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Molecular phylogenetic analyses reveal both underestimation and overestimation of species diversity in northern rain frogs (*Craugastor*)

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Abstract. Among direct-developing rain frogs of the genus *Craugastor* is a clade of 19 described species (*bocourti* series) that occur in Mexico and northern Central America. Many of these 19 species have been described based on subtle morphological differences and have never been examined using molecular data. Here, we used a multilocus dataset (one mitochondrial (mtDNA) and four nuclear (nDNA) gene fragments, totalling 3,048 concatenated base pairs) to investigate species limits and phylogenetic relationships among 60 northern rain frogs referable to 12 species, with a focus on species from Guatemala. We inferred phylogenies using maximum likelihood and Bayesian analyses on separate mtDNA and nDNA datasets. Concatenated and coalescent species-tree analyses support the monophyly of multiple species, with interspecific relationships mostly unresolved. These mtDNA and nDNA trees were often incongruent with morphology-based taxonomy. For example, two genetically shallow clades contained individuals referable to at least five described species, whereas a single described species contained deep divergences indicative of multiple cryptic species. These findings indicate that morphology-based taxonomy has both overestimated and underestimated actual species diversity (depending on the species), an interpretation supported by two molecular species-delimitation procedures. Based on these findings, we synonymise *C. glaucus* (Lynch, 1967) and *C. stuarti* (Lynch, 1967) with *C. xucanebi* (Stuart, 1941). We also synonymise *C. nefrens* (Smith, 2005) and *C. cyanochthebius* McCranie & Smith, 2006 with *C. campbelli* (Smith, 2005). The molecular data also support multiple undescribed species, notably within *C. decoratus* (Taylor, 1942). Overall, we show how morphology-based species delimitation can both underestimate and overestimate species richness in morphologically conservative groups.

Key words: *Craugastor*, *Hylactophryne*, Craugastoridae, Terraranae, Guatemala, Mexico, Honduras, Brachycephaloidea, cryptic species, phylogeny, species delimitation

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Introduction

Using DNA sequence variation to infer species boundaries has greatly increased our understanding of amphibian evolution. For many anurans (frogs and toads), these data have revealed vast underestimates of species richness (Stuart et al. 2006, Fouquet et al. 2007, Vieites et al. 2009, Funk et al. 2012, Rojas et al. 2018, Scherz et al. 2022). This underestimation has been particularly well-documented in a large radiation of New World direct-developing frogs called Brachycephaloidea (Padial et al. 2014) or Terraranae (Heinicke et al. 2018). In this clade, molecular data have revealed high levels of morphologically cryptic species diversity (Miyamoto 1983, Crawford 2003, Crawford et al. 2007, Padial & De La Riva 2009, Kieswetter & Schneider 2013, Fusinatto et al. 2013, Pie et al. 2018). Interestingly, the discovery of 'cryptic' taxa via molecular methods even extends to the familial level in this group, with many species traditionally placed in the genus *Eleutherodactylus* now distributed across multiple families (Heinicke et al. 2009). While the increasing affordability and ease of collecting DNA sequence data has shed much light on terraranan diversity, extensive molecular studies have yet to be conducted on many groups.

Northern rain frogs (*Craugastor bocourti* Species Series; *sensu* Hedges et al. 2008, hereafter '*bocourti* series') occur as far north as southern Tamaulipas in Mexico, as far west as central Guerrero in Mexico, as far south as Guatemala, and as far east as Honduras (Fig. 1). Most members of the *bocourti* series were previously included in the *Eleutherodactylus alfredi* group (Lynch 1966, 1967a, Campbell et al. 1989, Smith 2005). These frogs now belong to the family Craugastoridae (*sensu* Barrientos et al. 2021) and the subgenus *Hylactophryne* (*sensu* Hedges et al. 2008, Padial et al. 2014, Heinicke et al. 2018). Morphologically, species in the *bocourti* series are easily distinguished from congeners by the presence of large, expanded pads on fingers III and IV (Campbell et al. 1989; Fig. 2). The *bocourti* series currently contains 19 described species: *C. alfredi*, *C. batrachylus*, *C. bocourti*, *C. campbelli*, *C. cyanocthebius*, *C. decoratus*, *C. galacticorhinus*, *C. glaucus*, *C. guerreroensis*, *C. megalotympanum*, *C. nefrens*, *C. polymniae*, *C. silvicola*, *C. spatulatus*, *C. stuarti*, *C. taylori*, *C. uno*, *C. xucanebi* and *C. yucatanensis* (Frost 2023, Hedges et al. 2008). Conducting research on the *bocourti* series is challenging because most species are rarely encountered in the field (Streicher et al. 2011, Palacios-Aguilar 2017, Carbajal-Márquez et al. 2019). For example, 74% of these 19 species were described based on three or fewer specimens (Boulenger 1898, Taylor 1940, 1942, Stuart 1941, Shannon & Werler 1955, Lynch 1966, 1967a, b, Savage 1985 '1984', Campbell et al. 1989, Canseco-Márquez & Smith 2004). Furthermore, obtaining tissue samples for molecular analysis can be difficult because some species are considered vulnerable by the IUCN (e.g. *Craugastor uno*, Angulo et al. 2020; *Craugastor alfredi*, Luría-Manzano & Ramirírez-Bautista 2017). Despite these limitations, previous molecular studies have included up to ten described species from the *bocourti* series and supported its monophyly (Crawford & Smith 2005, Heinicke et al. 2007, Hedges et al. 2008, Pyron & Wiens 2011, Padial et al. 2014, Portik et al. 2023).

In addition to small sample sizes, another serious issue in the systematics of the *bocourti* series is that many species are distinguished by only subtle morphological differences. These differences include traits like palmar and plantar tubercle depth (Smith 2005), colour pattern (Canseco-Márquez & Smith 2004), and the relative widths of finger and toe pads (Shannon & Werler 1955, Lynch 1967a, McCranie & Smith 2006). Because of this overall morphological uniformity, assigning individuals to species, particularly juveniles and poorly preserved natural history specimens, is often a nontrivial task. This issue is exacerbated in locations where poorly known taxa are putatively sympatric (e.g. Central Mexico and southern Guatemala; Fig. 1).

In this study, we build on previous work by generating a multilocus dataset (one mitochondrial and four nuclear gene fragments) for 12 described species of the *bocourti* series with a focus on species from Guatemala and Honduras (Fig. 1). We used these data to produce gene and species trees and estimate species limits to compare to morphology-inferred species boundaries.

Material and Methods

Assigning specimens to species

As noted above, species in the *bocourti* series are challenging to identify because morphological differences among them are subtle, and many poorly known taxa occur in sympatry in some regions (e.g. Central Mexico and southern Guatemala; Fig. 1). Because we sampled several specimens from these challenging regions, we used comparisons of voucher specimens with type specimens to assign specimens to species. In cases where we could not confidently match vouchered material to types, we describe specimens using open nomenclature (*sensu*

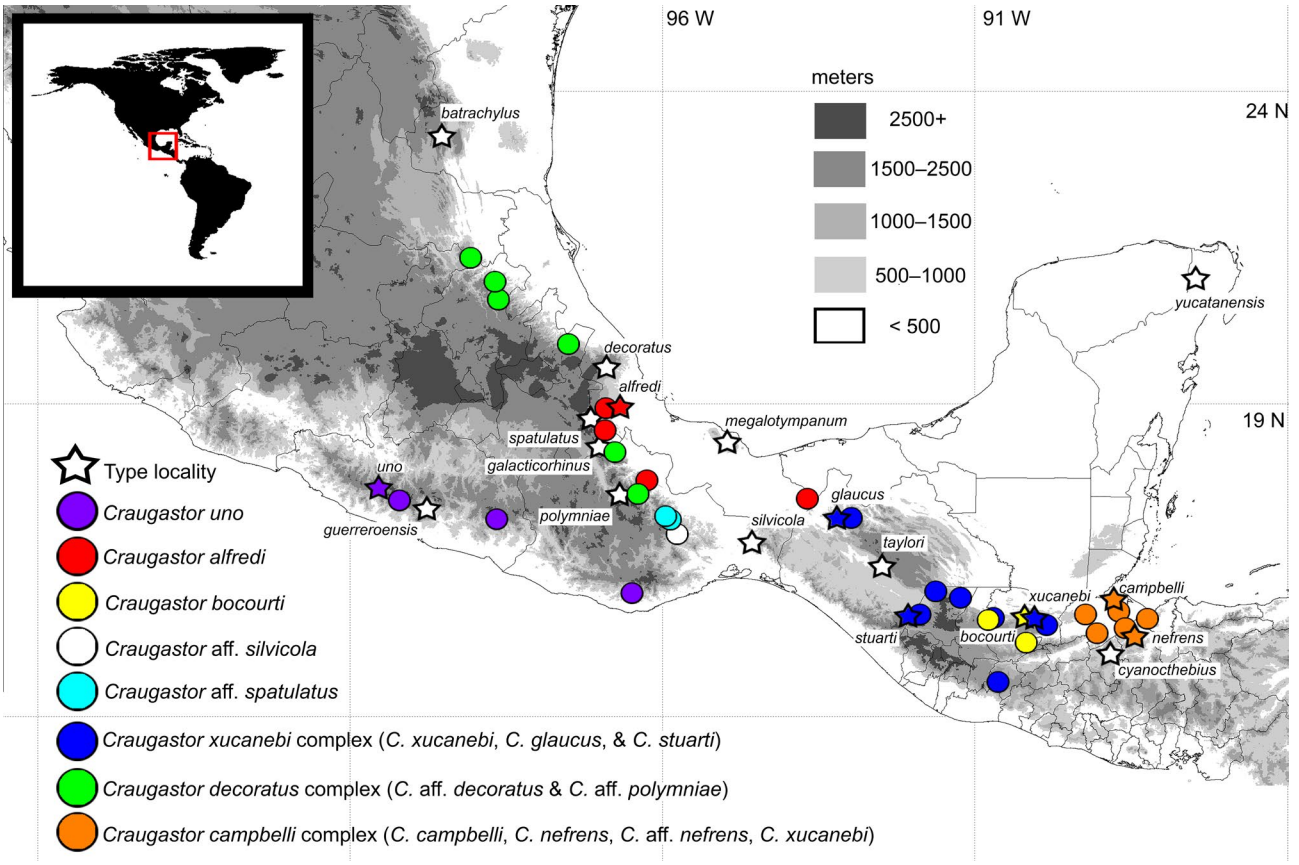

Fig. 1. Geographic distribution of samples used in the molecular analysis (circles) and type localities (stars) for the *Craugastor bocourti* series. Filled stars correspond to species with topotypic sampling.

Bengston 1988). Specifically, we used the designator *affinis* (aff.) when a specimen had a morphology that was highly similar to (but not completely congruent with) a described species.

We were able to confidently match specimens used for our molecular analyses to the following species based on type comparisons (type specimens used for comparisons in brackets; see acknowledgements for definitions of museum abbreviations): *C. alfredi* (BMNH 1947.2.15.54-55), *C. bocourti* (MNHNP 6413), *C. campbelli* (UTA A-33452), *C. glaucus* (TCWC 21463), *C. nefrens* (UTA A-45279), *C. stuarti* (UMMZ 126738), *C. uno* (UTA A-7984), and *C. xucanebi* (UMMZ 89914). We applied open nomenclature to several specimens originating from the Sierra Madre Oriental and Sierra Madre del Sur of Mexico (*C.* aff. *decoratus*, *C.* aff. *polymniae*, *C.* aff. *silvicola*, and *C.* aff. *spatulatus*) and eastern Honduras (*C.* aff. *nefrens*) because they did not convincingly match type specimens. These assignments resulted in 12 species names being associated with our molecular sampling, either through confident identification based on type specimens (eight species) or substantial morphological similarity to the type material (four species). Two of these species, *C. glaucus* and *C. nefrens*, have not previously had molecular data generated for them.

Taxonomic, genetic, and geographic sampling

We collected specimens from the field in Mexico, Guatemala, and Honduras and generated molecular data for 49 individuals assigned to eight morphology-based species and for 11 individuals with uncertain taxonomic affinities (open nomenclature). The sampling included specimens of *C. alfredi* (n = 4), *C. bocourti* (n = 3), *C. campbelli* (n = 3), *C.* aff. *nefrens* (n = 4), *C.* aff. *decoratus* (n = 7), *C. glaucus* (n = 1), *C. nefrens* (n = 1), *C.* aff. *polymniae* (n = 2), *C.* aff. *silvicola* (n = 1), *C.* aff. *spatulatus* (n = 2), *C. stuarti* (n = 3), *C. uno* (n = 4), and *C. xucanebi* (n = 25). Specific collection localities for all specimens are listed in Table 1. We obtained topotypic (or nearly topotypic) material for seven of the eight confidently identified species (*C. alfredi*, *C. bocourti*, *C. campbelli*, *C. glaucus*, *C. nefrens*, *C. stuarti*, and *C. uno*; Fig. 1).

We selected molecular markers used extensively in terraranan phylogenetics (e.g. Heinicke et al. 2007, Padial et al. 2014). For all 60 individuals, we sequenced a 460 base pair (bp) segment of the

Fig. 2. Examples of the *Craugastor bocourti* series. Clockwise from top left, palmar surface of the holotype of *Eleutherodactylus hidalgoensis* (FMNH 100094) demonstrating the expanded pads on fingers III and IV that is characteristic of the series; *C. uno* from Guerrero, Mexico; *C.* aff. *decoratus* from San Luis Potosí, Mexico; *C.* aff. *decoratus* from Hidalgo, Mexico; *C.* aff. *nefrens* from Cortés, Honduras; *C. campbelli* from Izabal, Guatemala.

mitochondrial 12S ribosomal subunit gene (12S). We obtained a multilocus nuclear DNA (nDNA) dataset for a subset of these individuals (n = 50), including representatives of all eight confidently identified species. Our nDNA dataset consisted of fragments of four genes: 351 bp of rhodopsin (exon 1; Rho), 653 bp of the recombination-activating protein 1 (RAG-1), 560 bp of tyrosinase precursor (Tyr), and 981 bp of the cellular-myelocitomatosis gene (c-*myc*). The c-*myc* gene fragment consisted of separate 403 bp and 578 bp sections that correspond to exons two and three, respectively. The outgroup taxa sampled included: 1) the sister group of the *bocourti* series (the *C. augusti* series; Hedges et al. 2008, Streicher et al. 2014), including one individual of *C. augusti* and one of *C. tarahumaraensis*; and 2) the species *C. daryi*, a representative of the *Craugastor* subgenus *Campbellius* (Hedges et al. 2008). The subgenus *Campbellius* was targeted as a distant outgroup because it has been supported as the earliest branching lineage of *Craugastor* in multiple studies (Crawford & Smith 2005, Hedges et al. 2008, Pyron & Wiens 2011, Padial et al. 2014, Portik et al. 2023). A complete list of voucher specimens, locality information, and GenBank numbers are provided in Table 1.

Laboratory methods and DNA sequencing

We obtained genomic DNA from muscle and liver tissues using Qiagen® kits. Before DNA extraction, tissue samples were stored in either 70-100% ethanol or an SDS-based lysis buffer. We used Promega reagents (e.g. polymerases, dNTPs, $MgCl_2$) to amplify DNA fragments. PCR amplification was performed using Green Taq Master Mix (Promega) and previously designed oligonucleotide primers (Table 2). To amplify 12S we used a standard thermal cycling profile with an annealing temperature of 50 °C. We amplified nuclear loci (c-*myc*, RAG-1, Rho, Tyr) using the touchdown thermal cycling protocol described in Streicher et al. (2009). Cycle sequencing was performed using Big-Dye terminator chemistry, and samples were sequenced by either SeqWright (Houston, Texas, USA) or the University of Texas at Arlington (UTA) genomics core facility (Arlington, Texas, USA). We used the PCR primers listed in Table 2 as sequencing primers and sequenced gene fragments in both directions for all loci except the c-*myc* exons, which were only sequenced in one direction.

We used the programs Sequencher v 4.1 (Gene Codes Corp; Ann Arbor, Michigan, USA) or Geneious v 7.1.3 (Biomatters; http://www.geneious.com) to clean the resulting DNA sequences. For nDNA loci, we assumed that a site was heterozygous (coded as a degenerate base) if equal chromatogram peaks were present for both bases (Hare & Palumbi 1999). We did not phase nuclear loci because putatively heterozygous sites were rare (< 10 observed) and thus unlikely to have a large influence on topological reconstructions.

Table 1. Specimen information and GenBank accession numbers for samples of *Craugastor* used in this study. MX = Mexico, GT = Guatemala, and HD = Honduras.

Taxon	Field No.	Museum No.	Locality	GPS N	GPS W	12S	RAG-1	Rho	Tyr	c-*myc* exon 2	c-*myc* exon 3
Genus *Craugastor*											
1. *alfredi*	JAC 21987	UTA A-62631	MX: Veracruz: near Córdoba	18.89417	–96.9347	JX001674	None	JX001826	None	None	None
2. *alfredi*	ENS 10031	ENEPI 6852	MX: Oaxaca: Jacatepec	17.77	–96.22	JX001688	JX001805	JX001870	JX001901	MW796431	MW815678
3. *alfredi*	JAC 24288	UTA A-62632	MX: Veracruz: Angelica	18.56667	–96.95	JX001699	JX001812	None	JX001908	MW796435	MW815694
4. *alfredi*	UOGV 379	MZFC HE-22131	MX: Tabasco: Cerro Las Flores	17.4753	–93.691	MW788659	None	None	None	None	None
5. *bocourti*	ENS 8245	UTA A-51245	GT: Quiché: Uspantán	15.51194	–90.7919	JX001711	JX001814	JX001854	None	MW796425	MW815699
6. *bocourti*	ENS 8060	UTA A-51220	GT: Quiché: Uspantán	15.51194	–90.7919	JX001713	JX001813	JX001871	None	MW796438	MW815693
7. *bocourti*	GAR 181	UTA A-55235	"Guatemala"	N/A	N/A	EF493713	JX001806	JX001824	None	MW796412	MW815686
8. *campbelli*	ENS 7069	UTA A-55228	GT: Izabal: Montañas del Mico	15.37	–89.06	JX001703	JX001781	JX001856	None	MW796423	MW815677
9. *campbelli*	JAC 20572	UTA A-53034	GT: Izabal: Montañas del Mico	?	?	JX001705	JX001793	JX001852	JX001897	MW796413	MW815687
10. *campbelli*	ENS 10239	UTA A-53035	GT: Izabal: Montañas del Mico	15.67	–88.68	JX001702	JX001790	JX001853	JX001902	MW796428	MW815674
11. aff. *decoratus*	JAC 26066	UTA A-62643	MX: Hidalgo: Tlanchinol	20.97563	–98.6694	JX001708	JX001797	JX001851	JX001907	MW796416	MW815689
12. aff. *decoratus*	JRM 4770	MZFC HE-16903	MX Oaxaca: S Vista Hermosa	17.58333	–96.4	JX001712	JX001798	JX001864	JX001906	MW796424	None
13. aff. *decoratus*	JAC 26659	UTA A-66137	MX: Hidalgo: Tlanchinol	20.9639	–98.6775	MW788660	None	None	None	None	None

Taxon	Field No.	Museum No.	Locality	GPS N	GPS W	12S	RAG-1	Rho	Tyr	c-*myc* exon 2	c-*myc* exon 3
14. aff. *decoratus*	UOGV 468	MZFC HE-23063	MX: Puebla: Tlatlauquitepec	19.9618	–97.5383	MW788661	None	None	None	None	None
15. aff. *decoratus*	UOGV 470	MZFC HE-23065	MX: Puebla: Tlatlauquitepec	19.9618	–97.5383	MW788662	None	None	None	None	None
16. aff. *decoratus*	SMR 1327	IBH 30456	MX: San Luis Potosí: Ahuacatlan	21.31886	–99.0526	JX001708	JX001799	None	None	None	None
17. aff. *decoratus*	UOGV 497	MZFC HE-20481	MX: Hidalgo: Zacuatipan	20.65	–98.6	MW788663	None	None	None	None	None
18. *glaucus*	JAC 24518	UTA A-62626	MX: Chiapas: Pueblo Nuevo Solistahuacan	17.20421	–92.9701	JX001675	JX001804	JX001844	JX001913	MW796444	MW815672
19. *nefrens*	ENS 7841	UTA A-51370	GT: Izabal: Sierra de Caral	15.433	–88.575	JX001707	JX001792	JX001825	JX001898	MW796417	MW815690
20. aff. *nefrens*	TS1	N/A	HD: Cortés: Cusuco National Park	15.577	–88.3	JX001740	JX001794	None	None	None	None
21. aff. *nefrens*	TS2	N/A	HD: Cortés: Cusuco National Park	15.577	–88.3	JX001749	JX001800	None	None	None	None
22. aff. *nefrens*	TS3	N/A	HD: Cortés: Cusuco National Park	15.577	–88.3	JX001746	JX001791	None	None	None	None
23. aff. *nefrens*	TS4	N/A	HD: Cortés: Cusuco National Park	15.577	–88.3	JX001742	JX001780	None	None	None	None
24. aff. *polymniae*	JAC 22728	UTA A-62653	MX: Oaxaca: Ejido Clemencia	18.244	–96.791	MW788665	None	None	None	None	None
25. aff. *polymniae*	JAC 22727	UTA A-62652	MX: Oaxaca: Ejido Clemencia	18.244	–96.791	MW788664	None	None	None	None	None
26. aff. *silvicola*	UOGV 423	MZFC HE-22140	MX: Oaxaca: San Lucas Camotlan	16.95	–95.7833	MW788666	None	None	None	None	None

Taxon	Field No.	Museum No.	Locality	GPS N	GPS W	12S	RAG-1	Rho	Tyr	c-*myc* exon 2	c-*myc* exon 3
27. *spatulatus*	JAC 21604	UTA A-62646	MX: Oaxaca: Sierra Mixe	17.1717	–95.9353	JX001700	JX001795	JX001858	JX001905	MW796437	None
28. *spatulatus*	JAC 21615	UTA A-62647	MX: Oaxaca: Sierra Mixe	17.1809	–95.9694	JX001701	JX001796	JX001833	JX001904	MW796429	MW815688
29. *stuarti*	ENS 7943	UTA A-51373	GT: Huehuetenango: La Democracia	15.624	–91.887	JX001676	JX001810	JX001839	JX001912	MW796448	MW815671
30. *stuarti*	ENS 7946	UTA A-51374	GT: Huehuetenango: La Democracia	15.624	–91.887	JX001692	None	JX001823	JX001911	MW796436	MW815671
31. *stuarti*	ENS 7940	UTA A-51371	GT: Huehuetenango: La Democracia	15.624	–91.887	JX001697	None	None	None	None	MW815664
32. *uno*	ENS 9494	ENEPI 6431	MX: Guerrero: Sierra Madre del Sur	17.45611	–100.198	GU576484	None	JX001846	None	MW796415	MW815681
33. *uno*	ENS 9496	ENEPI 6433	MX: Guerrero: Sierra Madre del Sur	17.45611	–100.198	GU576485	GU576494	JX001860	GU576490	MW796420	MW815696
34. *uno*	JAC 21377	UTA A-59250	MX: Oaxaca: San Jose-Candelaria	15.97894	–96.5167	GU576482	GU576496	JX001855	GU576492	MW796439	MW815676
35. *uno*	JAC 25673	UTA A-59252	MX: Guerrero: Malinal-Tepec	17.17068	–98.6807	GU576486	GU576493	None	GU576488	None	None
36. *xucanebi*	ENS 8890	UTA A-62636	GT: Huehuetenango: Barillas	15.85417	–91.2308	JX001699	JX001816	JX001872	None	None	None
37. *xucanebi*	ENS 8999	UTA A-62638	GT: Huehuetenango: Barillas	15.867	–91.233	JX001698	JX001750	JX001848	JX001921	MW796443	TBD
38. *xucanebi*	ENS 8891	UTA A-62637	GT: Huehuetenango: Barillas	15.867	–91.233	JX001673	JX001809	JX001849	JX001922	MW796440	MW815697

Taxon	Field No.	Museum No.	Locality	GPS N	GPS W	12S	RAG-1	Rho	Tyr	c-*myc* exon 2	c-*myc* exon 3
39. *xucanebi*	ENS 9000	UTA A-62639	GT: Huehuetenango: Barillas	15.867	–91.233	JX001668	JX001817	JX001837	JX001918	MW796432	MW815668
40. *xucanebi*	JAC 19442	UTA A-62633	GT: Huehuetenango: La Trinidad-Finca San Francisco	15.983	–91.6	JX001691	JX001782	JX001861	JX001910	MW796442	MW815675
41. *xucanebi*	MEA 3384	UTA A-62642	GT: Sacatepéquez: Santa Lucia	14.579	–90.67	JX001689	None	JX001842	JX001903	None	None
42. *xucanebi*	ENS 8853	UTA A-62635	GT: Huehuetenango: Barillas	15.883	–91.233	JX001704	JX001788	JX001834	None	MW796410	MW815691
43. *xucanebi*	ENS 9001	UTA A-62640	GT: Huehuetenango: Barillas	15.867	–91.233	JX001690	JX001821	JX001847	None	None	MW815700
44. *xucanebi*	ENS 8852	UTA A-62634	GT: Huehuetenango: Barillas	15.883	–91.233	JX001694	None	JX001850	JX001899	MW796433	None
45. *xucanebi*	ENS 8100	UTA A-51363	GT: Quiché: Uspantán	15.51194	–90.7919	JX001685	JX001783	JX001838	None	MW796430	MW815665
46. *xucanebi*	ENS 8117	UTA A-51364	GT: Quiché: Uspantán	15.51194	–90.7919	JX001696	JX001820	JX001840	None	MW796445	MW815667
47. *xucanebi*	JAC 20483	UTA A-53030	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001677	JX001801	JX001829	JX001919	MW796418	MW815682
48. *xucanebi*	JAC 20481	UTA A-53028	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001682	JX001785	JX001828	None	None	MW815666
49. *xucanebi*	JAC 20485	UTA A-53032	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001680	JX001810	JX001863	None	MW796434	MW815683
50. *xucanebi*	JAC 20482	UTA A-53029	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001683	JX001803	JX001827	None	MW796411	MW815698
51. *xucanebi*	JAC 20486	UTA A-53033	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001679	JX001784	JX001862	None	None	None

Taxon	Field No.	Museum No.	Locality	GPS N	GPS W	12S	RAG-1	Rho	Tyr	c-*myc* exon 2	c-*myc* exon 3
52. *xucanebi*	JAC 20484	UTA A-53031	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001678	JX001802	JX001830	JX001914	MW796427	MW815684
53. *xucanebi*	JAC 20480	UTA A-53027	GT: Alta Verapaz: Finca San Juan	15.467	–89.867	JX001681	JX001818	JX001835	JX001900	MW796446	MW815695
54. *xucanebi*	JAC 19267	UTA A-51368	GT: Huehuetenango: Sierra de los Cuchumatanes	15.883	–91.233	JX001686	JX001786	JX001841	JX001917	MW796441	None
55. *xucanebi*	JAC 19266	UTA A-51367	GT: Huehuetenango: Sierra de los Cuchumatanes	15.88278	–91.2467	JX001687	JX001787	JX001845	JX001920	MW796409	MW815679
56. *xucanebi*	JAC 19316	UTA A-51369	GT: Huehuetenango: Sierra de los Cuchumatanes	15.87	–91.2258	JX001684	JX001789	JX001832	JX001896	MW796447	MW815680
57. *xucanebi*	ENS 7804	UTA A-51316	?	?	?	JX001670	JX001819	JX001831	JX001915	MW796419	None
58. *xucanebi*	MEA 2024	UTA A-62641	'Guatemala'	N/A	N/A	JX001672	JX001808	JX001843	None	None	None
59. *xucanebi*	GAR 54	UTA A-51362	GT: Biotopo del Quetzal	15.214	–90.217	MW788667	MW809529	MW809533	MW809532	MW796421	MW815685
60. *xucanebi*	JAC 20552	UTA A-53048	GT: Izabal: Sierra de Santa Cruz	15.66	–89.25	JX001693	JX001815	JX001853	JX001902	MW796426	MW815673
Outgroups											
61. *augusti*	JRM 4651	UTA A-54930	MX: Guerrero: Chilpancingo	17.617	–99.083	JX001739	JX001772	JX001880	JX001926	MW796449	MW815709
62. *augusti*	JAC 23344	UTA A-57707	MX: Jalisco: Santa María de Las Angeles-Bolaños	21.98532	–103.347	JX001717	JX001764	JX001878	JX001929	MW796457	MW815720
63. *tarahumaraensis*	JAC 29189	UTA A-62630	MX: Chihuahua: Guadalupe y Calvo	25.94136	–106.775	JX001735	JX001771	JX001891	None	None	None
64. *milesi*	MEA 622	UTA A-62648	GT: Alta Verapaz: Chisec	?	?	JX001695	MW809530	MW809534	MW809531	MW796414	MW815692

Table 2. Primers used to amplify and sequence *Craugastor* mitochondrial (mtDNA) and nuclear (nDNA) gene fragments.

Locus (Genome)	Primer name	Direction	Sequence (5′ to 3′)	Reference
12S (mtDNA)	L1091	F	AAA CTG GGA TTA GAT ACC CCA CTA T	Bossuyt & Milinkovitch 2000
12S (mtDNA)	12E	R	ACA CAC CGC CCG TCA CCC TC	Liu et al. 2000
Rhod (nDNA)	Rhod.ma	F	AAC GGA ACA GAA GGY CC	Hoegg et al. 2004
Rhod (nDNA)	Rhod.md	R	GTA GCG AAG AAR CCT TC	Hoegg et al. 2004
Tyr (nDNA)	Tyr1C	F	GGC AGA GGA WCR TGC CAA GAT GT	Hedges et al. 2008
Tyr (nDNA)	Tyr1G	R	TGC TGG GCR TCT CTC CAR TCC CA	Hedges et al. 2008
RAG-1 (nDNA)	R182	F	GCC ATA ACT GCT GGA GCA TYA T	Hedges et al. 2008
RAG-1 (nDNA)	R270	R	AGY AGA TGT TGC CTG GGT CTT C	Hedges et al. 2008
c-*myc* (nDNA)	L4437	F	AAG CTT TCG GGC CCA TAC C	Crawford & Smith 2005
c-*myc* (nDNA)	H5934	R	AGR GTG CCA ATG TCT TTG TGR TT	Crawford & Smith 2005

Instead, we coded these sites as degenerate bases for analysis and GenBank submission.

Sequence alignments were performed using MUSCLE (Edgar 2004) software executed in MEGA 5.1 (Tamura et al. 2011). We identified reading frames for protein-coding genes (RAG-1, Tyr, Rho, c-*myc*) and performed multilocus concatenation using Geneious v 7.1.3. All alignments used in this study were deposited in the Natural History Museum Data Portal (http://data.nhm.ac.uk).

Separate phylogenetic analyses of mitochondrial and nuclear DNA

We separately analysed mtDNA and concatenated nDNA data using maximum likelihood (ML) and Bayesian phylogenetic methods. To select an appropriate model of nucleotide evolution, we used MEGA 5.1 for mtDNA (12S), treating the entire gene fragment as a single partition. For nDNA, we used PartitionFinder v1.1 (Lanfear et al. 2012) with codon partitions specified for all loci (including for exons 1 and 2 of *c-myc* separately). The nucleotide models selected for each partition are listed in Table 3.

We performed ML analyses using MEGA 5 using a heuristic tree search criterion with nearest-neighbour interchange. We assessed ML branch support using bootstrapping analysis (1,000 pseudoreplicates). We performed Bayesian Markov Chain Monte Carlo (MCMC) analyses using MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003). For Bayesian MCMC analyses we unlinked all parameters between the identified partitions and used a variable rate prior. We used default settings for Bayesian MCMC concatenated analyses except that we increased the sampling frequency to 1,000 generations and ran the analyses for 10 million generations. We used the online software 'Are we there yet?' (AWTY; Wilgenbusch et al. 2004) to select an appropriate number of sampled trees to discard as burn-in before summarising the paired Bayesian MCMC runs and calculating posterior probabilities, using the majority rule consensus of the post-burnin trees. We visualised trees using the software FigTree v 1.4 (available at http://tree.bio.ed.ac.uk/software/figtree/).

Combined Bayesian species-tree inference

We performed a Bayesian species-tree analysis using all five markers (1 mtDNA + 4 nDNA) with the software *BEAST v 1.6.0 (Heled & Drummond 2010) and BEAST 1.7.5 (Drummond & Rambaut 2007). As in our MrBayes 3.2.1 concatenated analysis of nDNA, we treated the two c-*myc* exons as separate data partitions. While recombination within *c-myc* is unlikely, we chose to treat the exons as separate partitions because model selection identified different codon model schemes for each exon (Table 3). We used the seven matched mtDNA and nDNA clusters of individuals as our *a priori* 'species' for the *BEAST analysis (Table 4). Clusters were identified using the following criteria: 1) they contained the

Table 3. Models of nucleotide evolution selected for alignments of *Craugastor* mitochondrial (mtDNA) and nuclear (nDNA) gene fragments. Partition column indicates regions of nDNA that were included in the same partition because of Model overlap.

Region	Genome	Model	Partition	Model Selection Tool
1. 12S	mtDNA	GTR+G	mtDNA 1	MEGA 5.1 (BIC)
2. Tyr_{pos1}	nDNA	HKY+G	nDNA 1	PartitionFinder (BIC)
3. Tyr_{pos2}	nDNA	K80+G	nDNA 3	PartitionFinder (BIC)
4. Tyr_{pos3}	nDNA	HKY+G	nDNA 1	PartitionFinder (BIC)
5. $RAG\text{-}1_{pos1}$	nDNA	HKY+G	nDNA 1	PartitionFinder (BIC)
6. $RAG\text{-}1_{pos2}$	nDNA	HKY+G	nDNA 1	PartitionFinder (BIC)
7. $RAG\text{-}1_{pos3}$	nDNA	K80+G	nDNA 3	PartitionFinder (BIC)
8. Rho_{pos1}	nDNA	HKY+G	nDNA 2	PartitionFinder (BIC)
9. Rho_{pos2}	nDNA	HKY+G	nDNA 2	PartitionFinder (BIC)
10. Rho_{pos3}	nDNA	K80+G	nDNA 3	PartitionFinder (BIC)
11. c-myc-exon 2_{pos1}	nDNA	K80+G	nDNA 3	PartitionFinder (BIC)
12. c-myc-exon 2_{pos2}	nDNA	JC	nDNA 4	PartitionFinder (BIC)
13. c-myc-exon 2_{pos3}	nDNA	HKY+G	nDNA 2	PartitionFinder (BIC)
14. c-myc-exon 3_{pos1}	nDNA	HKY+G	nDNA 2	PartitionFinder (BIC)
15. c-myc-exon 3_{pos2}	nDNA	K80+I+G	nDNA 5	PartitionFinder (BIC)
16. c-myc-exon 3_{pos3}	nDNA	K80+G	nDNA 3	PartitionFinder (BIC)

same individuals, 2) they were monophyletic in both nDNA and mtDNA analyses, and 3) their monophyly received high Bayesian statistical support. We also included two individuals of *C. augusti* and one individual of *C. tarahumaraensis* as outgroups.

We used the same model-partitioning scheme and models employed for the separate analyses of mtDNA and nDNA when using MrBayes (Table 3). We used a Yule tree prior and unlinked loci and substitution models across the dataset. We used an uncorrelated relaxed-clock model to account for rate variation among branches. Species-trees were selected and scored based on a 10 million generation MCMC analysis sampled every 1,000 generations. We used Tracer v 1.5 (Rambaut & Drummond 2007) to ensure that all parameters had reached effective sample sizes over 200. We used a burn-in of 10% (1 million generations) before summarising species trees to determine the 95% Highest Posterior Density (HPD) of posterior distributions. To visualise the 95% HPD tree set, we used the program DensiTree v 2.1 (Bouckaert 2010).

Species delimitation procedures

To infer species boundaries in each dataset, we used two approaches: ML Poisson Tree Process (PTP) species delimitation (Zhang et al. 2013) and a

Table 4. Matched mitochondrial (mtDNA) and nuclear (nDNA) DNA clusters of *Craugastor* used in species tree analysis.

Matched Cluster	mtDNA (Fig. 3) BAYES / ML	nDNA (Fig. 4) BAYES / ML	Taxa
1	1.0/100	1.0/95	*C. glaucus*, *C. stuarti*, and *C. xucanebi*
2	1.0/100	1.0/98	*C. campbelli*, *C. nefrens*, *C.* aff. *nefrens*, and *C. xucanebi*
3	1.0/82	1.0/94	*C. alfredi*
4	1.0/100	1.0/99	*C. bocourti*
5	1.0/100	1.0/99	*C. spatulatus*
6	0.94/NS	1.0/89	*C.* aff. *decoratus*, *C.* aff. *polyminae**
7	1.0/100	1.0/99	*C. uno*

*Included in mtDNA analysis only; NS = No Support.

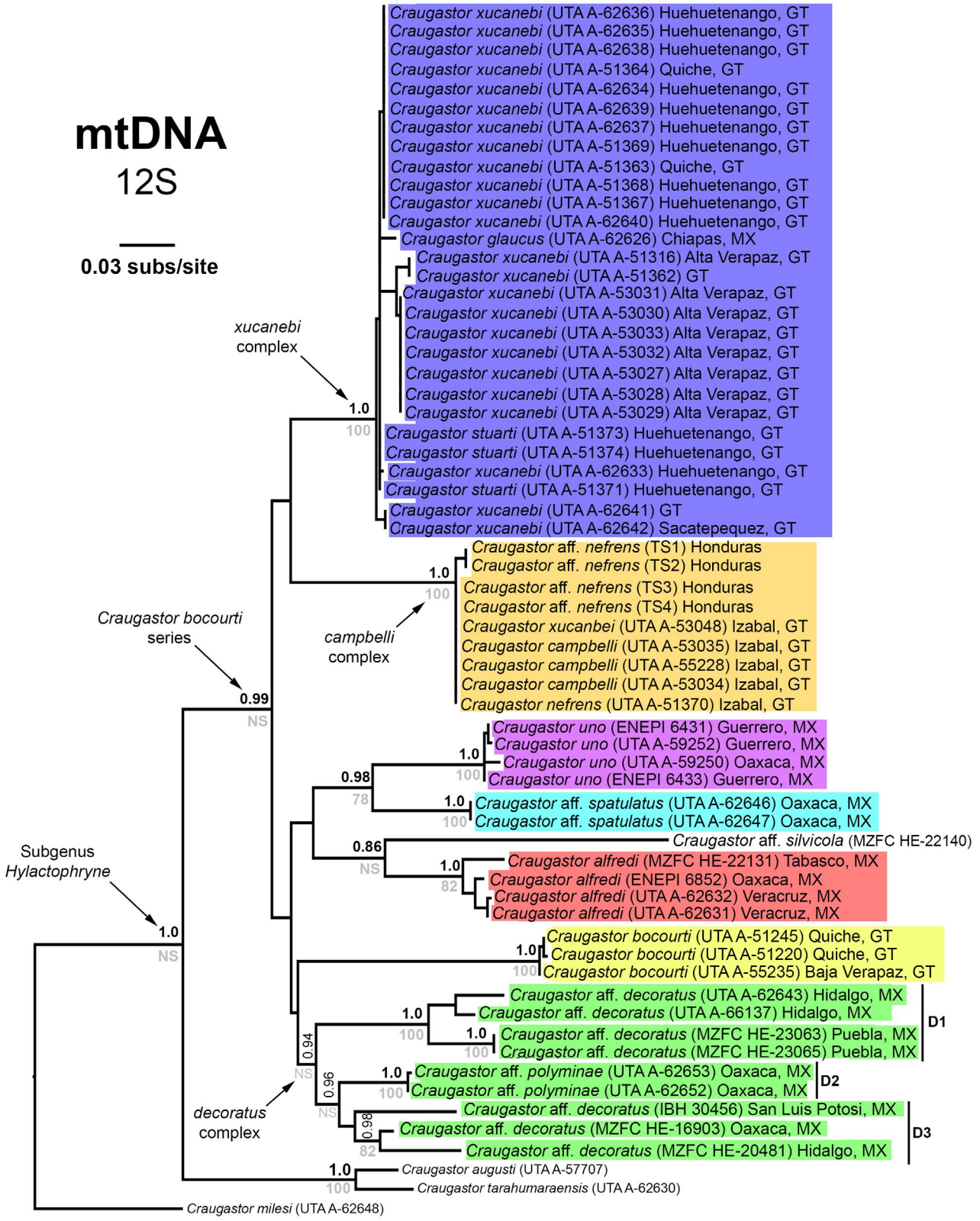

Fig. 3. Mitochondrial phylogram for the *Craugastor bocourti* series resulting from Maximum Likelihood analysis. Nodal support values from ML bootstrapping appear below branches (grey) and Bayesian inference appears above branches (black). NS indicates branches with no statistical support.

generalised mixed Yule-coalescent (GMYC) model (Fujisawa & Barraclough 2013). We ran PTP and GMYC separately on the mtDNA ML tree and the combined nDNA ML tree (both rooted with *C. daryi*) using the online species delimitation server (https://species.h-its.org/). This server implements a Bayesian version of PTP where Bayesian support values are added to the best species-partitioning schemes to

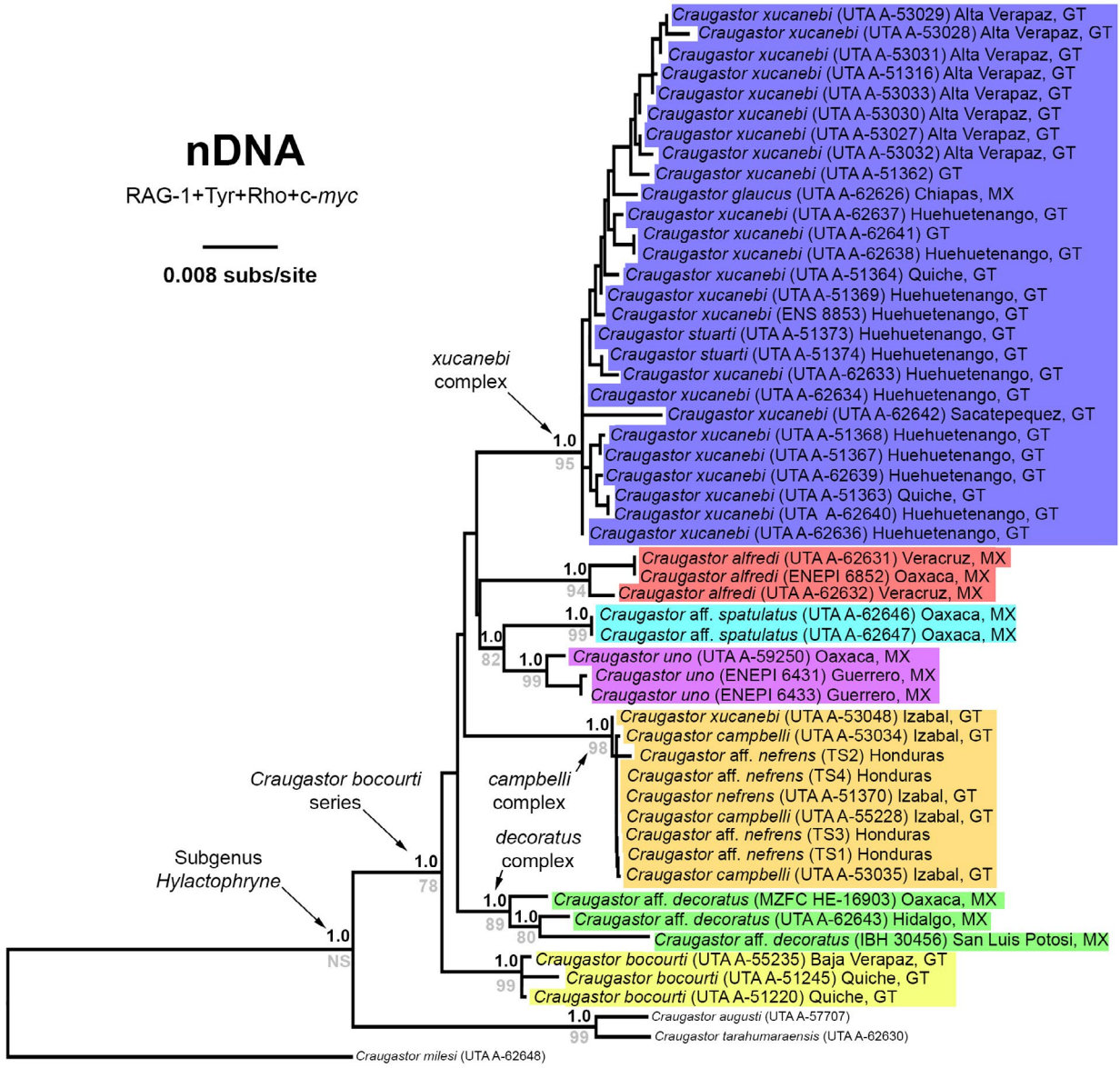

Fig. 4. Nuclear phylogram for the *Craugastor bocourti* series resulting from concatenated Maximum Likelihood analysis of four nuclear fragments. Nodal support values from ML bootstrapping appear below branches (grey) and Bayesian inference appear above branches (black). NS indicates branches with no statistical support.

quantify the relative support for each inferred species delimitation. We used the following parameter settings to generate these support values: a search across 100,000 generations, a thinning value of 100, and burn-in of 10%. We ran a single-threshold model of GMYC. To time-calibrate each phylogeny for the GMYC analysis, we used treePL (Smith & O'Meara 2012) and two calibrations from Portik et al. (2023); 1) the divergence between the subgenera *Campbellius* and *Hylactophryne* (estimated at 39.1 million years ago, mya) and 2) the divergence between the *C. augusti* series and *C. bocourti* series (estimated at 20.3 mya). For the treePL analyses, we used the thorough setting with a smoothing value of 0.1, as determined by cross-validation analyses run on both trees.

Results

Mitochondrial DNA phylogeny

Using the Bayesian Inference Criterion (BIC) in MEGA 5.1, the GTR+Γ model was selected as the most appropriate model of nucleotide evolution for the mtDNA dataset (Table 3). The AWTY examination of topological congruence (using both symmetrical difference and agreement scores) suggested that paired MrBayes 3.2.1 searches in the mtDNA analysis either did not converge (symmetrical-difference scores) or converged after 5 million generations (agreement scores). Thus, to be conservative we discarded 50% of the retained trees as burn-in (leaving 5,000 trees in the final sample). As in previous studies

(Crawford & Smith 2005, Hedges et al. 2008), our Bayesian analysis strongly supported the monophyly of the *bocourti* series and the subgenus *Hylactophryne* (Fig. 3). Although deep and shallow nodes in the Bayesian consensus tree were well supported (posterior probability > 0.95), nodes at intermediate depths were often weakly supported. Two exceptions to this pattern were the strongly supported nodes uniting 1) *C. uno* with *C.* aff. *spatulatus* and 2) *C.* aff. *decoratus* with *C.* aff. *polymniae*. Hereafter, we refer to the clade of *C.* aff. *decoratus* + *C.* aff. *polymniae* as the *C. decoratus* complex (Fig. 3). Interestingly, we did not recover a monophyletic *C. decoratus* complex in our ML analysis. However, the ML analysis did show moderate support for the clade of *C. uno* + *C.* aff. *spatulatus* (bootstrap support (BS) = 78, Fig. 3).

We found that two genetically shallow clades contained all individuals of five putative species: 1) a clade containing *C. campbelli*, *C.* aff. *nefrens*, *C. nefrens*, and some sampled populations previously identified as *C. xucanebi* (from the Guatemalan department of Izabal) and 2) a clade containing *C. glaucus*, *C. stuarti*, and *C. xucanebi* (from central and western Guatemala). Hereafter, we refer to the first clade as the *C. campbelli* complex and the second clade as the *C. xucanebi* complex. We selected names for these complexes using the oldest available name in the clade, which also included samples from the type locality. Although individuals of *C. xucanebi* are present in the *C. campbelli* complex clade, those individuals are from Izabal, Guatemala, far away from the type locality of *C. xucanebi* in Alta Verapaz, Guatemala (Fig. 1). For the *C. xucanebi* complex, several samples of *C. xucanebi* are from Alta Verapaz, Guatemala.

Importantly, these results render the described species *C. xucanebi* as non-monophyletic. The *C. campbelli* complex and *C. xucanebi* complex were found to be sister taxa (although with low support values, BS < 70). We also observed deeper levels of divergence within the *C. decoratus* complex than expected, with *C.* aff. *polyminae* rendering *C.* aff. *decoratus* non-monophyletic. The mitochondrial tree supported the monophyly of the sampled individuals of the species *C. uno*, *C.* aff. *spatulatus*, *C. alfredi*, and *C. bocourti*.

Nuclear DNA phylogeny

The best-fitting PartitionFinder scheme (lnL = –6749.92; BIC score = 14464) for the concatenated nuclear genes had five partitions with four models

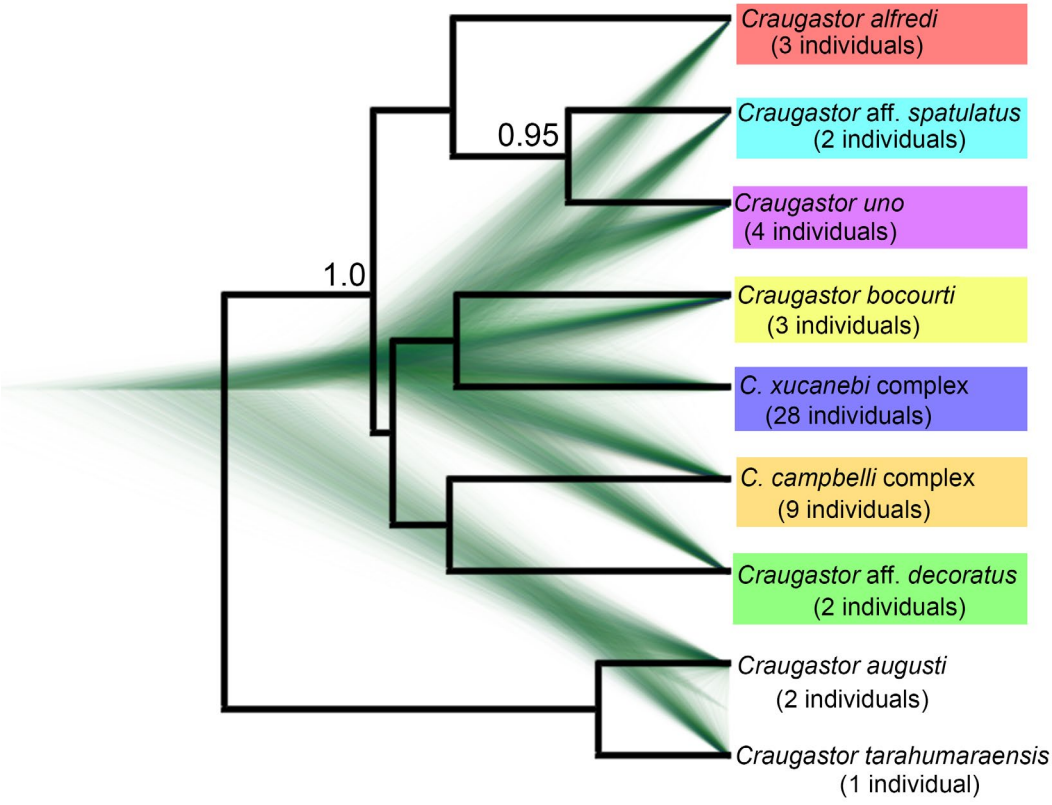

Fig. 5. Coalescent species tree estimated using *BEAST. Posterior probabilities appear below branches. Dark lines correspond to the consensus tree, and light blue trees in the background are the posterior distribution of species trees, all visualised using DensiTree. Only posterior probabilities > 0.90 are indicated on the consensus tree.

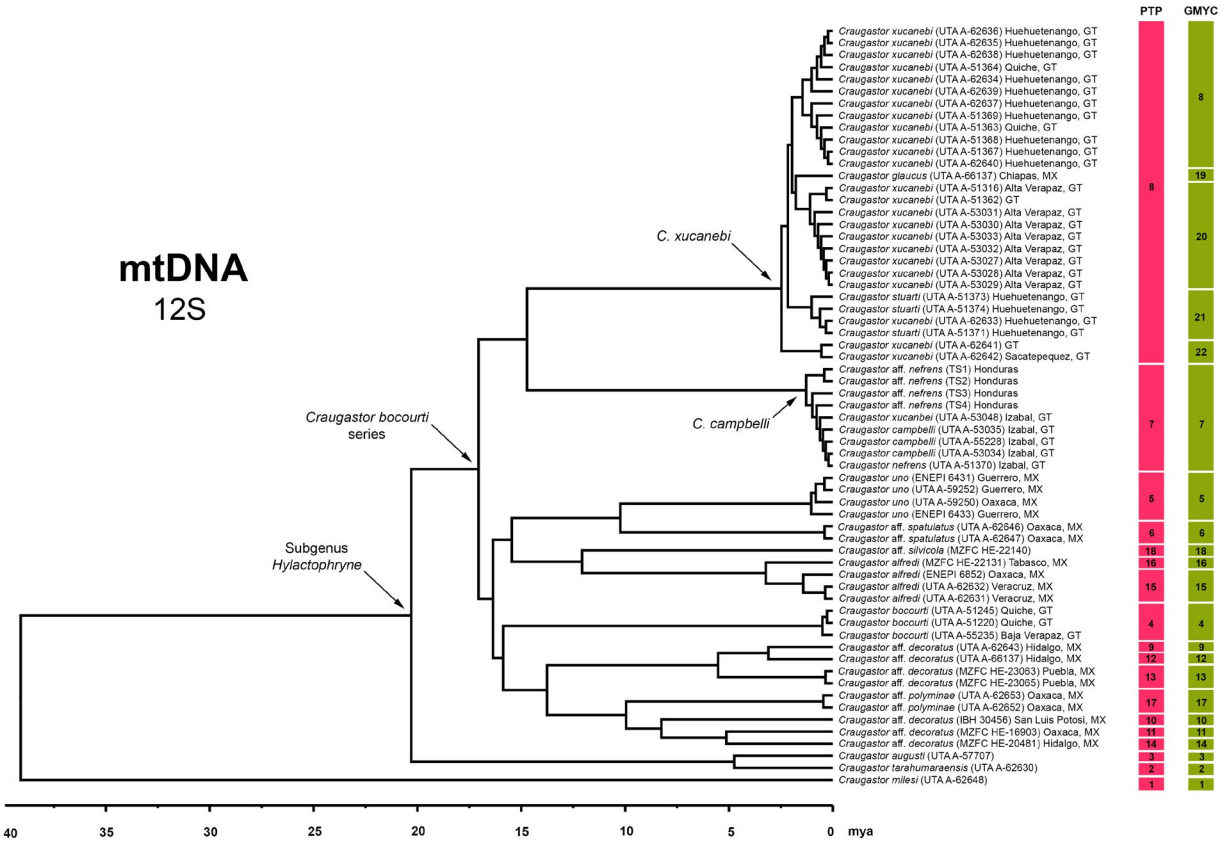

Fig. 6. Results of Poisson Tree Process (PTP) and generalised mixed Yule coalescent (GMYC) species delimitation indicating that the mitochondrial (mtDNA) dataset supported 18-22 species. Boxes adjacent to tip labels reference the delimitation scheme described in Table 5.

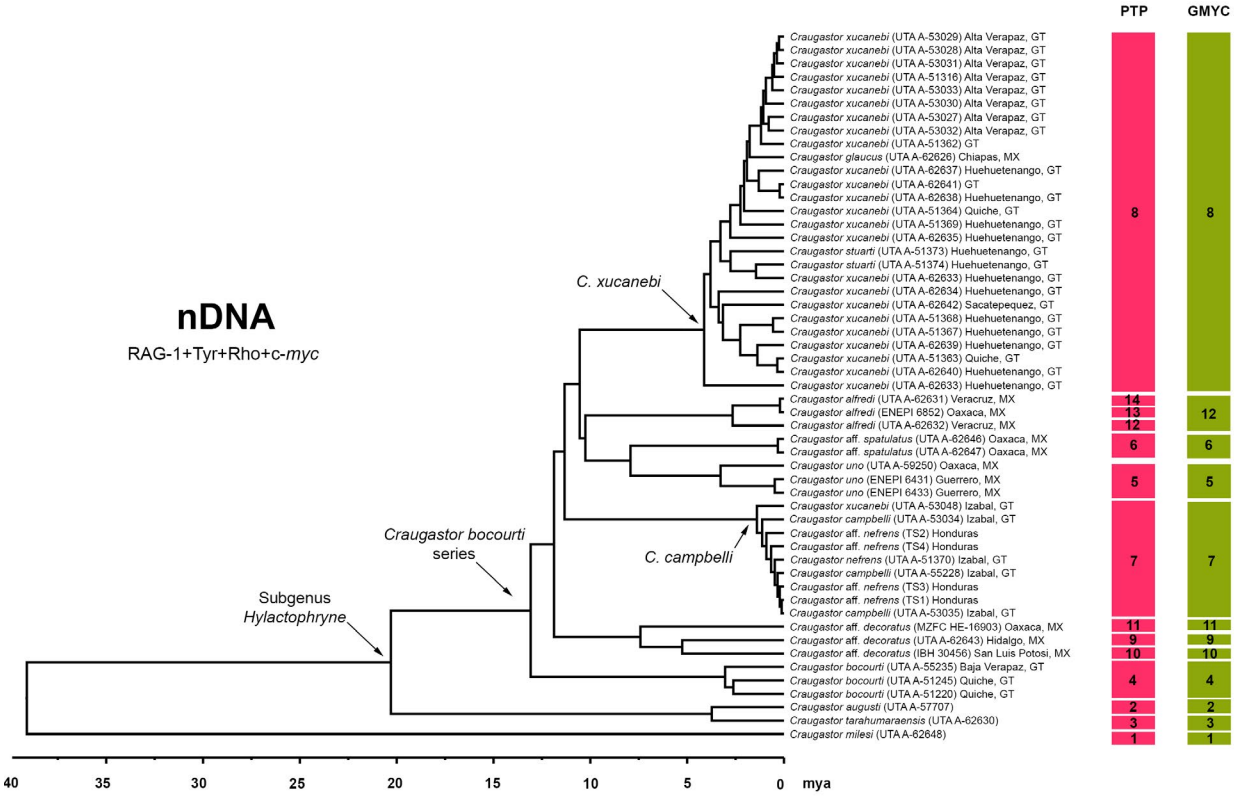

Fig. 7. Results of Poisson Tree Process (PTP) and generalised mixed Yule coalescent (GMYC) species delimitation indicating that the concatenated nuclear dataset (nDNA) supported 12-14 species. Boxes adjacent to tip labels reference the delimitation scheme described in Table 5.

Table 5. Results of Poisson Tree Process (PTP) species delimitation among different species of the *Craugastor bocourti* series and three outgroup species. Support for different species delimitations is in Bayesian support (BS) as determined by PTP. Sample sizes (n) are the number of individuals included in each putative species. GMYC column described if Generalised mixed Yule coalescent delimitation detected the same entity as PTP. Results in bold indicate those species delimited consistently in all delimitation analyses of mitochondrial (mtDNA) and nuclear (nDNA). See text for definitions of *C. campbelli* complex and *C. xucanebi* complex.

	mtDNA				nDNA			
Delimitation result	n	Taxa included	PTP BS	GMYC	n	Taxa included	PTP BS	GMYC
Species 1	**1**	***C. daryi***	**1.00**	**Yes**	**1**	***C. daryi***	**1.00**	**Yes**
Species 2	**1**	***C. augusti***	**0.96**	**Yes**	**1**	***C. augusti***	**0.97**	**Yes**
Species 3	**1**	***C. tarahumaraensis***	**0.96**	**Yes**	**1**	***C. tarahumaraensis***	**0.97**	**Yes**
Species 4	**3**	***C. bocourti***	**0.80**	**Yes**	**3**	***C. bocourti***	**0.68**	**Yes**
Species 5	**4**	***C. uno***	**0.47**	**Yes**	**3**	***C. uno***	**0.39**	**Yes**
Species 6	**2**	***C.* aff. *spatulatus***	**0.47**	**Yes**	**2**	***C.* aff. *spatulatus***	**0.49**	**Yes**
Species 7	**9**	***C. campbelli* complex**	**0.16**	**Yes**	**10**	***C. campbelli* complex**	**0.78**	**Yes**
Species 8	**28**	***C. xucanebi* complex**	**0.09**	**No**	**30**	***C. xucanebi* complex**	**0.42**	**Yes**
Species 9	**1**	***C.* aff. *decoratus* (JAC 26066)**	**0.84**	**Yes**	**1**	***C.* aff. *decoratus* (JAC 26066)**	**0.99**	**Yes**
Species 10	**1**	***C.* aff. *decoratus* (SMR 1327)**	**0.99**	**Yes**	**1**	***C.* aff. *decoratus* (SMR 1327)**	**0.99**	**Yes**
Species 11	**1**	***C.* aff. *decoratus* (JRM 4770)**	**0.95**	**Yes**	**1**	***C.* aff. *decoratus* (JRM 4770)**	**1.00**	**Yes**
Species 12	1	*C.* aff. *decoratus* (JAC 26659)	0.84	Yes	1	*C. alfredi* (JAC 24288)	0.86	No
Species 13	2	*C.* aff. *decoratus*	0.52	Yes	1	*C. alfredi* (JAC 21987)	0.41	No
Species 14	1	*C.* aff. *decoratus* (UOGV 497)	0.95	Yes	1	*C. alfredi* (ENS 10031)	0.41	No
Species 15	3	*C. alfredi*	0.60	Yes				
Species 16	1	*C. alfredi* (UOGV 379)	0.73	Yes				
Species 17	2	*C.* aff. *polymniae*	0.83	Yes				
Species 18	1	*C.* aff. *silvicola* (UOGV 423)	0.99	Yes				

(Table 3). In total our concatenated nDNA alignment contained 2,545 bp. The AWTY examination of topological congruence (using both symmetrical difference and agreement scores) suggested that the paired Bayesian nDNA runs converged between 3 and 4 million generations. Therefore, we discarded 50% of the trees (first 5 million generations) as burn-in. As in the mtDNA analysis, our nDNA Bayesian analysis strongly supported the monophyly of the *bocourti* series and of the subgenus *Hylactophryne* (Fig. 4). However, similar to the mtDNA dataset, ML bootstrap analysis did not show high support for these groupings. ML bootstrap analysis did show strong support for two clades: 1) the sister relationship between *C. uno* and *C.* aff. *spatulatus*, and 2) the monophyly of the *C. decoratus* complex. Two additional patterns in the nDNA tree were similar to the mtDNA tree: 1) individuals of the *C. campbelli* and *C. xucanebi* complexes were placed within two shallow clades that each contained multiple species and 2) nodes at intermediate depths (i.e. nodes between species) were weakly supported. While there were fewer individuals of *C.* aff. *decoratus* included in the nDNA phylogeny, deep levels of divergence among the three individuals were evident, as in the mtDNA topology.

Species-tree analysis

The 95% HPD set of species trees (Fig. 5) was congruent with the separate mtDNA and concatenated nDNA analyses (Figs. 3 and 4) in that the only relationships supported by most of the trees (i.e. posterior probability > 0.90) were the clade uniting *C. uno* and *C.* aff. *spatulatus* and the monophyly of the *bocourti* series relative to the outgroups (*C. augusti* and *C. tarahumaraensis*).

Species-delimitation analysis

The PTP delimitation procedure inferred 18 species in the mtDNA dataset and 14 species in the nDNA dataset (Table 5). The GMYC delimitation procedure inferred 22 species in the mtDNA dataset

(confidence interval, CI; 16-24) and 12 species (CI; 10-14) in the nDNA dataset (Figs. 6 and 7). Comparisons between the delimitation procedures revealed many similarities. First, all delimitation procedures supported recognising the *C. campbelli* complex as a single species (Figs. 6 and 7, Table 5). The *C. xucanebi* complex was recognised as a single species in all but the GMYC mtDNA delimitation (Fig. 6; see explanation below). In PTP analyses of these two complexes, Bayesian support for a single species within each complex was low in the mtDNA delimitation but higher in the nDNA delimitation (PTP; *C. xucanebi* complex, mtDNA = 0.09, nDNA = 0.42; *C. campbelli* complex, mtDNA = 0.16, nDNA = 0.78). Second, all delimitation procedures supported the recognition of each of the following taxa as single species: *C. bocourti* (PTP; mtDNA = 0.80, nDNA = 0.68), *C. uno* (PTP; mtDNA = 0.47, nDNA = 0.39), and *C.* aff. *spatulatus* (PTP; mtDNA = 0.47, nDNA = 0.49). Finally, all delimitations procedures recognised the three outgroup taxa as species: *C. daryi* (PTP; mtDNA = 1.00, nDNA = 1.00), *C. augusti* (PTP; mtDNA = 0.96, nDNA = 0.97), *C. tarahumaraensis* (PTP; mtDNA = 0.96, nDNA = 0.97).

There were also differences between PTP and GMYC species delimitation results (Table 5). The PTP and GMYC mtDNA delimitations were similar, except for the *C. xucanebi* complex, where GMYC inferred five species. A similar result was observed for the PTP and GMYC nDNA delimitations, where GMYC inferred a single species in *C. alfredi*, whereas PTP inferred three different species within the *C. alfredi* clade. A related and difficult-to-interpret result is that two of the three individuals of *C. alfredi* in the PTP delimitation with nDNA had zero (or close to zero) differences in branch length. We would not expect two individuals with such shallow divergence to be different species (Fig. 7).

Discussion

Analyses of mtDNA and nDNA revealed four noteworthy patterns: 1) deep levels of divergence within the *C. decoratus* complex, 2) low levels of genetic divergence among named species within the *C. campbelli* complex and *C. xucanebi* complex, 3) evidence for the distinctiveness of three species (*C. alfredi*, *C. bocourti*, and *C. uno*), and 4) an inability to strongly resolve interspecific relationships near the base of the *bocourti* series. Importantly, the first two patterns, coupled with the results of the species delimitation analyses, indicate that morphology-based taxonomy has both over-delimited and under-delimited species in this group, depending on the taxon. Below we discuss our results and their implications for the taxonomy and future study of northern rain frogs.

Craugastor decoratus complex: under-delimited

Taylor (1942) described *C. decoratus* (as *Eleutherodactylus decoratus*) from near 'Banderia' (Banderilla) in the Mexican state of Veracruz, and *Eleutherodactylus hidalgoensis* from Tianquistengo, Hidalgo. Lynch (1967c) described latitudinal variation in *C. decoratus*, synonymised *E. hidalgoensis* with *C. decoratus*, and designated two subspecies, *C. d. decoratus* (from Hidalgo and Veracruz) and *C. d. purpurus (*from Gomez Farias, Tamaulipas, México). These were originally described as *E. d. decoratus* and *E. d. purpurus*, respectively. Campbell et al. (1989) described *C. polymniae* (as *Eleutherodactylus polymniae*) from the Sierra Juárez near Vista Hermosa in the Mexican state of Oaxaca. Bayesian MCMC (MrBayes 3.2.1) analysis of mtDNA recovered three well-supported clades within our *C. decoratus* complex (D1-D3; Fig. 3). While clade D2 contains individuals that we assigned to *C.* aff. *polymniae*, clades D1 and D3 (which are not sister clades in our mtDNA analysis) contain specimens that are morphologically similar to the *C. decoratus* material examined by Lynch (1967c), indicating that there are likely multiple species within the current concept of *C. decoratus*. Indeed, the species delimitation results for this complex support this interpretation (Table 5). Notably, clade D3 contains an individual from approximately 6 km from the type locality of *E. hidalgoensis*, suggesting that this name may be available for the inferred species.

Our nDNA dataset lacked many individuals in the mtDNA dataset, so future nDNA sequencing will provide valuable information for delimiting candidate species. Although we lacked genetic samples to include it here, we suspect that *C. batrachylus* is also a member of the *C. decoratus* complex given the geographic proximity of the type locality in southern Tamaulipas to the type locality of *C. decoratus purpurus* and populations of *C. decoratus* in Hidalgo and San Luis Potosí (Fig. 1). This possible relationship is also suggested by the overall morphological similarity between *C. batrachylus* and *C. decoratus* (see Taylor 1940).

Craugastor campbelli complex: over-delimited

Smith (2005) described *C. campbelli* and *C. nefrens* (as *Eleutherodactylus campbelli* and *E. nefrens*) from Guatemala in the montane wet forests of the Montañas del Mico and Sierra de Caral, respectively.

He also examined specimens of *C. xucanebi* from the Sierra de Santa Cruz in the western Izabal department and concluded that they differed from *C. campbelli* and *C. nefrens*. Our results suggest that all northern rain frogs from southern Izabal (south of Lake Izabal and the Polochic and Dulce rivers), adjacent eastern Honduras, and some populations in the Sierra de Santa Cruz, form a clade with shallow within-clade divergence, not including *C. xucanebi* from central and western Guatemala (also inhabiting the Sierra de Santa Cruz) and the adjacent state of Chiapas in Mexico (Figs. 3 and 4). The occurrence of geographic variation in the morphology of young northern rain frog lineages is not unprecedented (Streicher et al. 2011) and is a likely contributor for the incongruence between morphological and molecular variation in the *C. campbelli* complex.

The *C. campbelli* complex occurs in the Guatemalan department of Izabal across the Motagua-Polochic fault system, an important biogeographic boundary for many terrestrial vertebrates (Castoe et al. 2009, Daza et al. 2010). However, much like a study on *Bolitoglossa* salamanders (Rovito et al. 2012), we found that the *C. campbelli* complex ranges across the faults, indicating either that 1) these features have not limited dispersal or 2) recent range expansions have obscured the historical importance of this barrier.

Based on our phylogenetic results (Figs. 3 and 4) and species delimitation results (Figs. 6 and 7), we recommend that *C. nefrens* be considered a junior synonym of *C. campbelli* (the first species described in Smith (2005)). We also recommend that some populations of *C. xucanebi* inhabiting the Sierra de Santa Cruz and Puerto Barrios regions of Izabal be referred to as *C. campbelli*, those with an expanded toe V tip. We also consider the sampled individuals of *C.* aff. *nefrens* (frogs from eastern Honduras) to be *C. campbelli*. McCranie & Smith (2006) reported that *C. cyanocthebius* from western Honduras was closely allied to *C. campbelli* and *C. nefrens*. Although we lack molecular data from the type locality of *C. cyanocthebius* (Fig. 1), the synonymisation of *C. nefrens* with *C. campbelli* renders *C. cyanocthebius* morphologically undiagnosable as there is now overlap in the total number of vomerine teeth (0–8 in *C. cyanochthebius* versus 0–14 in *C. campbelli*), the shape of the proximal subarticular tubercle of Toe V (rounded in *C. cyanochthebius* versus pointed to rounded in *C. campbelli*), and the ratio between proximal and distal subarticular tubercles of Toe V (see McCranie & Smith 2006). As such, we recommend that *C. cyanocthebius* also be considered a junior synonym of *C. campbelli*.

Craugastor xucanebi complex: over-delimited

Stuart (1941) described *C. xucanebi* (as *Eleutherodactylus xucanebi*) from the department of Alta Verapaz in Guatemala. Lynch (1967a) described *C. glaucus* (as *E. glaucus*) and *C. stuarti* (as *E. stuarti*) from the state of Chiapas in Mexico and the department of Huehuetenango in Guatemala, respectively. Lynch (1967a) compared these two species with other *Craugastor* species, but he did not compare *C. glaucus* and *C. stuarti* to one another. Furthermore, both species described by Lynch (1967a) exclusively used specimens collected by L.C. Stuart and the descriptions relied heavily on colour pattern. Based on our phylogenetic results (Figs. 3 and 4) and three out of four species delimitation results (Figs. 6 and 7), we recommend that *C. glaucus* and *C. stuarti* be considered junior synonyms of *C. xucanebi*. The mtDNA GMYC species delimitation analysis inferred five species within the *C. xucanebi* complex, including a species containing a single individual of *C. glaucus* and a species containing a clade of mostly *C. stuarti* (Fig. 6). Nevertheless, we consider this delimitation scheme to be 'over-split' given 1) the shallow genetic divergence levels within the *C. xucanebi* clade and 2) the results of the three other molecular species delimitation analyses which all inferred a single species for this clade.

We suspect that *C. taylori*, although not included in our molecular analysis, may be closely related to (or a member of) the *C. xucanebi* complex, given its geographic distribution (Fig. 1) and given overlap in the diagnostic morphological characters that are supposed to separate *C. taylori* from other *Craugastor*. Specifically, Lynch (1966) reported that *C. taylori* was separated from other species by having large tympana (3/4 of the eye diameter), a smooth dorsum, pallid venter, tarsal fold, and lack of vocal slits. However, other species in the *C. xucanebi* complex often share these morphological characteristics. For example, *C. glaucus* lacks vocal slits in males, has a tympanum that is 3/4 of the eye, and has smooth skin (Lynch 1967a).

Evidence of rapid diversification and comparisons to previous studies

In systematics, a pattern of long branches among very short internodes is often interpreted as evidence of an ancient rapid diversification (e.g. Rothfels et al. 2012). In this study, we observed this pattern among

clearly differentiated species of the *bocourti* series using mtDNA (Fig. 3) and concatenated nDNA (Fig. 4). Viewing the species-trees simultaneously via DensiTree demonstrates this pattern particularly well (Fig. 5). Thus, the species-tree inferences amongst species in the *bocourti* series are effectively a cloud-like polytomy. Given our phylogenetic results, one interpretation of this pattern is that northern rain frogs underwent a rapid radiation (Schluter 2000) early in their evolution.

Another study that included most species in the *bocourti* series was recently published by Portik et al. (2023). Their anuran-wide tree included ten species from the *bocourti* series. While the tree of Portik et al. (2023) strongly supports the monophyly of the *bocourti* series, it also has low branch support for relationships amongst species, as in our results here. Thus, at least two studies suggest that early, rapid radiation may have occurred in the *bocourti* series, leading to low branch support for most interspecific relationships. A possible way to improve support in the future would be to obtain phylogenomic data for these species.

There are other consistencies between our study and the tree of Portik et al. (2023), including the sister relationship of *C. alfredi* and *C. silvicola* (which supports that our *C.* aff. *silvicola* is indeed *C. silvicola*), and a shallow, strongly supported branch uniting *C. xucanebi* and *C. stuarti*. The most notable difference between our trees and the tree of Portik et al. (2023) tree is their placement of *C. spatulatus* and *C. polymniae* as sister taxa (with strong support), whereas in our mtDNA analysis we found these species to be distantly related. However, further work is needed to determine if what we call *C.* aff. *polymniae* and *C.* aff. *spatulatus* here is equivalent to the *C. polymniae* and *C. spatulatus* of Portik et al. (2023).

In a molecular study of many terraranan species, Padial et al. (2014) did not support the reciprocal monophyly of the *bocourti* and *augusti* series. Yet, these two groups were supported by many other molecular studies (Crawford & Smith 2005, Hedges et al. 2008, Portik et al. 2023). We find the recommendation of Padial et al. (2014) to 'lump' the *bocourti* and *augusti* series to be untenable, given the large amount of morphological and molecular data that support the reciprocal monophyly of these two series.

Misdiagnosed diversity?

We found that previous taxonomy based on morphological data overestimated species richness in some northern rain frog lineages. This finding contrasts with many previous molecular analyses of anuran species, in which morphology-based species contained one or more cryptic species (e.g. Padial & de la Riva 2009, Kieswetter & Schneider 2013). Specifically, we found that individuals from five species recognised based on morphology are contained within two shallow molecular clades (*C. campbelli* complex and *C. xucanebi* complex). Based on these low levels of genomic divergence and based on two explicit molecular species delimitation analyses (Table 5, Figs. 6 and 7), we conclude that the populations referable to *C. campbelli*, *C. glaucus*, *C. nefrens*, *C. stuarti*, and *C. xucanebi*, should be considered only two species (*C. campbelli* and *C. xucanebi*) and not five. Except for *C.* aff. *nefrens*, which we identified as *C. campbelli*, and the implied undiagnosability of *C. cyanochthebius*, which we also identify as *C. campbelli*, the taxonomy of other samples to which we applied open nomenclature remains uncertain and will need to be examined in future research.

Biologists familiar with terraranan systematics may not be surprised by our report of overestimated biodiversity because many species possess intraspecific polymorphisms in morphology (see Savage 1987, Lynch 1993). As such, delimiting species in terrarananas may involve greater taxonomic uncertainty than in many other vertebrate groups. Indeed, Stuart (1941) referenced the uncertainty felt by terraranan systematists when he wrote, "I have overcome my hesitancy to further multiply Eleutherodactylid names and herein describe them…" in his description of *C. xucanebi*. While molecular data have led to 'multiplied names' in many terraranan groups (e.g. Crawford et al. 2010) and have revealed several undescribed species in the *bocourti* series, our study is also an important reminder that molecular data can also play an important role in countering overestimation of species diversity, especially in groups that are morphologically conservative, polymorphic, and rarely encountered.

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Author Contributions

J. Streicher and E. Smith designed the study and examined museum specimens; J. Streicher generated and analysed the molecular data; J. Streicher, J. Wiens and E. Smith contributed to the analytical design; O. García-Vázquez, M. Jocqué, J. Streicher and E. Smith participated in fieldwork; J. Streicher, J. Wiens and E. Smith wrote the paper with input from O. García-Vázquez and M. Jocqué. All authors approved of the final version of the manuscript.

Data Availability Statement

DNA alignments and phylogenetic trees are available on the NHM Data Portal at https://doi.org/10.5519/7mmve3n2.

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