

SUPPLEMENTARY INFORMATION

Historical biogeography highlights the role of Miocene landscape changes on the diversification of a clade of Amazonian tree frogs

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Sequencing of 16S rRNA

We obtained new mitochondrial sequences of a 16S rRNA fragment from recent field collections ($n = 52$) using two methods: one specifically targeting 16S (Illumina MiSeq high throughput sequencing; $n = 36$), and the second one corresponds to 16S sequences derived from the sequencing of entire mitogenomes ($n = 16$) (GenBank accessions are in Electronic Supplement 2). For both methods, we first extracted DNA from muscle or liver tissue (preserved in 100% ethanol) using the Wizard® Genomic extraction protocol (Promega). The mitogenome sequencing is described in ‘Dated mitogenomic phylogeny’ under Materials and Methods in the main text.

For the MiSeq (Illumina) method, we targeted a ~400 bp fragment for 16S. We used primers N16F/N16R (Salducci et al., 2005), to which we added NNN + 8-nucleotide labels (hereafter designated as ‘tags’) for sample identification as all resulting PCR products were mixed into single libraries: 32 tags for forward primer (N16R) and 36 tags for reverse primer (N16F). We performed PCRs in 20 µl reaction volumes, with 2 µl of template DNA (50 ng/µl), 10 µl of AmpliTaq Gold® 360 Master Mix (Life Technologies), 5.84 µl of Nuclease Free Ambion Water (Thermo Fisher Scientific), 1 µl of each forward and reverse primers (0.25 µM), and 0.16 µl of Bovine Serum Albumin (3.2 µg) (Roche Diagnostic). We ran the PCR in

duplicate for each sample and included blank PCR controls in the analyses using nuclease-free water as template. PCR cycles involved steps I = 95°C for 10 min; II = 95°C for 30 seconds; III = 55°C for 30 seconds; IV = 72°C for 1 minute (steps II–IV x 40 cycles); V = 72 °C for 7 minutes. Libraries of mixed PCR products were sequenced using 2 x 250 paired-end reads sequencing technology using MiSeq high throughput sequencing (Illumina) at the Génopole facility (Toulouse, France). The resulting outputs were analysed with the OBITOOLS software suite (Boyer et al., 2016). Paired-end reads were assembled and merged, and we used the tag attached to the primer to assign the reads to its label. Then we removed low quality reads (alignment scores < 50, containing Ns or shorter than 50 bp). The resulting batch of reads was dereplicated while keeping the coverage information (number of reads merged). All sequences < 100 bp were discarded. Finally, all sequences included in our dataset were > 380 bp long.

Genetic distances (16S)

The uncorrected p-distances (mean and range) calculated for a 16S rRNA fragment (583 bp) among our 50 putative species are in Table S1 (below). The comparisons are presented for eight species groups (the genera *Dryaderces* and *Tepuihyla*, and six *Osteocephalus* species groups). For four of these groups, the mean uncorrected p-distances (in %) among species which correspond to Operational Taxonomic Units (OTUs) after the species delimitation analysis ranged as: *Dryaderces* (0.5–5.2), *O. alboguttatus* group (1.7–4.5), *O. planiceps* group (1.4–6.9), and ‘*O. mimeticus* group’ (2.4). On the other hand, the other four groups contained both species delimited as different OTUs [*Tepuihyla* (4.4–7.7), *O. taurinus* group (1.9–2.8), and *O. buckleyi* group (0.7–5.7)], and species that clustered within the same OTU [*Tepuihyla* (*T. edelcae* vs. *T. aeci* vs. *T. rodriguezi* vs. *T. obscura* = 0.6–1.1), *O. taurinus* group (*O. taurinus* vs. *O. oophagus* = 2.3), *O. leprieurii* group (*O. leprieurii* vs. *O. yasuni* = 1.3), and *O. buckleyi* group (*O. helenae* vs. *O. aff. helenae* 2 = 1.2)].

Summarising, the mean uncorrected p-distance among species that were delimited as separate OTUs ranged from 0.5–7.7, and for species that clustered within the same OTU it ranged from 0.6–2.3. This suggest that our delimitation (led by ABGD method) incorporated clade-specific properties to identify the ‘gap’ existing between inter- and intraspecific distances. Nevertheless, our results corroborate that phenotypically recognized species with low 16S distances are frequent in this group (Jungfer et al., 2013). This may either be due to many recent speciation events (Fig. 1a) and to a slowdown in the evolution rate of this fragment in this group.

References

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Table S1 Interspecific, uncorrected p-distances for a 16S rRNA fragment (583 bp) among OTUs and recognized species clustering within the same OTU for eight ‘species groups’: the genera *Dryaderces* and *Tepuihyla*, and six clades within *Osteocephalus* (Tables S1.1–8). See also ‘Species delimitation (16S)’ under Results and ‘Taxonomic account’ under Appendix. Below the diagonal is the mean genetic distance (in %), and above the diagonal is the range. The order of groups and putative species within groups follows Figure 1

Table S1.1 *Dryaderces* genus

	<i>D. pearsoni</i>	<i>D. inframaculata</i>	<i>D. aff. inframaculata 3</i>	<i>D. aff. inframaculata 4</i>	<i>D. aff. inframaculata 2</i>	<i>D. aff. inframaculata 1</i>
<i>D. pearsoni</i> (<i>n</i> = 2)	-	3.6–4.7	3.8–3.8	3.7–3.7	5.2–5.2	4.0–4.2
<i>D. inframaculata</i> (<i>n</i> = 2)	4.2	-	2.5–3.3	2.3–3.1	2.4–2.9	2.3–3.1
<i>D. aff. inframaculata 3</i> (<i>n</i> = 1)	3.8	2.9	-	0.5–0.5	1.9–1.9	1.6–1.7
<i>D. aff. inframaculata 4</i> (<i>n</i> = 3)	3.7	2.7	0.5	-	1.7–1.7	1.7–1.9
<i>D. aff. inframaculata 2</i> (<i>n</i> = 2)	5.2	2.6	1.9	1.7	-	1.9–2.1
<i>D. aff. inframaculata 1</i> (<i>n</i> = 5)	4.0	2.6	1.6	1.8	1.9	-

Table S1.2 *Tepuihyla* genus (*Tepuihyla edelcae*, *T. aecii*, *T. rodriguezi*, and *T. obscura* clustered within the same OTU)

	<i>T. exophthalma</i>	<i>T. warreni</i>	<i>T. tuberculosa</i>	<i>T. edelcae</i>	<i>T. aecii</i>	<i>T. rodriguezi</i>	<i>T. obscura</i>
<i>T. exophthalma</i> (<i>n</i> = 7)	-	5.7–6.3	5.7–6.2	4.7–5.3	4.3–4.7	4.5–5.7	4.4–5.3
<i>T. warreni</i> (<i>n</i> = 3)	5.9	-	7.7–7.8	5.2–5.8	5.0–5.1	5.2–6.0	4.8–5.2
<i>T. tuberculosa</i> (<i>n</i> = 1)	5.8	7.7	-	5.9–6.0	5.7–5.7	6.0–6.8	6.0–6.4
<i>T. edelcae</i> (<i>n</i> = 5)	4.9	5.5	6.0	-	0.8–0.9	0.8–1.3	0.7–1.3
<i>T. aecii</i> (<i>n</i> = 1)	4.4	5.0	5.7	0.9	-	0.4–0.8	0.5–1.0
<i>T. rodriguezi</i> (<i>n</i> = 13)	4.9	5.6	6.4	1.1	0.6	-	0.5–1.4
<i>T. obscura</i> (<i>n</i> = 4)	4.7	5.0	6.2	1.0	0.7	0.9	-

Table S1.3 *Osteocephalus taurinus* species group (*Osteocephalus taurinus* and *O. oophagus* clustered within the same OTU)

	<i>O. aff. taurinus</i>	<i>O. taurinus</i>	<i>O. oophagus</i>
<i>O. aff. taurinus</i> (<i>n</i> = 2)	-	1.2–3.1	2.1–4.0
<i>O. taurinus</i> (<i>n</i> = 230)	1.9	-	0.7–4.2
<i>O. oophagus</i> (<i>n</i> = 48)	2.8	2.3	-

Table S1.4 *Osteocephalus alboguttatus* species group

	<i>O. alboguttatus</i>	<i>O. heyeri</i>	<i>O. subtilis</i>	<i>O. aff. subtilis</i>
<i>O. alboguttatus</i> (<i>n</i> = 2)	-	4.0–4.3	2.9–3.3	4.3–4.6
<i>O. heyeri</i> (<i>n</i> = 2)	4.2	-	1.7–1.7	3.4–3.4
<i>O. subtilis</i> (<i>n</i> = 1)	3.1	1.7	-	2.7–2.7
<i>O. aff. subtilis</i> (<i>n</i> = 2)	4.5	3.4	2.7	-

Table S1.5 *Osteocephalus planiceps* species group

	<i>O. castaneicola</i>	<i>O. vilarsi</i>	<i>O. planiceps</i>	<i>O. aff. planiceps</i>	<i>O. leoniae</i>	<i>O. aff. leoniae 2</i>	<i>O. aff. leoniae 1</i>	<i>O. fuscifacies</i>	<i>O. deridens</i>	<i>O. aff. deridens</i>
<i>O. castaneicola</i> (<i>n</i> = 7)	-	3.2–5.4	1.4–3.5	2.4–3.3	4.7–6.4	4.3–6.6	3.5–5.0	3.1–5.4	4.2–6.1	3.8–5.7
<i>O. vilarsi</i> (<i>n</i> = 15)	3.9	-	2.0–3.5	3.5–4.0	4.5–6.1	5.5–7.2	3.9–5.2	3.9–5.4	3.9–5.4	3.7–5.2
<i>O. planiceps</i> (<i>n</i> = 13)	2.4	2.6	-	1.4–3.3	3.8–5.2	3.8–5.9	2.4–3.9	2.4–4.1	3.1–3.9	3.1–4.0
<i>O. aff. planiceps</i> (<i>n</i> = 3)	2.6	3.7	2.4	-	5.2–5.4	5.9–6.1	3.8–4.0	3.8–4.7	5.0–5.2	4.0–4.2
<i>O. leoniae</i> (<i>n</i> = 1)	5.1	4.8	4.3	5.3	-	3.8–4.5	3.3–3.3	4.9–5.7	5.7–5.9	4.9–5.1
<i>O. aff. leoniae 2</i> (<i>n</i> = 7)	5.7	6.4	5.0	6.0	4.3	-	2.9–3.5	4.3–6.1	5.7–7.3	5.4–6.8
<i>O. aff. leoniae 1</i> (<i>n</i> = 1)	3.8	4.2	3.3	3.9	3.3	3.4	-	3.6–4.0	4.2–4.3	4.2–4.4
<i>O. fuscifacies</i> (<i>n</i> = 4)	4.2	4.6	3.4	4.2	5.4	5.6	3.8	-	4.0–4.8	4.3–5.2
<i>O. deridens</i> (<i>n</i> = 2)	5.0	4.4	3.4	5.0	5.8	6.9	4.2	4.5	-	1.4–1.5
<i>O. aff. deridens</i> (<i>n</i> = 2)	4.6	4.2	3.4	4.1	5.0	6.5	4.3	4.8	1.4	-

Table S1.6 *Osteocephalus leprieurii* species group (*Osteocephalus leprieurii* and *O. yasuni* clustered within the same OTU)

	<i>O. yasuni</i>	<i>O. leprieurii</i>
<i>O. yasuni</i> (<i>n</i> = 12)	-	0.5–2.6
<i>O. leprieurii</i> (<i>n</i> = 71)	1.3	-

Table S1.7 ‘*Osteocephalus mimeticus* species group’

	<i>O. mimeticus</i>	<i>O. aff. mimeticus</i>
<i>O. mimeticus</i> (<i>n</i> = 6)	-	1.9–3.3
<i>O. aff. mimeticus</i> (<i>n</i> = 3)	2.4	-

Table S1.8 *Osteocephalus buckleyi* species group (*Osteocephalus helenae* and *O. aff. helenae* 2 ‘morph *cabrerai*’ clustered within the same OTU)

	<i>O. festae</i>	<i>O. mutabor</i>	<i>O. carri</i>	<i>O. camufatus</i>	<i>O. aff. helenae</i> 1	<i>O. helenae</i>	<i>O. aff. helenae</i> 2	<i>O. vilmae</i>	<i>O. buckleyi</i>	<i>O. cabrerai</i>	<i>O. aff. cabrerai</i>	<i>O. verruciger</i>	<i>O. cf. omega</i>	<i>O. cannatellai</i>
<i>O. festae</i> (n = 1)	-	4.4–4.4	4.1–4.1	3.5–3.7	4.4–4.4	3.5–5.2	4.1–5.7	4.3–4.6	3.5–3.6	3.7–3.7	4.7–4.7	4.6–4.8	4.1–4.1	3.7–3.7
<i>O. mutabor</i> (n = 1)	4.4	-	2.4–2.4	3.5–3.6	4.9–4.9	3.6–5.9	4.2–5.9	4.3–4.3	3.6–3.7	3.8–3.8	5.7–5.7	4.7–5.2	4.2–4.2	3.5–3.5
<i>O. carri</i> (n = 1)	4.1	2.4	-	3.5–3.6	4.9–4.9	3.8–5.4	4.2–5.4	4.3–4.7	3.6–3.7	3.8–3.8	5.0–5.0	4.7–5.2	4.2–4.2	3.5–3.5
<i>O. camufatus</i> (n = 2)	3.6	3.6	3.6	-	1.7–1.9	1.1–3.1	1.9–3.1	1.7–2.3	0.7–0.9	1.2–1.4	2.4–2.6	2.1–2.6	1.6–1.7	1.2–1.4
<i>O. aff. helenae</i> 1 (n = 1)	4.4	4.9	4.9	1.8	-	1.3–2.6	1.7–2.6	2.6–2.9	1.9–1.9	2.1–2.1	3.5–3.5	2.6–2.9	2.4–2.4	2.1–2.1
<i>O. helenae</i> (n = 40)	4.4	4.9	4.7	2.3	2.0	-	0.5–2.8	1.7–3.3	1.2–2.4	1.4–2.6	2.6–3.8	2.1–3.8	1.7–3.1	1.1–2.6
<i>O. aff. helenae</i> 2 (n = 11)	4.9	5.1	4.8	2.6	2.2	1.2	-	0.4–3.3	1.8–3.1	2.0–3.3	2.5–4.0	2.9–4.2	2.4–3.8	1.5–2.8
<i>O. vilmae</i> (n = 3)	4.4	4.3	4.4	1.9	2.7	2.5	2.8	-	1.0–1.4	1.6–1.9	2.8–3.3	2.4–3.1	1.9–2.3	1.6–1.9
<i>O. buckleyi</i> (n = 3)	3.5	3.7	3.7	0.8	1.9	1.9	2.2	1.2	-	0.9–0.9	1.9–1.9	1.7–2.1	1.2–1.2	0.9–0.9
<i>O. cabrerai</i> (n = 2)	3.7	3.8	3.8	1.3	2.1	2.1	2.5	1.7	0.9	-	1.7–1.7	1.6–1.8	1.0–1.0	0.7–0.7
<i>O. aff. cabrerai</i> (n = 1)	4.7	5.7	5.0	2.5	3.5	3.1	3.3	3.0	1.9	1.7	-	2.6–2.8	2.1–2.1	1.7–1.7
<i>O. verruciger</i> (n = 3)	4.7	4.9	4.9	2.3	2.8	3.1	3.5	2.7	1.9	1.7	2.7	-	1.2–1.5	1.6–1.8
<i>O. cf. omega</i> (n = 3)	4.1	4.2	4.2	1.6	2.4	2.5	2.9	2.0	1.2	1.0	2.1	1.3	-	1.0–1.0
<i>O. cannatellai</i> (n = 2)	3.7	3.5	3.5	1.3	2.1	2.0	2.0	1.7	0.9	0.7	1.7	1.7	1.0	-

Table S2 Age of nodes (in million years ago) estimated from a time-calibrated, Maximum Clade Credibility phylogeny of *Osteocephalus*, *Tepuihyla*, and *Dryaderces* (Anura: Hylidae) resulting from the analysis of mitogenomes (up to 14,098 bp) in BEAST. Node numbers correspond to Figure 1. The outgroup is *Trachycephalus coriaceus*

Node	Mean (95% CI)	Node	Mean (95% CI)
Outgroup - Ingroup	22.3 (15.0–29.4)	25	6.3 (3.5–9.2)
1	19.5 (11.8–26.9)	26	5.3 (2.7–8.1)
2	11.3 (5.6–17.6)	27	5.3 (2.9–7.7)
3	3.3 (1.4–5.3)	28	3.1 (1.6–4.8)
4	2.0 (0.9–3.7)	29	2.2 (1.0–3.5)
5	0.3 (0.08–0.7)	30	4.5 (2.4–6.7)
6	1.6 (0.7–2.7)	31	1.3 (0.5–2.1)
7	17.4 (10.4–24.2)	32	10.4 (5.9–14.8)
8	9.8 (5.3–14.7)	33	1.8 (0.7–3.1)
9	9.0 (4.7–13.5)	34	9.8 (5.6–14.1)
10	8.0 (4.1–12.2)	35	4.6 (1.9–7.6)
11	3.8 (1.5–6.2)	36	7.4 (4.0–10.8)
12	2.2 (1.1–3.5)	37	6.5 (3.4–9.7)
13	1.9 (0.8–3.0)	38	3.1 (1.2–5.1)
14	1.6 (0.7–2.6)	39	4.2 (2.3–6.2)
15	13.1 (7.6–18.4)	40	3.1 (1.6–4.7)
16	5.7 (2.7–9.2)	41	2.2 (1.1–3.5)
17	2.6 (1.0–4.4)	42	1.8 (0.8–2.9)
18	11.7 (6.9–16.6)	43	3.8 (2.1–5.6)
19	10.4 (6.0–14.9)	44	2.1 (0.8–3.4)
20	4.6 (2.3–7.1)	45	3.5 (1.9–5.2)
21	4.0 (1.8–6.2)	46	2.6 (1.2–4.1)
22	3.2 (1.3–5.3)	47	1.9 (0.9–2.9)
23	7.0 (3.9–10.1)	48	1.5 (0.6–2.5)
24	6.5 (3.6–9.4)	49	1.1 (0.4–1.9)

Table S3 BioGeoBEARS results for model selection of ancestral area reconstruction of *Osteocephalus*, *Tepuihyla*, and *Dryaderces* (Anura: Hylidae) in Amazonia (three areas analysed: western Amazonia, Guiana Shield, and Brazilian Shield). Models include parameters of dispersal (d), extinction (e), founder-event (j), and values of log-likelihood (LnL) and Akaike's information criterion with sample size correction (AICc). ^ = best-fitting model

Model	No. parameters	LnL	d	e	j	AICc
^DEC	2	-41.89	0.015	1.0E-12	0	88.04
DEC+J	3	-41.84	0.015	1.0E-12	0.0028	90.21
DIVALIKE	2	-46.04	0.022	1.0E-12	0	96.34
DIVALIKE+J	3	-45.53	0.019	1.0E-12	0.01	97.58
BAYAREALIKE+J	3	-51.99	0.013	1.0E-07	0.036	110.5
BAYAREALIKE	2	-61.16	0.019	0.038	0	126.6

Comments on dispersals within *Osteocephalus*

The nodes associated to all dispersals out of western Amazonia for *Osteocephalus* (within the *O. taurinus*, *O. buckleyi*, and *O. lepriurii* groups; last ~6 My) display some ambiguity on the inferred areas when comparing the probability of different states in DEC model (Fig. 2a) and across models (Electronic Supplement 5: ‘Fig. S3a–f’). The codes for the three analysed areas are: western Amazonia (W), Guiana Shield (G), and Brazilian Shield (B). The results of ancestral areas correspond to states including one of these areas, or combinations of them. The crown age of the *O. taurinus* group (node 16; mean 5.7 Mya) recovered WGB with the highest probability, whereas WG and W were slightly less likely in DEC and DEC+J models (Figs. 2a, S3a–b); these two latter states had the highest probability in other models (Fig. S3c–f). Similarly, the area for the ancestor of *O. taurinus* + *O. oophagus* (node 17; mean 2.6 Mya) was WGB with the highest probability and WG with the second highest in DEC and DEC+J models (Figs. 2a, S3a–b). In the rest of models at this node, G had the highest probability in three of them, and WB in one (Fig. S3c–f).

Within the *O. buckleyi* group, node 39 (mean 4.2 Mya) recovered WB with the highest probability in four models, including DEC (Figs. 2a, S3a–d); while W was a secondary state in these models, and dominant in the remaining two models (Fig. S3e–f). Node 40 (mean 3.1 Mya) recovered WB with the highest probability in half models, including DEC (Figs. 2a, S3a–b, e); while WGB and B were secondary states in those models, and B was dominant in the other three models (Fig. S3c–d, f). In nodes 41 and 42 (means 2.2 and 1.8 Mya, respectively), WGB had the highest probability in DEC and DEC+J models (Figs. 2a, S3a–b); while for other models either GB, G, and B were states with the highest probability at those nodes (Fig. S3c–f). The crown age of the *O. lepriurii* group (node 33; mean 1.8 Mya) included WGB with the highest probability but not as a dominant state in DEC and DEC+J models (Figs. 2a, S3a–b); while W was dominant in the other four models (Fig. S3c–f).

Combined, the models suggest that the colonization sequence of *Osteocephalus* species groups out of W was: *O. taurinus* group first, from W to G and subsequently to B; *O. buckleyi* group second, from W to B and subsequently to G; and the terminal of *O. lepriurii* third, from W to G or B equally likely.