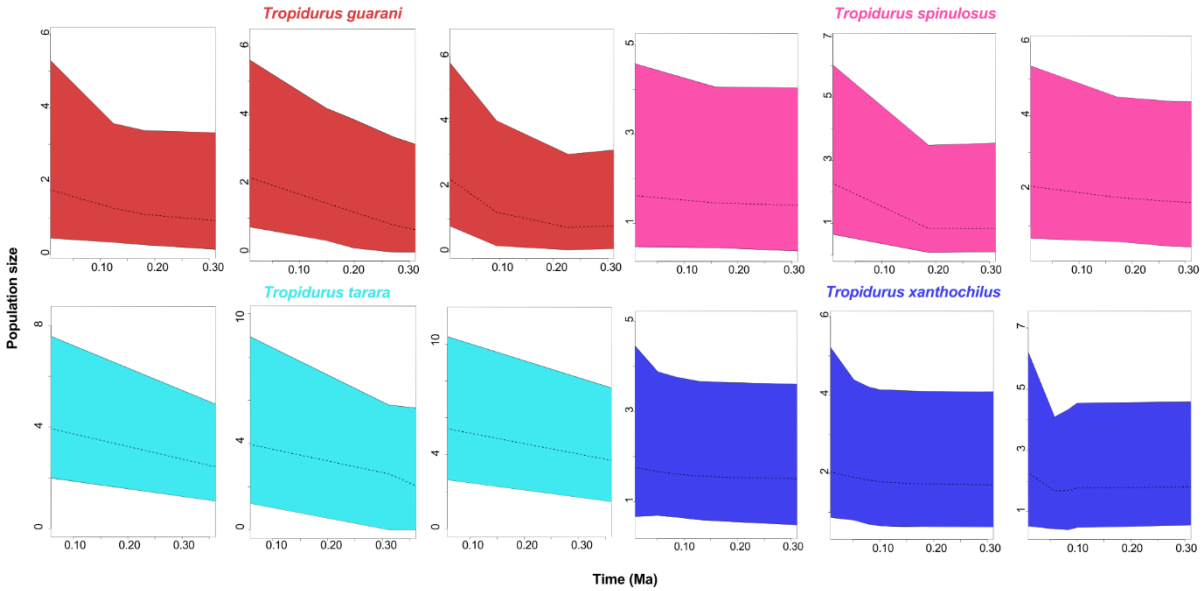


Fig. 4. EBSP results showing changes in effective population size through time for lineages in the *Tropidurus spinulosus* species group, based on three mitochondrial partitions (from left to right: cytb, 12 + 16s, and COX1, 2 and 3). Y-axis shows the effective population size with respective mean (dashed lines) and 95% HPD limits (the surrounding colored area delimited by the continuous lines). Assuming a generation time of 1 year, the current effective population size estimated at 1 would be equivalent to around 1 million. While this number appears large, it serves only as an illustration. Actual values will vary depending on the chosen mutation rate and generation time. The x-axis shows time in Ma as time goes backwards from left to right.

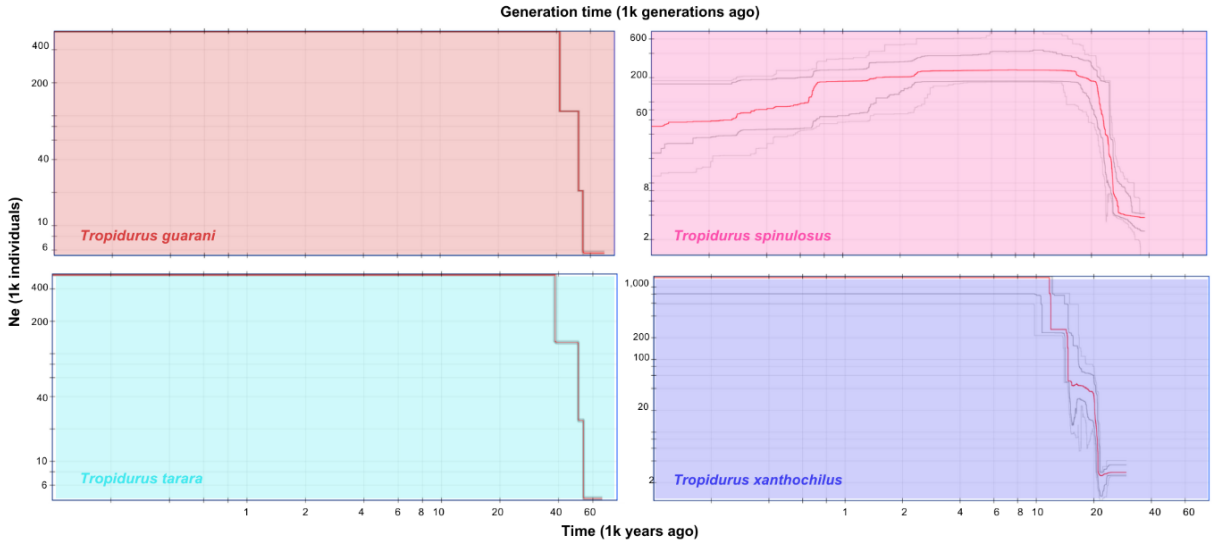


Fig. 5. Stairway Plot demographic inferences of four lineages within the *Tropidurus spinulosus* species group. The red line shows the median estimate of the effective population size (Ne). The gray lines indicate the 75% (dark) and 95% (light) confidence intervals.

315 **Discussion**

316 Our genomic analysis of the *Tropidurus spinulosus* species group reveals a complex
317 evolutionary history characterized by: (a) significant discordance between nuclear and
318 mitochondrial phylogenetic groupings, with very low levels of nuclear introgression; (b) at
319 least two major mitochondrial capture events, specifically involving ancient *T. xanthochilus*
320 populations and non-sister groups such as the *T. spinulosus* + *T. guarani* clade and *T. tarara*;
321 (c) no signs of strong positive selection acting on mtDNA; and (d) demographic evidence of
322 recent population expansions in both nuclear and mitochondrial genomes across various
323 species in the group, along with differences in the sizes of ancestral populations that may have
324 reinforced introgression events directionality. Given these findings, we suggest that the
325 mitonuclear discordance observed in this study likely originated from admixture among
326 ancient populations of the *T. spinulosus* species group that experienced long-term isolation,
327 followed by secondary contact.

328

329 *Methodological challenges in investigating introgression as a cause of mitonuclear*
330 *discordance*

331 Some caution is warranted when interpreting our results, especially given the nature
332 of our dataset. UCEs, by definition, represent highly conserved genomic regions shared across
333 evolutionarily distant taxa (Faircloth et al. 2012). While this might suggest that such data are
334 primarily suited for addressing 'deep' phylogenetic questions, multiple studies have
335 demonstrated their utility at the population and individual levels (Smith et al. 2014; Raposo
336 do Amaral et al. 2018). Additionally, the introgression and gene flow tests we used are able to
337 capture events on different time scales. For instance, the ABBA-BABA test assumes that
338 multiple substitutions at a given site are rare, as an excess of substitutions can distort patterns

339 of site discordance. This assumption tends to break down in deeply divergent taxa (Hibbins
340 and Hahn 2022), making the ABBA-BABA test more suitable for detecting recent introgression
341 events. In our study, only one significant case of interspecific gene flow was detected (both in
342 the case of the PhyloNetworks analysis and the ABBA-BABA tests), though it warrants caution
343 (as discussed below). Conversely, phylogenetic networks, which analyze discordance between
344 gene trees and other concordant factors, are less impacted by multiple substitutions at
345 individual sites, which makes them better suited for detecting older introgression events
346 (Hibbins and Hahn 2022).

347 Therefore, as we used different methodologies capable of detecting signals across
348 distinct time scales, our results cannot be considered totally dependent on the nature of the
349 used markers—even considering that the accuracy of phylogenetic network methods may also
350 depend on the number of gene trees used. In this regard, previous studies have shown that a
351 similar number of gene trees compared to our dataset is sufficient to reliably recover an
352 accurate network topology, although the exact direction of reticulation events may remain
353 uncertain depending on the evolutionary scenario (Solís-Lemus and Ané 2016). Moreover, the
354 consistency of recovered topologies across multiple analyses, with varying levels of data
355 completeness, suggests that the majority of our UCE gene trees are not either unresolved or
356 phylogenetically uninformative. Therefore, although other genomic markers may offer
357 greater sensitivity for detecting introgression, the consistency of our results across multiple
358 analytical frameworks—each suited to detecting gene flow over different temporal scales—
359 supports the conclusion of minimal or no nuclear introgression within the *Tropidurus*
360 *spinulosus* group. In any case, future comparative studies are warranted to evaluate how the
361 choice of genomic markers may influence the detection of complex evolutionary processes
362 such as introgression.

363

364 *Decoupled histories: mitonuclear discordance with minimal nuclear introgression*

365 Reports of mitonuclear discordance are not unexpected, given that mitochondrial and
366 nuclear genomes evolve semi-independently. In this context, while previous studies have
367 documented nuclear introgression associated with mitonuclear discordance (e.g., Zieliński et
368 al. 2013; Myers et al. 2022), our findings suggest a different pattern. For instance, while we
369 identified one significant nuclear reticulation event involving *Tropidurus*
370 *callathelys* (Supplementary Figure S14) and one significant D-statistic result involving *T.*
371 *teyumirim* (Supplementary Table S2), these findings require cautious interpretation. First,
372 there is no evidence suggesting contemporary hybridization within the *T. spinulosus* group.
373 The known biology and distribution of these specific lineages do not suggest the occurrence
374 of hybridization events between them (Carvalho et al. 2013; Carvalho 2013; 2016), although
375 this may reflect a distinction between contemporary gene flow and the ability of our methods
376 to detect introgression over evolutionary timescales. Second, the extreme sampling disparity
377 (n=1 for both *T. teyumirim* and *T. callathelys* versus larger samples for other taxa) could have
378 contributed to false positives in ABBA-BABA tests, which have reduced sensitivity at low
379 admixture levels (Durand et al. 2011), and PhyloNetworks analyses due to stochastic effects.

380 ILS is another phenomenon that can contribute to mitonuclear discordance, and its
381 signals can easily be mistaken for those caused by introgression (Andersen et al. 2021; DeRaad
382 et al. 2023). In our case, if some samples of a species coalesced deep in the tree, before this
383 lineage diverged from its common ancestor, a significant portion should have coalesced if ILS
384 were the predominant factor driving the discordance with mitochondrial data. However, even
385 our phylogenetic network analyses explicitly accounting for ILS (particularly PhyloNetworks
386 and PhyloNet) did not detect substantial nuclear introgression events. Moreover, the

387 agreement between partitioned (based on IQ-TREE) and ASTRAL analyses of nuclear markers
388 also suggests that ILS has a limited impact on the phylogenetic signal (Supplementary Figures
389 S1-S6), as ASTRAL is designed to recover the correct topology even under high levels of ILS
390 (Zhang et al. 2018).

391 Finally, it is important to acknowledge the lack of methods specifically designed to
392 detect mitochondrial genome introgression with the same level of precision available for
393 nuclear markers. The approach adopted in this study—particularly the BPP-based framework
394 using both mitochondrial and nuclear topologies as guide trees and integrating the
395 mitochondrial partition with multiple nuclear loci (Figure 3)—was designed to illustrate how
396 gene flow may differ between nuclear and mitochondrial genomes due to their distinct
397 inheritance patterns and evolutionary dynamics. Furthermore, we also aimed at emphasizing
398 the value of a genome-wide analytical perspective for understanding the role of introgressive
399 hybridization in animal speciation—an approach still lacking in many biological systems (Good
400 et al. 2015).

401 By doing that, we discovered that while our nuclear data largely dismiss the occurrence
402 of prominent introgression within the *Tropidurus spinulosus* group, the mitochondrial genome
403 presents a contrasting picture. Migration rate estimates increase when mitochondrial DNA is
404 included—either as an additional partition or by using the mitochondrial topology as the guide
405 tree (Tables S3-S4). Specifically, while BPP analyses of 100 UCE loci produced minimal
406 migration rate estimates (under the nuclear-guided topology), incorporating the mitogenome
407 into the dataset increased the estimated values despite the much larger size of the nuclear
408 dataset. Importantly, higher migration rate estimates appeared only in scenarios involving
409 ancestral populations (regardless of whether the nuclear or mitochondrial topology was used
410 as the guide tree), producing more realistic values (Figure 3). These findings not only

411 underscore the analytical value of including mitochondrial data in gene flow estimates but
412 also support the conclusion that the nuclear phylogenies more accurately reflect species
413 history in the *T. spinulosus* system.
414
415 *Proximate explanations of mitonuclear discordance in the Tropidurus spinulosus species group*
416 Evidence supporting a demography-related hypothesis regarding introgression
417 typically falls into three main categories (e.g., Hinojosa et al. 2019; Palacios et al. 2023; Shen
418 et al. 2025): (i) prolonged periods of isolation; (ii) population growth leading to secondary
419 contact; and (iii) a biogeographic signal related to the discordance. Below, we discuss how our
420 findings align with this framework, as the primary cause of the mitonuclear discordance
421 reported in this study is mitochondrial capture associated with the demography of the
422 lineages within the *Tropidurus spinulosus* group. Specifically, our results indicate ancient gene
423 flow events occurring in populations that were likely isolated for extended periods before
424 undergoing secondary contact, although we detected limited evidence of nuclear
425 introgression. Additionally, both nuclear and mitochondrial datasets reveal widespread
426 historical fluctuations in population size (Figures 4 and 5). Similar cases have been
427 documented in lizards (e.g., Myers et al. 2022), where geographic isolation, followed by range
428 and demographic expansions, led to secondary contact and gene flow between previously
429 diverged groups.
430 First, our divergence time estimates support a gradual and prolonged separation
431 among species in the *Tropidurus spinulosus* group, with divergence events between species
432 occurring from 20 Ma onward. Specifically, the initial split occurred between 20 and 15 Ma,
433 with *T. melanopleurus* diverging first. Around 10 Ma, *T. callathelys* separated from the
434 remaining taxa, while most subsequent speciation events took place between 8 and 2.5 Ma

435 (Figure 2). Related to that, it is important to note that the evolutionary history of South
436 America's dry diagonal has been shaped by multiple Neogene (23 Ma onward) geological and
437 climatic processes, with individual taxa responding uniquely to local conditions (Guillory et al.
438 2024). Deep divergences among Squamates, including those within the *T. spinulosus* group,
439 likely stem from Miocene events such as the uplift of the Central Brazilian Plateau and marine
440 incursions in the southern dry diagonal (Guillory et al., 2024; Bezerra et al., 2025).

441 If such geological events led to prolonged geographic isolation of lineages, their
442 gradual cessation would have increased opportunities for secondary contact and potential
443 introgression, particularly when coupled with demographic and range expansions. In such a
444 scenario, periods of secondary contact may have led to the formation of hybrid zones with
445 varying levels of interbreeding, followed by contrasting patterns of gene flow between the
446 nuclear and mitochondrial genomes. During periods of geographic and/or demographic
447 expansion, nuclear genomes—subject to recombination and meiotic segregation—undergo
448 widespread admixture and tend to homogenize over time, diluting distinct traces of past
449 isolation and secondary contact. In contrast, divergent mitochondrial haplotypes, which are
450 non-recombining and inherited solely from the females, may persist due to genetic drift,
451 particularly if introgression occurred early in the population expansion or when the population
452 sizes were relatively low.

453 The directionality of gene flow events is another factor that could have played a crucial
454 role in facilitating neutral mitochondrial capture. Because genetic drift is more pronounced in
455 small populations, neutral mutations in mitogenomes are more likely to become fixed (Moore
456 1995; Després, 2019). Consequently, unidirectional introgression from a larger, resident
457 population into a smaller, invading one would be more probable during the early stages of
458 contact. If the ancestral populations of any of *Tropidurus spinulosus* species were more

459 restricted in distribution at that point, such conditions would further facilitate the fixation of
460 captured mitochondrial variants through drift. The repeated instances of discordance
461 involving *T. xanthochilus*, due to ancient introgression, may suggest that this lineage had a
462 smaller population size compared to others. Our ancestral population size estimates are
463 consistent with this scenario. For instance, in the model where migration rates are higher (in
464 this case, using the mitochondrial topology as the guide tree; Supplementary Tables S3-S4), Θ
465 estimates of *T. xanthochilus* are considerably lower than those of the ancestor involved in the
466 gene flow event—in this case M (*T. xanthochilus* → ancestor (*T. guarani*, *T. spinulosus*)) =
467 0.3629, with Θ values of 0.0006258 and 0.02017, respectively (Supplementary Table S5). A
468 similar pattern was observed when the nuclear topology was used as the guide tree, with the
469 ancestors of the *T. tarara* + *T. spinulosus* + *T. guarani* clade exhibiting higher Θ values than
470 those of the ancestral *T. xanthochilus* populations (Supplementary Table S6). Under this
471 scenario, it would be plausible to suggest that the mitogenomes of some *T. xanthochilus*
472 individuals could have been incorporated by the larger ancestral populations of the *T. guarani*
473 + *T. spinulosus* clade, and also by ancestral populations of *T. tarara*.

474 It is also noteworthy that our main demographic analyses (EBSP and Stairway Plot)
475 might have captured a shorter time scale than that over which the gene flow events occurred.
476 Even so, relatively recent demographic shifts, during the Pleistocene (2.5 Ma – 12 kya), may
477 have contributed to the discordance pattern as well, either by facilitating the fixation of
478 mitochondrial variants or reducing detectable signals of nuclear introgression. Population
479 expansion events, in particular, appear to have occurred from at least 100 kya onward (Figures
480 4 and 5), supporting this interpretation. Similar patterns of demographic fluctuation shaping
481 phylogeographic histories have been reported for other squamates as well (Guillory et al.
482 2024). Moreover, the observed differences in temporal estimates of species demography

483 between those two approaches (Figures 4 and 5) are likely attributable to the distinct
484 mutation rates we applied to the mitochondrial and nuclear datasets. As with many non-
485 model organisms, species-specific substitution rate estimates for *Tropidurus* are unavailable,
486 adding uncertainty to time-based inferences.

487 The two particular instances of mtDNA capture involving *Tropidurus xanthochilus* are
488 also associated with biogeographic signals: one possibly occurring to the west of *T.*
489 *xanthochilus*' distribution, involving ancestral populations of the *T. spinulosus + T. guarani*
490 clade, and another to the south, involving *T. tarara*. Broadly speaking, *T. spinulosus* is found
491 west of the Paraguay River, primarily within the Chaco region (Carvalho 2013). Specifically, its
492 distribution spans from north-central Argentina and northwestern Paraguay to southeastern
493 and central Bolivia, covering both the Chaco and dry forest zones (Frost et al. 1998; Carvalho
494 2013). Although distributional data remain limited, the known ranges of *T. spinulosus* and *T.*
495 *xanthochilus* are allopatric. The nearest known populations of *T. spinulosus* are located at least
496 350 km south of the type locality of *T. xanthochilus,* near to the '*Serranía de Huanchaca*'
497 (Harvey and Gutberlet, 1998). Even so, Harvey and Gutberlet (1998) proposed that *T.*
498 *xanthochilus* and *T. spinulosus* might be parapatric in the area where the forests of the Tarvo
499 and Paraguá Rivers merge with the semideciduous *'Chiquitania'* dry forest. On the other hand,
500 *T. tarara* is currently documented from locations in the northern portion of Eastern Paraguay,
501 east of the Paraguay River, and its range extends into the Brazilian state of Mato Grosso do
502 Sul (near the southern limit of the distribution of *T. xanthochilus*).

503 In this context, future studies—whether empirical or based on modelling scenarios—
504 will be crucial to assess the potential overlap (past or present) in the distributions of *T.*
505 *xanthochilus* with *T. spinulosus* + *T. guarani* clade, and *T. tarara* as well. Such overlap would
506 provide additional biogeographical evidence for mitochondrial capture among these lineages.

507 Environmental changes can increase opportunities for hybridization and introgression through
508 neutral demographic processes (Excoffier et al. 2009; Phuong et al. 2016), with mitonuclear
509 discordance of this nature often linked to climatic instability and its effects on demographic
510 dynamics (Phuong et al. 2016). Phylogenetic niche modeling, which combines past and
511 present climate data with occurrence records and time-calibrated phylogenies to reconstruct
512 historical geographic ranges (e.g., Guillory and Brown, 2021), may be particularly useful for
513 testing these hypotheses in the future.

514 Finally, our findings suggest that mitochondrial capture in the *Tropidurus spinulosus*
515 group likely occurred during a period when the current lineages were not yet fully
516 differentiated and still overlapped in both space and time. This interpretation is supported by
517 our migration rate results, which show that the highest estimated values were associated with
518 scenarios involving ancestral populations (Figure 3). Particularly, this aligns with the "gray
519 zone of speciation" concept—a transitional phase where populations shift from widespread
520 admixture to complete genetic isolation (Roux et al. 2016; Burbrink et al. 2021), facilitated by
521 pre and/or post-zygotic barriers (Stankowski and Ravinet, 2021).

522

523 *Mitonuclear discordance beyond neutral processes: a case for natural selection?*

524 Our analyses did not reveal strong evidence of positive selection acting on the
525 mitochondrial genome of the *Tropidurus spinulosus* species group. Site-model tests across the
526 phylogeny identified only a few individual codons under selection, indicating that these
527 methods captured limited signals of episodic or pervasive positive selection acting on the
528 mitogenome. However, it is important to note that these approaches are designed to detect
529 selection at the level of specific codons or phylogenetic branches, rather than to assess the
530 rapid fixation of mitochondrial haplotypes following introgression (see the HyPhy

531 documentation for a detailed explanation). In such a scenario, an entire haplotype could be
532 fixed in a new species, a process that might not be directly detectable by the methods adopted
533 here.

534 Nonetheless, complementary analyses, including nucleotide diversity estimates and
535 Tajima's D test results, do not provide support for natural selection either. First, comparable
536 π levels across the lineages involved in the discordance (Table S7) are not indicative of
537 selective forces strongly acting on the mitogenomes of *Tropidurus spinulosus* species.
538 Similarly, the negative Tajima's D values (Table S8), while theoretically compatible with both
539 selection and population expansion, are here interpreted as indicative of historical
540 demographic changes in light of the additional evidence previously discussed. Furthermore,
541 in lizards—where mitochondrial function is less constrained by high metabolic demands
542 compared to endotherms (Chung and Schulte 2020)—the selective pressure for rapid fixation
543 of an introgressed mitogenome is likely lower. Nonetheless, while selective factors are unlikely
544 to be the primary drivers of mitonuclear discordance in *T. spinulosus* lineages, adaptive
545 explanations may be still underrepresented in the literature (e.g., Mao and Rossiter 2020),
546 which may partly reflect the inherent challenges in reliably detecting selection on mtDNA
547 (Bonnet et al. 2017).

548

549 *Sex-biased dispersal in Tropiduridae and its potential role in mitonuclear discordance*

550 Finally, future investigations could look further into the role of mitonuclear
551 incompatibilities and mating systems in explaining contrasting signals between nuDNA and
552 mtDNA. Asymmetries between sexes in mating behavior, offspring production, and dispersal
553 are well-known demographic factors contributing to mitonuclear discordance (Dessi et al.
554 2022). For example, in species where males exhibit greater dispersal, nuDNA is expected to

555 show higher genetic homogeneity across distant populations, as male-mediated gene flow
556 facilitates the spreading and mixing of their genetic material. Conversely, if females are more
557 philopatric (i.e., remain near their birthplace), mtDNA may display greater population
558 structure, preserving historical patterns of isolation. As a result, while nuDNA may indicate a
559 single, cohesive population over a broad geographic range, mtDNA could reveal distinct
560 subpopulations shaped by limited female dispersal. This process can lead to geographically
561 structured mitochondrial lineages that persist despite widespread nuclear gene flow (see
562 reviews in Toews and Brelsford 2012; Bonnet et al. 2017). Currently, empirical data on the
563 dispersal behavior of *Tropidurus* species are lacking. However, research on certain groups
564 closely related to Tropiduridae (e.g., Phrynosomatidae) suggest that females exhibit strong
565 philopatry and possibly territoriality, while males may disperse further from contact zones,
566 facilitating nuclear gene flow but limiting mitochondrial inheritance (Qi et al. 2013). Although
567 limited studies have been conducted on squamate reptiles, some evidence indicates that sex-
568 biased differences in behavior and fitness can influence dispersal patterns. Therefore, we
569 recommend future research to investigate whether sex-related dispersal contributes to the
570 mitonuclear discordance observed in *Tropidurus*.
571
572 **Conclusion**
573 The growing number of reports documenting deep mitochondrial lineages without
574 corresponding nuclear differentiation highlights the need for comprehensive molecular
575 analyses, ideally employing phylogenomic approaches that incorporate both mitochondrial
576 and nuclear markers. This is especially relevant for large genomic datasets, which can provide
577 robust branch support, while also obscuring discordance among individual markers. Thus,
578 integrative approaches are crucial for disentangling complex evolutionary processes that

579 cause mitonuclear discordance, such as introgression, and for highlighting the unique role of
580 mtDNA in detecting gene flow events that may be overlooked in nuclear data.

581 In this study, we explored a biological system where mitochondrial capture events
582 were detected, offering insights and a foundation for future research into the evolutionary
583 mechanisms underlying these patterns in Neotropical squamates. By analyzing different types
584 of genetic markers, we provided a thorough investigation that contributes to the
585 understanding of the complex evolutionary history of the *Tropidurus spinulosus* species group.
586 Beyond mapping a clear case of mitonuclear discordance, we suggest that certain
587 mitochondrial genomes within this group have undergone ancestral introgression, likely
588 followed by complete capture during secondary contact events. These events left minimal
589 traces in the nuclear genome, due to the species' historical demography. Accurately
590 quantifying these patterns and understanding their underlying mechanisms are crucial for a
591 deeper comprehension of speciation processes.

592

593 **Methods**

594 *Sampling and Lab work*

595 The phylogenomic approaches used in this study were based on anchored sequencing
596 methods, specifically Ultraconserved Elements (UCE) as described by Faircloth et al. (2012).
597 We sequenced a total of 43 individuals (Supplementary Material, Table S9), representing all
598 species of the *Tropidurus spinulosus* group. RAPiD Genomics LLC (Gainesville, FL) was tasked
599 with sample library preparation and target enrichment of UCEs using the tetrapod 5k probe
600 set (Faircloth et al. 2012), followed by multiplexed paired-end (PE) sequencing (2 × 100 bp) of
601 UCEs on an Illumina HiSeq 3000 PE100 platform. Our samples are mainly distributed around

the Pantanal Basin, with some extending to dry environments of Paraguay and Argentina (Figure 6).

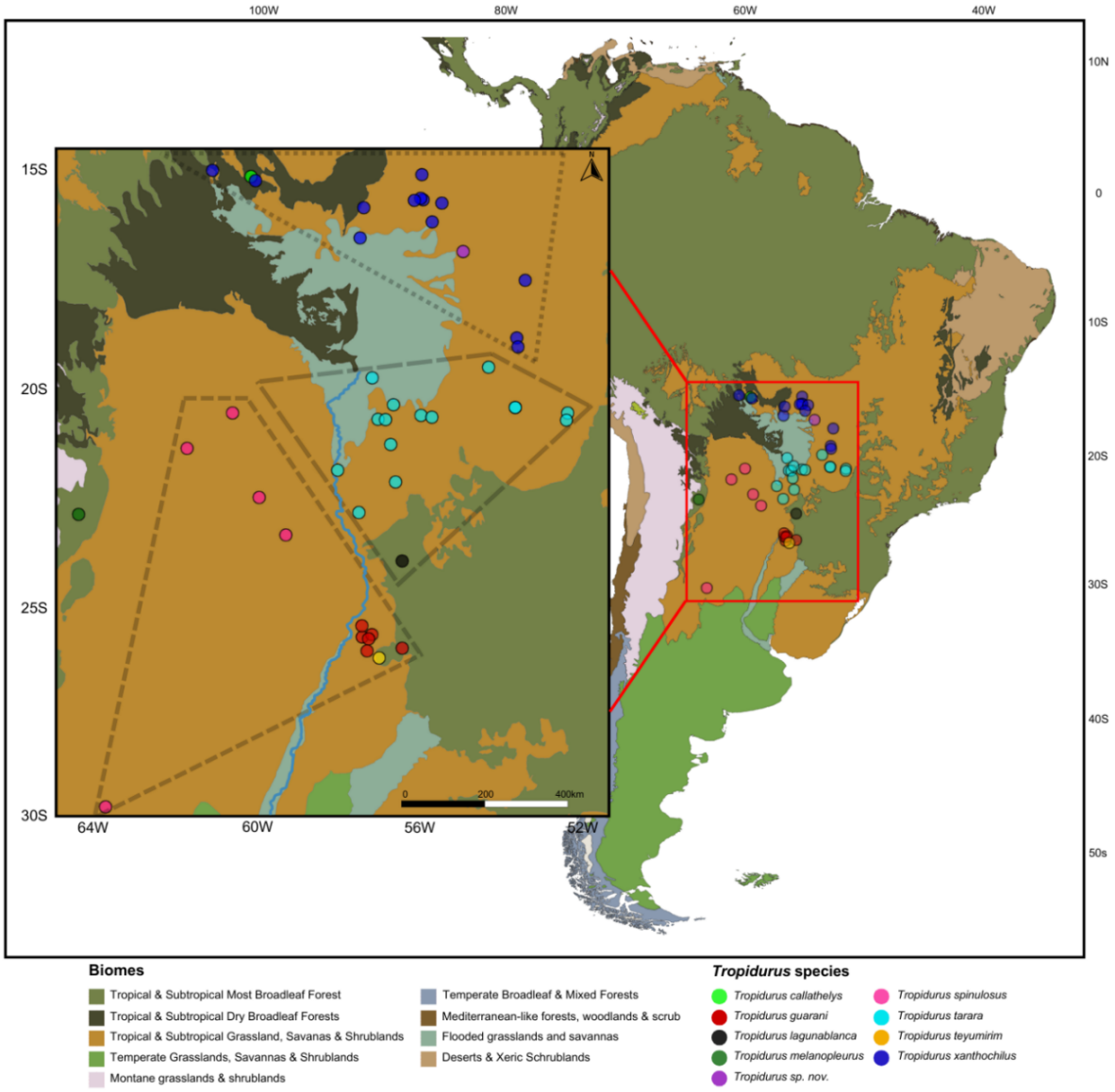


Fig. 6. Sample distribution with species assignments based on our concatenated phylogenetic analysis. The map at a larger scale shows the distribution of samples across various South American biomes, according to the classification of "Terrestrial Ecoregions of the World" adopted by the World Wildlife Fund. For a detailed review of the resolution and delimitation differences in these ecoregions, see Olson et al. (2001). The smaller-scale map highlights the specific region where the analyzed samples are distributed. Phylogenetically identified groups are marked with three distinct dashed line patterns, emphasizing their allopatric distribution: (1) the *T. spinulosus* + *T. guarani* + *T. teyumirim clade* (multi-species), (2) the *T. tarara clade* (with *T. lagunablanca* tentatively considered a synonym of *T. tarara*), and (3) the *T. xanthochilus* + *T. sp. nov.* clade. The map on the smaller box also shows part of the course of the Paraguay River (light blue), which separates the *T. spinulosus* lineages to the west from *T. guarani* and *T. tarara* to the east.

620 *UCEs and mtDNA pipeline*

621 We assembled the reads using the Phyluce v1.7.1 pipeline (Faircloth 2016).
622 Demultiplexed reads were cleaned to remove low quality bases and adapter sequences in
623 Trimmomatic (Bolger et al. 2014), using the wrapper program illumiprocessor in Phyluce. To
624 accelerate assembly and render difficult datasets tractable, we normalized read depth at a
625 minimum of 5 using the script bbnorm.sh available in the BBTools suite (Bushnell 2018).
626 Trimmed and normalized reads were inspected for quality and adapter contamination using
627 FastQC v0.11.9 (Andrews 2010), and then assembled into contigs with SPAdes v3.15.5
628 (Bankevich et al. 2012). We used Phyluce to align assembled contigs back to their associated
629 UCE loci, remove duplicate matches, create a taxon-specific database of contig-to-UCE
630 matches and extract UCE loci for all individuals. Specifically, contigs matching UCE loci were
631 identified and extracted using the program LastZ v1.0 (Harris 2007) within Phyluce, both in
632 incomplete and complete (75% and 95% of completeness) matrices (see Faircloth 2016). After
633 probes and UCEs were matched, we aligned UCE contigs with MAFFT v7.471 (Katoh and
634 Standley 2013) using specific customized settings (-globalpair, --maxiterate 1000, --
635 adjustdirection), and trimmed the resulting alignments using the internal-trimming algorithm
636 (Gblocks: Castresana 2000). As a final step, AMAS (Borowiec 2016) was used to compute final
637 summary statistics for all alignments.

638 Due to the high abundance of mtDNA in samples and the less-than-perfect efficiency
639 of target enrichment methods, mitochondrial data, including entire mitogenomes are often
640 generated as a byproduct of the UCE sequencing process (Allio et al. 2020). Thus, we extracted
641 mtDNA from our contig assembly produced through Phyluce using MitoFinder (Allio et al.
642 2020), which can find and extract multiple mitochondrial genes or even entire mitogenomes

643 from assembled sets of bulk sequences. In general terms, the same alignment process
644 described above for UCEs was adopted for mitochondrial data.
645
646 *SNP calling workflow*
647 We selected the sample with the largest number of UCE loci among all individuals
648 ('MTR 29586', with 4800 loci) retrieved during the extraction step described above as the main
649 reference during the process of calling single nucleotide polymorphisms (SNPs), as
650 recommended in the Zarza et. al. (2016) pipeline to extract SNPs from UCE reads. Then, we
651 followed the sequence capture data from population-level samples workflow developed by
652 Harvey et al. (2016). The first step involves BWA, which was used to map raw reads from
653 individuals to contigs (Li et al. 2009; Li 2013). Thus, SAM files were converted to BAM with
654 SAMtools (Li et al. 2009). Alignments were checked for BAM format violations, read group
655 header information was added, and PCR duplicates were marked for each individual using
656 Picard (v. 1.106). The resulting BAM files for each individual in a lineage were merged into a
657 single file with Picard, which was then indexed with SAMtools. The Genome Analysis Toolkit
658 (GATK; v. 3.4-0; McKenna et al. 2010) was used to locate and realign around indels, which was
659 followed by calling SNPs using the 'UnifiedGenotyper' tool in GATK. SNPs and indels were then
660 annotated and indels were masked. Finally, we used GATK to restrict our datasets to high-
661 quality SNPs (Q30 filter) and performed read-backed phasing. After that, we used the R
662 packages SNPfiltR (DeRaad 2022) and vcfR (Knaus and Grunwald 2017) to visual and iteratively
663 filter our SNP dataset based on specific quality and completeness metrics. For analyses
664 requiring unlinked SNPs, a linkage disequilibrium-based filtering approach was applied using
665 PLINK (Purcell et al. 2007) with fixed parameters (--indep-pairwise 50 10 0.5). At the end of
666 this process, two datasets were generated. The first, referred to as the '*unlinked SNP dataset*

667 *I*', included 42 samples and excluded the *Tropidurus melanopleurus* sample, retaining only one
668 sample external to the largest clade within the group, namely *T. callathelys* (used in D-suite
669 analysis; 40,265 SNPs, 25.34% missing data). The second one, '*unlinked SNP dataset II',* with
670 37 samples, excluding the *T. melanopleurus, T. callathelys, T. teyumirim* and *T. sp. nov.*
671 samples, and assigning the *T. lagunablanca* sample as *T. tarara* (used in Stairway Plot analysis;
672 27,454 SNPs, 24.71 % missing data).
673
674 *Gene and species tree analysis*
675 Gene trees for all UCE loci and the mtDNA locus were inferred using IQ-TREE v2.2.6
676 (Minh et al. 2020), and support was inferred using 1000 ultrafast bootstraps (≥ 95 considered
677 as strong support). For partitioned ML analyses, ModelFinder (Kalyaanamoorthy et al. 2017),
678 part of the IQ-TREE package, was used to select the best fit model for each partition followed
679 by tree reconstruction (-m TESTNEWMERGE option), allowing partitions to have different
680 evolutionary rates (-spp option). Support was inferred using 1000 ultrafast bootstraps.
681 Additionally, species trees were inferred for the UCE dataset using ASTRAL v5.15.4 (Zhang et
682 al. 2018), which does this from gene trees and provides internal branch lengths in coalescent
683 units of gene tree discordance, as well as branch support values in the form of local posterior
684 probabilities (LPPs). In our ASTRAL analyses, we used the topology resulting from the
685 partitioned approach produced through IQ-TREE as a reference. Finally, we used BPP v.4.6
686 (Flouri et al., 2018) to estimate a species tree for the *Tropidurus spinulosus* group, based on
687 the full dataset of 820 UCE loci.
688 In the case of mitochondrial data, besides partitioning by gene, the alignment was also
689 partitioned by third codon positions (in the case of coding sequences), and we did an
690 additional analysis including two outgroup species to evaluate the monophyly of the

691 *Tropidurus spinulosus* group — *Plica plica* and *T. torquatus* (Supplementary Figure S6;
692 GenBank accession numbers: AB218961 for *P. plica*, and KU245273, KU245300, KU245090 and
693 KU245062 for *T. torquatus*). We present only the trees (both based on nuDNA and mtDNA)
694 featuring species from the *T. spinulosus* group (our in-group) in the results section. In all cases,
695 trees were manually rooted in *T. melanopleurus* using FigTree (Rambaut 2014), following the
696 topology obtained in the previously described analysis.
697
698 *Divergence time estimation*
699 A time-tree was inferred using two different approaches. The first one was the
700 RelTime-Branch Lengths method (Tamura et al. 2018), available in MEGA11 (Tamura et al.
701 2021), which uses a phylogenetic tree previously estimated branch lengths to infer divergence
702 dates. In this case, the time-tree was computed using the UCE-based phylogenetic tree
703 (complete matrix) with one calibration constraint, namely the node separating *Tropidurus*
704 *callathelys* from all the other species in the group (and using *T. melanopleurus* as the
705 outgroup). We used 10.74 Ma as the reference value for this constraint following the
706 estimates obtained by Zheng and Wiens (2016) regarding the node separating *T. callathelys*
707 and the rest of the species in our ingroup. The method described in Tao et al. (2020) was used
708 to estimate confidence intervals and set a normal distribution on the node for which
709 calibration densities were provided, and a normal distribution was used with the mean
710 centered on 10.74 and standard deviation = 0.5.
711 Alternatively, divergence time estimates were also estimated using the MCMCTree
712 v4.10 program (dos Reis and Yang 2019), which uses an approximate likelihood calculation
713 approach. In this case, the 100 most clocklike UCE loci within our dataset were selected,
714 particularly the loci with the least root-to-tip variance. Clock-likeness was assessed using the

715 program SortaDate (Smith et al. 2018), which measures root-to-tip variance within gene trees
716 and then sorts them from highest to lowest variance. The calibration in this case was
717 performed by constraining the age of the same node described above by > 10 and < 11 Ma,
718 using a fixed topology. Molecular clock estimates were obtained using the independent rates
719 model and we used the HKY85 model. The gamma prior on the mean substitution rate for
720 partitions (rgene_gamma) was set to G (7, 9.75), which means a substitution rate of
721 approximately 0.00717 substitutions/site/Ma (value available for the phylogenetically closest
722 species to *Tropidurus* in the germline mutation rate study of Bergeron et al. 2023). The gamma
723 rate variance (sigma2_gamma) was specified with G (1, 10). Two independent runs were
724 performed, each consisting of 550.000 MCMC iterations, with 10.000 as burn-in, 'nsample' =
725 100.000, and 'sampfreq' = 5. The ESS of the MCMCTree runs were examined in Tracer v.1.7
726 (Rambaut et al. 2018) to determine convergence, and values > 200 were retained.
727
728 *Gene flow and phylogenetic network estimation*
729 Failing to consider the potential influence of gene flow and introgressive hybridization
730 among species can negatively impact phylogenetic inference (e.g. Solís-Lemus and Ané 2016;
731 Solís-Lemus et al. 2016). Therefore, given the mitonuclear discordance observed in the
732 *Tropidurus spinulosus* clade, we evaluated reticulate evolution using five approaches: three
733 phylogenetic network methods, ABBA-BABA tests, and migration rate estimates.
734 Regarding the network approaches, we first used the PhyloNetworks program (Solís-
735 Lemus et al. 2017), in which the inference of phylogenetic networks is done with maximum
736 pseudolikelihood from gene trees or multi-locus sequences (SNaQ; Solís-Lemus and Ané
737 2016). We supplied SNaQ with unrooted gene trees from 820 UCE loci containing
738 representatives for 32 focal samples; tests were also carried out with 23 and 30 samples, in

739 order to verify the possible influence of the number of tips on the estimation of reticulation
740 events. In all of these alternative tests, we did not use all 43 samples from the UCEs dataset
741 in order to lighten the computational load of the analyses. As results were similar in all
742 configurations, here we only report the estimates with 32 and 30 samples (Supplementary
743 Figures S13 and S14). Observed concordance factors (CFs) were calculated from the final 820
744 gene trees dataset (complete matrix, see previous section) and used to infer phylogenetic
745 networks with different maximum numbers of reticulation events (*hmax* up to 12, using 5
746 independent runs per h-value) and the proportion (γ) of genomic ancestry in the hybrid
747 lineage contributed by each parental species/lineage. As the default setting for SNaQ
748 considers that each allele in a gene tree corresponds to a specific tip in the network, we also
749 estimated a species-level network, in which each allele/individual was mapped to a particular
750 species, with the assignment done according to the patterns identified through IQ-TREE's
751 partitioned approach. In both cases, we performed a goodness-of-fit test to check whether a
752 given network could explain the quartet concordance factor data adequately (Cai and Ane
753 2021).

754 For the PhyloNet analysis (Wen et al. 2018), we applied the MPL algorithm to infer the
755 network assigning the maximum number of reticulations from 0 to 5, with 10 runs for each
756 reticulation value. We used the '-a taxa map' option to associate taxa with species in the
757 species tree, also following the patterns identified through IQ-TREE's partitioned approach. In
758 this case all individuals present the 820 UCE loci dataset were included in the analysis, but
759 using rooted gene trees (using *Tropidurus melanopleurus* as the outgroup). In both
760 PhyloNetworks and PhyloNet analyses, support for each value of *h* (number of hybridization
761 events) was evaluated using the graphical slope heuristic, which involves plotting the
762 pseudolikelihood scores against *h* (referred to as *hmax*), following the framework proposed

763 by Solís-Lemus et al. (2017). Finally, the same dataset used for PhyloNetworks was also used
764 in the NetRAX program (Lutteropp et al. 2022), but the input of NetRAX is the partitioned
765 alignment itself, not the gene trees.

766 We used D-suite (Malinsky et al. 2021) as a complementary analysis to get gene flow
767 estimates obtained from Patterson's D and f-4 ratio analyses between populations/species. In
768 this approach, ABBA-BABA proportions were obtained with the Dtrios' program for all samples
769 in the *unlinked SNP dataset I,* and applying different assignment configurations of samples to
770 populations/species (Table S10). This program fixes the outgroup and then estimates ABBA-
771 BABA in all possible combinations of three populations. For example, in a tree with four
772 branches plus a fixed outgroup, Dtrios estimates four independent ABBA-BABA by excluding
773 one of the populations at a time, and assigning the remaining three populations as P1, P2, and
774 P3. Moreover, regarding ABBA-BABA positions, in D-suite P1 and P2 are ordered so that nABBA
775 ≥ nBABA and the resultant D statistic is always positive. Therefore, all the results, including
776 the f4-ratio and other statistics reflect evidence of excess allele sharing between P3 and P2
777 for each trio. Additionally, when testing for introgression among highly divergent species or
778 for ancient introgression events, the D-statistic might indicate false signals of introgression
779 due to substitution rate variation among branches. To address this issue, we used the '--
780 ABBAclustering' option available in the D-suite program, where greater clustering of sites
781 would indicate a real gene flow event rather than a false positive caused by homoplasy.

782 As a final approach to assess how gene flow differentially impacted nuclear and
783 mitochondrial genomes, we used the migration model (MSC-M) available in BPP v.4.6 (Flouri
784 et al. 2018; 2023). This allowed us to estimate the number of migrants per generation and the
785 direction of gene flow for species showing the strongest mitonuclear discordance in our
786 phylogenetic trees. This was done in two stages, either using the nuclear species tree or the

787 mitochondrial genealogy as the guide tree. In the first case, we used only a portion of the
788 nuDNA dataset—specifically, the 100 most clocklike UCEs (as previously described). A second
789 migration analysis was performed using that same dataset, but this time incorporating
790 mitochondrial data alongside nuclear data. In other words, two analyses were conducted
791 using the nuclear topology as the guide tree: one using nuclear markers only (100 UCE loci,
792 93,246 bp) and another combining nuclear data with mitochondrial data (100 UCE loci, 93,246
793 bp + 1 mitochondrial partition, 13,578 bp).

794 In both cases we tested bidirectional migration scenarios with the following species:
795 *Tropidurus xantochilus* ↔ *T. guarani*, *T. xanthochilus* ↔ *T. spinulosus,* and *T. xanthochilus* ↔ *T.*
796 *tarara*. Scenarios involving ancestral populations or species of the following groups were also
797 tested. For analyses using the mitochondrial genealogy as the guide tree, these included: (A)
798 *T. tarara* and one *T. xanthochilus* group; (B) *T. guarani* and *T. spinulosus*; (C) *T. guarani, T.*
799 *spinulosus* and another *T. xanthochilus* group. When using the nuclear topology as the guide
800 tree, the same scenarios with current species mentioned above were tested, while also
801 considering the following scenarios involving ancestral nodes: (B) *T. guarani* and *T. spinulosus*;
802 (D) *T. sp. nov.* and *T. xanthochilus*; (E) *T. tarara, T. guarani*, *T. spinulosus* and *T. teyumirim*; (F)
803 *T. guarani*, *T. spinulosus* and *T. teyumirim.* Population sizes (θ) were also estimated and
804 recorded for each species and ancestral node involved in the migration pairs. In all cases, we
805 used an inverse gamma prior of G(3, 0.04) for the Θ parameter and G(3, 0.2) for the τ
806 parameter, fixing the species model prior on the topologies described above. The gamma
807 migration prior used was 0.1 (α = 1, β = 10). We ran analyses for 4 x 10^5 MCMC generations,
808 taking samples every five and using 5 x 10^3 burn-in generations. To check for consistency of
809 results, we conducted two independent runs for each analysis.

810 Furthermore, we also used POPART v. 1.7 (Leigh et al. 2015) to generate a TCS
811 haplotype network (Clement et al. 2002), based on 3 mitochondrial genes - cytochrome c
812 oxidase I, II, and III (COX1, COX2, and COX3).
813
814 *Tests for positive selection and demographic changes through time*
815 To detect significant deviations from the hypothesis of neutral evolution in the mtDNA
816 dataset, we tested for the evidence of positive selection using MEME (Mixed Effects Model of
817 Evolution; Murrell et al. 2012), BUSTED (Branch-Site Unrestricted Statistical Test for Episodic
818 Diversification; Murrell et al. 2015), aBSREL (adaptive Branch-Site Random Effects Likelihood;
819 Smith et al. 2015), and FUBAR (Fast, Unconstrained, Bayesian Approximation; Murrell et al.
820 2013). MEME is designed to identify sites underlying episodic selection, BUSTED provides a
821 gene-wide (not site-specific) test for positive selection by asking whether a gene has
822 experienced positive selection at least one site on at least one branch, aBSREL focuses on tree
823 branches and can be used to test if positive selection has occurred on a proportion of
824 branches, while FUBAR uses a Bayesian approach to infer nonsynonymous (dN) and
825 synonymous (dS) substitution rates on a per-site basis for a given coding alignment and
826 corresponding phylogeny. All of these tests were performed utilizing the HYPHY package
827 through the DataMonkey server, using default parameters, and the threshold of $p < 0.05$ for
828 all four analyses. The same alignment of mitochondrial genes used for the phylogenetic
829 analyses was utilized here, with the particularity that in order to perform the selection
830 analyses, stop codons of each coding gene were removed, manually. Besides, Tajima's D was
831 calculated to evaluate evidence of natural selection and population size changes in the
832 mitogenome using the R function 'tajima.test', from the 'pegas' package (Paradis 2010).

833 Mitochondrial nucleotide diversity was estimated for populations of *T. guarani*, *T. spinulosus*,
834 *T. tarara*, and *T. xanthochilus* using the 'nuc.div' function, also from the 'pegas' package.
835 Because selection tests indicated neutrality in the mtDNA dataset (see results), we
836 then performed the multilocus coalescent-based EBSP (Heled and Drummond 2008)
837 implemented in BEAST v2.7.6 (Bouckaert et al. 2019) to estimate changes in effective
838 population sizes over time. Four phylogenetic groups were used in this analysis, namely:
839 *Tropidurus guarani*, *T. spinulosus*, one *T. tarara* clade and *T. xanthochilus*. Regarding this, we
840 highlight that demographic analyses typically assume that each analyzed group is
841 monophyletic and below the species level. While *T. guarani* is consistently recovered as
842 paraphyletic in our nuclear phylogenies, it forms a monophyletic clade sister to *T. spinulosus*
843 in mitochondrial analyses. Therefore, for the EBSP analysis, which was performed exclusively
844 with mitochondrial data, *T. guarani* was treated as a single lineage. A similar issue arises with
845 *T. tarara*, which appears as two distinct mitochondrial groups. In this case, analyses were
846 conducted separately for each cluster, but since the results were identical, only one is
847 presented in the text, as it effectively represents both *T. tarara* groupings. In the remaining
848 cases, samples were divided according to the clades recovered in the mtDNA partitioned
849 approaches previously described.
850 To reduce computational burden and facilitate convergence, we conducted separate
851 analyses on three sets of mitochondrial genes rather than using the entire mitochondrial
852 genome. These sets included: 1) cytochrome b (cytb); 2) COX1, COX2, and COX3; 3) 12S and
853 16S ribosomal RNAs. For coding gene analyses, alignments were partitioned by gene and
854 codon position, with tree and clock models linked across all partitions. Site models were
855 treated independently, with BEAST Model Test performed separately for each partition to
856 estimate mutation rates, and restricting potential models to the "namedExtended" set.

857 Regarding substitution rates, we set 1.0×10^{-2} as the mean for the COX partition, 6.339×10^{-3}
858 for the 12s + 16s partition, and 1.94×10^{-2} for the cytb partition, all reference values already
859 used in previous studies which used similar approaches when dealing with *Tropidurus'* mtDNA
860 in BEAST (e.g., Werneck et al. 2015; Camurugi et al. 2022; Carvalho et al. 2024). An
861 uncorrelated relaxed molecular clock was adopted in all three cases (with a normal
862 distribution for the 'ucldMean'; Sigma = 0.0025). Because the default of the hyper-prior of the
863 mean of the population size distribution ("populationMean.alltrees") is a sensitive option and
864 takes a long time to converge (requiring longer runs), we modified the prior to a Normal
865 distribution centered on 1.0 (Mean) with a standard deviation of 0.1 (Sigma) to improve
866 convergence. Initial tests were carried out to determine the optimum number of MCMC
867 simulations required for each of the populations. In most tests, we ran 500,000,000 MCMC
868 simulations, sampled every 50,000 chains. In all cases, three independent runs were carried
869 out and we checked for convergence using Tracer 1.7 (Rambaut et al. 2018) by visually
870 checking effective sample size (ESS > 200). In specific cases where convergence was not
871 achieved with this number of simulations, we combined logs with LogCombiner (Rambaut and
872 Drummond 2014) and used those files to generate EBSP plots.

873 To infer the demographic history using the nuDNA dataset, we first generated a folded
874 SFS file for the SNP dataset with easySFS (Gutenkunst et al. 2009). We then used Stairway Plot
875 v. 2.18 (Liu and Fu 2020) to estimate historical effective population size changes of *Tropidurus*
876 *xanthochilus*, *T. tarara*, *T. spinulosus* and *T. guarani*. For this last group, only the 4 samples
877 that form a clade in the partitioned phylogenetic analyses described above were used
878 (Supplementary Figures S1-S6). The parameters for the Stairway Plot analysis were as follows:
879 67% for the training sites, and one year was assumed as generation time (A. Carvalho, pers.
880 commun.). The nuclear mutation rate was assumed to be 7.17×10^{-9}

881 substitutions/site/generation, which is the value available for the phylogenetically closest
882 species to *Tropidurus* in the germline mutation rate study of Bergeron et al. (2023).
883 Table 1 provides a summary of all analyses conducted in this study, along with their
884 respective objectives and main results.

885 Table 1. Overview of the analytical workflow used in this study, detailing the rationale behind each step. SNP = single nucleotide
886 polymorphism; MSA = multiple sequence alignment; nuc = nuclear; mt = mitochondrial.

Method	Data	Analysis	Objectives	Main results
IQ-TREE	MSA-nuc (100, 95 and 75% UCE matrices) MSA-mt (coding + non-coding regions)	Individual-level phylogenies	What are the phylogenetic relationships of sampled individuals? Do nuc and mt topologies agree?	nuc: Three main clades detected: (*Tropidurus xanthochilus* + *T. sp. nov.*), (*T. tarara* + *T. lagunablanca*), (*T. teyumirim* + *T. guarani* + *T. spinulosus*), in addition to *T. melanopleurus* and *T. callathelys* as external lineages to the clade comprising all those species. mt: Strong disagreement between mitochondrial and nuclear-based phylogenies.
ASTRAL	MSA-nuc (100, 95 and 75% UCE matrices)	Species-level phylogenies	Does the species tree agree with the relationships inferred in the individual-level phylogenies?	Overall, it agrees with the IQ-TREE nuclear phylogenies.
BPP	MSA-nuc (100% matrix)	Species tree		
RelTime-Branch Lengths	Nuclear phylogenetic tree (100% UCE complete matrix)	Divergence times	When did the diversification of the *T. spinulosus* group occur? Could this temporal pattern be linked to the species' demographic history, or might it help explain gene flow events throughout their evolution?	Major divergence events occurring from 20 Ma onward. The initial split occurred between 20 and 15 Ma, with *T. melanopleurus* diverging first. Around 10 Ma, *T. callathelys* separated from the remaining taxa, while most subsequent speciation events took place between 7.5 and 2.5 Ma.
MCMCTree	MSA-nuc (100 most clocklike UCE loci selected through SortaDate)			
PhyloNetworks (SNaQ)	Unrooted nuclear gene trees (100% UCE complete matrix) + concordance factors	Phylogenetic Network	Can we detect introgression in the nuclear genome? Is evolutionary history better explained by networks or bifurcating trees in this case?	All phylogenetic network results suggest minimal or no introgression within the *T. spinulosus* species group.
PhyloNet	Rooted nuclear gene trees (100% UCE complete matrix)			
NetRAX	Unrooted nuclear gene trees (100% UCE complete matrix)			
POPART (TCS haplotype network)	Three mtDNA coding genes (COX1, COX2, and COX3)	Population structure	How is mtDNA structured in the group's lineages?	The clusters align with the groupings in the mt-phylogenetic tree, exhibiting the same pattern of discordance with the nuclear data.
D-suite (ABBA-BABA tests)	SNP	Gene flow	Is gene flow occurring between species within the *Tropidurus spinulosus* group?	Minimal interspecific nuclear admixture, suggesting that introgressive hybridization has had limited impact on the nuclear genomes of these species.
BPP (MSC-M model)	MSA-nuc (100 most clocklike UCE loci selected through SortaDate) + MSA-mt (coding + non-coding regions)	Multiple parameter estimation (mainly ancestral population sizes	Assess how gene flow differentially impacted nuclear and mitochondrial genomes	Which phylogenetic reconstruction more accurately reflects the true history of population divergence: the nuclear species tree or the mitochondrial genealogy? In

		and migration rates)		other words, is the mitochondrial genealogy a reliable representation of species history, with the nuclear tree being discordant due to factors such as incomplete lineage sorting or other processes? Or, conversely, does the nuclear tree capture the actual species relationships, while the mitochondrial genealogy is discordant due to processes like introgression or historical gene flow?
HYPHY	MSA-mt (coding regions)	Selection tests	Is there evidence of positive selection in the mitogenomes of *Tropidurus spinulosus* lineages, supporting an adaptive-based process leading to mitonuclear discordance?	Overall, there is no strong evidence of adaptive processes influencing mtDNA, with positive selection detected at only a few sites in the mitogenome.
Skyline Plot (BEAST)	MSA-mt (three partitions, comprehending six genes)	Demography tests	Estimate changes in effective population sizes over time	Demographic expansions in all analyzed species, particularly from 70 kya onward.
Stairway Plot	SNP			

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Author contributions

Matheus Salles: study design, analyses, lead writing. Fabricius Domingos and André Carvalho: study design, field sampling, genome sequencing, writing final draft. Nicolas Martinez, Frederick Bauer, Martha Motte, Viviana Espínola, Miguel T. Rodrigues, Carla Piantoni, Guarino R. Colli, Fernanda P. Werneck: field sampling efforts and/or provision of collection materials and infrastructure. Márcio Pie, André Olivotto, Erik Choueri, Adam Leache: lab work, genome sequencing. All authors read, commented and approved the final draft of the manuscript.

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925 **Data availability**

926 The data underlying this artic https://doi.org/10.5281/zenodo.15284000le, including

927 phylogenetic datasets, corresponding trees, input and output files for all analyses, and any

928 other relevant supplementary files are available in Zenodo, at

929 https://doi.org/10.5281/zenodo.15284000.

930

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