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Phylogeny, biogeography, and electric signal evolution of Neotropical knifefishes of the genus *Gymnotus* (Osteichthyes: Gymnotidae)

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ABSTRACT

The Neotropical knifefish genus *Gymnotus* is the most broadly distributed and the most diverse (34 + species) gymnotiform genus. Its wide range includes both Central and South American drainages, including the Amazon, Orinoco, and La Plata Basins. Like all gymnotiforms, *Gymnotus* species produce weak electric fields for both navigation and communication, and these fields exhibit interspecific variation in electric waveform characteristics. Both biogeography and electric signal evolution can profitably be analyzed in a phylogenetic context. Here, we present a total evidence phylogeny for 19 *Gymnotus* species based on data from the mitochondrial cytochrome *b* and 16S genes (1558 bp), the nuclear RAG2 gene (1223 bp), and 113 morphological characters. Our phylogenetic hypothesis resolves five distinct *Gymnotus* lineages. In a previous morphology-based analysis, the Central American *Gymnotus cylindricus* lineage was hypothesized as the sister group to all other *Gymnotus* species. In our analysis, the *G. cylindricus* lineage is nested within South American species, and molecular age estimates support a relatively recent origin for the clade in Central America. Phylogenetic optimization of electric signal waveforms indicate that the ancestral state in *Gymnotus* is a multiphasic (4 + phases of alternating polarity) condition, and independent phase loss has occurred in multiple lineages. *Gymnotus* is a model group for understanding Neotropical diversification and the evolution of communication at a continental scale.

1. Introduction

The Neotropical freshwater fish family Gymnotidae comprises the weakly electric banded knifefishes of the genus *Gymnotus* and the strongly electric eel, *Electrophorus electricus* (Albert and Campos-da-Paz, 1998; Albert, 2001). Of all gymnotiform taxa, *Gymnotus* species are the most widely distributed. They occur in lowland freshwater habitats from the Pampas of Argentina (36°S) to southern Chiapas, Mexico (18°N) and are also found on the island of Trinidad (Mago-Leccia, 1994; Albert, 2001). Within this area, *Gymnotus* species are known from a variety of habitats including blackwater and whitewater rivers, terra firme streams, and varzea (whitewater floodplains), and generally inhabit vegetation such as floating meadows, root masses, or leaf litter (Crampton, 1998a). Like other gymnotiforms, *Gymnotus* species produce weak, species-specific electrical signals that function in electrolocation and communication in their nocturnal environment (Bullock et al., 2005; Crampton and Albert, 2006). The diversity of *Gymnotus* species, in addition to their widespread geographical distribution, diverse habitat use, and use of weak electrical signaling, make the group well-suited to species level studies of biogeography and diversification in the Neotropics.

Until recently, little was known regarding the systematic relationships among the species of *Gymnotus*. This was largely due to the lack of adequate species descriptions, with many *Gymnotus* species lumped under the title *Gymnotus carapo* (Albert et al., 1999; Albert and Crampton, 2003). However, ongoing efforts to describe the knifefish fauna of South America have revealed that *Gymnotus* species diversity is far greater than previously thought (e.g., Albert et al., 1999; Albert and Crampton, 2001, 2003; Campos-da-Paz, 2002; Crampton et al., 2003, 2005; Maldonado-Ocampo and Albert, 2004; Fernandes et al., 2005; Cognato et al., 2007; Richer-de-Forges et al., 2009). Currently 34 valid species of *Gymnotus* are recognized, and several more morphologically distinct species taxa await formal description. Additional species diversity in *Gymnotus* is suggested by reports of morphologically cryptic forms

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of *G. carapo sensu stricto* with distinct chromosome arrangements that likely enforce post-zygotic reproductive isolation (Milhomen et al., 2008).

Based on an analysis of 113 phenotypic characters, Albert et al. (2004) proposed a phylogenetic hypothesis for *Gymnotus* species (Fig. 1). The results supported three major clades: the *Gymnotus cylindricus* species group, the *G. pantherinus* species group, and the *G. carapo* species group. The *G. cylindricus* group comprised the two *Gymnotus* species distributed in Central America (*G. cylindricus*, and *G. maculosus*), while the *G. pantherinus* and *G. carapo* groups included species that occur both east and west of the Andes in South America. The *G. cylindricus* group was hypothesized to be the sister group of all other *Gymnotus* species (Fig. 1).

The presence of *Gymnotus* in drainage basins in the northwestern portion of South America (Colombia) and in both the Atlantic and Pacific drainages of Central America make this genus particularly appropriate for studies of South and Central American biogeography. The biogeography of freshwater fishes in Central America has long been of interest (Miller, 1966; Myers, 1966; Bussing, 1976; Rosen, 1975; Briggs, 1984; Bermingham and Martin, 1998; Reeves and Bermingham, 2006), resulting in a variety of hypotheses to explain distribution patterns. Given that an Isthmian landbridge has been missing for most of the history of the region, scenarios have centered on dispersal during brief periods of terrestrial connection between South and Central America. Myers (1966) suggested that Central American gymnotiforms are the product of recent invasions from South America that occurred only after the establishment of the Isthmus of Panama, approximately 3 million years ago (mya) (Duque-Caro, 1990; Coates and Obando, 1996). In contrast, Bussing (1985) suggested that Central American *Gymnotus* were part of a much earlier invasion by an 'Old Southern Element' that reached Central America from the south during the late Cretaceous or Paleocene ~65 mya, at which time a land connection between North and South America is hypothesized to have existed (e.g., Rage, 1981; Pitman et al., 1993; Briggs, 1994; Marshall et al., 1997).

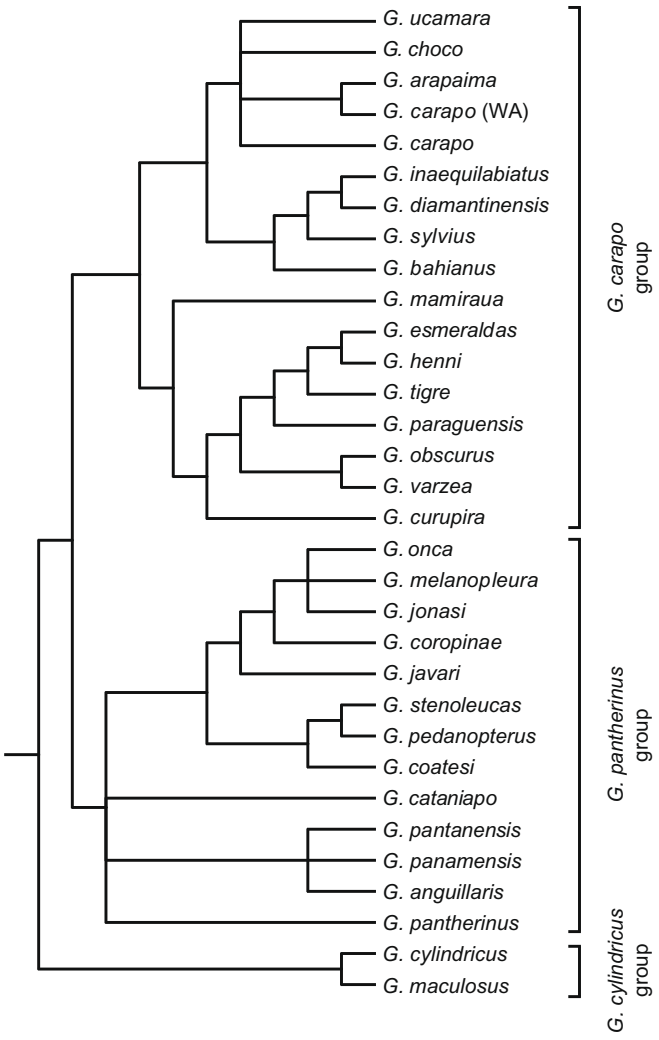

Fig. 1. Phylogenetic hypothesis for *Gymnotus* based on morphology (after Albert et al., 2004).

The previously proposed hypotheses regarding *Gymnotus* dispersal into Central America make specific temporal and phylogenetic predictions. Bussing's (1985) 'Old Southern Element' hypothesis posits that Central American *Gymnotus* diverged from South American *Gymnotus* approximately 65 mya. Given that most diversification in the genus likely occurred after this date (Albert et al., 2004), the 'Old Southern Element' scenario predicts that the Central American *Gymnotus* lineage would branch off at or near the base of the *Gymnotus* tree. Alternatively, if Central American *Gymnotus* species dispersed to Central America via the Isthmus of Panama 3 mya during the Pliocene, as proposed by Myers (1966), Central American *Gymnotus* would be predicted to have diverged more recently from South American *Gymnotus* species. Intriguingly, the current morphology-based hypothesis of *Gymnotus* relationships indicates that a pair of Central American species, *G. maculosus* and *G. cylindricus*, are the sister group of all other Gymnotus species (Albert et al., 2004), supporting the ancient invasion hypothesis. If accurate, *Gymnotus* would be a biogeographical outlier among Central American primary freshwater fishes (Reeves and Bermingham, 2006). The molecular dataset for *Gymnotus* we report here is therefore needed to confirm the phylogenetic position of Central American *Gymnotus* species, and to provide an alternative method of age estimation using molecular dating techniques.

A robust phylogeny of *Gymnotus* species will also be useful for an exploration of the evolution of electric waveform production. Like other weakly electric knifefishes, *Gymnotus* generate species-specific electrostatic fields from specialized electric organs. These electric organ discharges (EODs) permit electrolocation, the detection of objects within the electrostatic field, and also electrocommunication, including mate-attraction (reviews in Caputi, 1999; Bullock et al., 2005; Crampton and Albert, 2006). *Gymnotus* EODs consist of a continuous series of discrete pulsed waveforms, where each waveform has one or more phases of alternating polarity, depending upon the morphology, organization, and patterns of innervation of the electrocytes composing the electric organ (Bennett, 1961, 1971; Macadar et al., 1989). Larval *Gymnotus* produce single phase (monophasic) EODs, but quickly undergo transition (within several weeks) to a characteristic adult waveform with 1–6 phases (Crampton and Hopkins, 2005; Pereira et al., 2007).

Adult *Gymnotus* exhibit species-specific variation in various aspects of the EOD waveform, including the number and relative amplitude and duration of phases (Crampton, 2006; Crampton et al., 2008), and some of these EOD features have profitably been examined in other electric fishes using phylogenetic methods (Sullivan et al., 2000; Turner et al., 2007; Lavoué et al., 2008). Here, we focus on the evolution of phase number across *Gymnotus* phylogeny. While all South American *Gymnotus* species possess multiphasic adult EODs, Central American species have a monophasic adult EOD (Stoddard, 1999; Crampton and Albert, 2006), as does the electric eel, the presumed sister group of *Gymnotus*. Monophasic EODs have been considered plesiomorphic (Stoddard, 2002a), and this view has contributed to the placement of the Central American *G. cylindricus* lineage as the sister group to all other *Gym-*

notus species. Here, we explicitly test this hypothesis by reconstructing the evolution of EOD phase number on *Gymnotus* phylogeny.

The objectives of this study were to produce a phylogeny of *Gymnotus* species based on both nucleotide and morphological data and use it to: (1) evaluate the hypothesis of *Gymnotus* relationships proposed by Albert et al. (2004); (2) test the previously proposed biogeographic hypotheses regarding *Gymnotus* dispersal into Central America (Myers, 1966; Bussing, 1985); and (3) examine the evolution of the number of EOD waveform phases in *Gymnotus*.

2. Materials and methods

2.1. Taxon sampling

Tissues for 19 gymnotid (ingroup) and 6 outgroup species were included in the analysis (see Table 1 for a complete list of specimens sequenced). An effort was made to include representatives from each of the three species groups recognized by Albert et al. (2004). The sample therefore reflects the morphological and taxonomic diversity within *Gymnotus*. Whenever possible two individuals of each species were sequenced. In the case of two geographically widespread species, *G. coropinae* and *G. carapo*, multiple geographic variants were sequenced, which brought the number of operational taxonomic units (OTUs) including outgroup taxa, to 28. Representative outgroups were selected from different gymnotiforms families, and included individuals from the families Rhamphichthyidae (*Rhamphichthys*), Hypopomidae (*Hypopomus*, *Brachyhypopomus*) and Sternopygidae (*Sternopygus*). Outgroup selection was also designed to provide a calibration point for estimating divergence times. *Brachyhypopomus diazi* and *B.* n. sp. PAL, are known, respectively, from Atlantic drainages in Venezuela, and Pacific drainages in western Ecuador, and are assumed to have diverged as a result of the uplift of the Eastern Cordillera of the Andes (see Section 2.5).

Specimens were collected in the field by colleagues and by the authors, using an electric-fish detector (which consisted of a differential amplifier and loud-speaker connected to electrodes which were placed in the water on the end of a makeshift pole) and sampled using dip-nets with a 3–4 mm mesh size. Muscle tissue was excised and stored in either 95–100% ethanol or a buffered solution consisting of 20% DMSO and 0.25 M EDTA at pH 8, saturated with NaCl (Seutin et al., 1991).

2.2. DNA isolation, PCR, and sequencing

Total genomic DNA was isolated from muscle tissue using DNeasy Tissue Kits (QIAGEN). The polymerase chain reaction (PCR) was used to obtain 1223 bp of the nuclear recombinase activating gene-2 (RAG2), 1106 bp of the mitochondrial DNA (mtDNA) gene cytochrome *b* (cyt *b*) and 553 bp of the 16S ribosomal mtDNA gene using combinations of the primers listed in Table 2. Cytochrome *b* was amplified using primers in the adjacent glutamine (GLUDG.L) and threonine (CytbR) transfer RNAs (Palumbi et al., 1991), with PCR carried out in 50 μl volumes including 10× PCR buffer (50 mM KCL, 20 mM Tris–HCl, pH 8.4), 200 μM of each dNTP, 3 mM $MgCl_2$, 0.4 μM of each primer, 1 U of Taq DNA Polymerase (Invitrogen) and 1 μl of DNA extract. A fragment of the 16S mtDNA gene was amplified using the 16sar and 16sbr primers of Palumbi (1996), with PCR carried out in 50 μl volumes consisting of 10× PCR buffer (50 mM KCl, 20 mM Tris–HCl, pH 8.4), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 1 U of Taq DNA Polymerase and 1 μl of DNA extract. The amplification of RAG2 was accomplished using previously designed primers RAG2F1 and RAG2R6 (Lovejoy and Collette, 2001), in addition to two primers designed specifically for this study RAG2GY-F and RAG2GY-R (Table 2). PCR volumes and concentrations followed those outlined for 16S with the exception that 2–5 μl of DNA extract was used. For mitochondrial genes, thermal cycling conditions were: 95 °C for 30 s denaturation, 50–58 °C for 60 s annealing, 72 °C for 60–90 s extension. This profile was run for 35 cycles(16S) or 35–40 cycles (cyt *b*), with hold steps of 95 °C for 30 s for the first cycle, and 72 °C for 300 s after the last cycle. Thermal cycling conditions for RAG2 followed a touch down protocol of 95 °C for 30 s denaturation, 58 °C, 56 °C, 54 °C, 52 °C, for two cycles each, then 50 °C for 32 cycles annealing, followed by extension at 72 °C for 90 s.

PCR products were purified using the Montage PCR purification kit (Millipore). Samples were then sequenced using either an ABI 377 or ABI 3730 Genetic Analyzer, using dye terminators (BigDye version 3.1, Applied Biosystems). Sequencing was accomplished with external primers, except for two internal primers, GYRAG2ISP1 and GYRAG2ISP2 (Table 2) which were designed based on an initial *Gymnotus* alignment and used for RAG2 sequencing.

2.3. Alignment

Sequences were edited and preliminary alignments generated using Sequencher 4.2.2 (Gene Codes Corp.). For the protein coding genes RAG2 and cytochrome *b*, alignment was trivial and no insertions/deletions were observed. For 16S, further alignment was completed using Clustal X (Thompson et al., 1997). Positions of stem and loop regions for 16S sequences were estimated by comparison to the published secondary structures of *Pygocentrus natteri* (Ortí et al., 1996). Alignments were performed using several gap opening and extension cost combinations (7/5, 10/5, 20/5, 10/10). Alignments were then compared and those portions of the 16S alignment for which homology assessment differed were excluded from further analyses following the practice of Gatesy et al. (1993). This 16S alignment was used for parsimony analyses, ML, and Bayesian analyses, and is available on request. However, to test the effect of this data exclusion, we also generated total evidence trees using parsimony-based direct optimization methods (see online Supplementary material).

2.4. Phylogenetic analysis

Parsimony analysis of the total evidence dataset was conducted in PAUP* (Swofford, 2002). Nucleotide and morphological character data were combined into a single matrix (a total of 2894 characters for 28 OTUs). Data partitions were defined as: nuclear (RAG2), mtDNA (cyt *b* and 16S combined), and morphology (113 phenotypic characters from Albert et al. (2004)). The parsimony-based incongruence length difference test (ILD or partition homogeneity test of PAUP*) was employed to test the combinability of data partitions (Farris et al., 1994; Swofford, 2002). Pairwise ILD comparison between nuclear and mitochondrial partitions indicated congruence ($p > 0.05$), but pairwise comparisons of morphology with each molecular partition (mtDNA and nuclear) indicated incongruence ($p < 0.05$). Combining incongruent data partitions may contribute to greater phylogenetic accuracy (e.g., Yoder et al., 2001). Therefore, we proceeded with total evidence analysis (all partitions combined), but also assessed the phylogenetic contribution of different partitions. For each analysis, we used the heuristic search algorithm with 100 replicates of random addition of taxa, and TBR branch swapping. All trees were rooted using *Sternopygus macrurus*, and gaps were treated as missing data. Bootstrap values (Felsenstein, 1985a) were calculated in PAUP* using the heuristic search option (1000 replicates, 10 random taxon additions), and decay indices (or Bremer support values, Bremer,

Table 1
List of specimens included in study.

Species[a]	Specimen	Voucher	Genbank Accession No.			Locality
			RAG2	Cyt *b*	16S	
Brachyhypopomus brevirostris	2617	UF 116556	GQ862536	GQ862588	GQ862640	Rio Nanay, Peru
Brachyhypopomus diazi	305	UF 174334	GQ862537	GQ862589	GQ862641	Rio Las Marias, Venezuela
Brachyhypopomus diazi	2408	UF 174334	GQ862538	GQ862590	GQ862642	Rio Alpargatón, Venezuela
Brachyhypopomus n. sp. PAL	2432	UF 148572	GQ862539	GQ862591	GQ862643	Rio Palenque, Ecuador
Electrophorus electricus	2619	UF 116585	GQ862540	GQ862592	GQ862644	Rio Nanay, Peru
Electrophorus electricus	2026	MZUSP 103218	GQ862541	GQ862593	GQ862645	Lago Secretaria, Brazil
Gymnotus sp. cf. *anguillaris*	2091	AUM 36616	GQ862542	GQ862594	GQ862646	Rio Aponwao, Guyana
Gymnotus arapaima	2002	MZUSP 75179	GQ862543	GQ862595	GQ862647	Lago Mamirauá, Brazil
Gymnotus arapaima	2003	MZUSP 103219	GQ862544	GQ862596	GQ862648	Lago Mamirauá, Brazil
Gymnotus carapo (OR)	2040	UF 174335	GQ862545	GQ862597	GQ862649	Rio Guaratico, Venezuela
Gymnotus carapo (OR)	2041	UF 174335	GQ862546	GQ862598	GQ862650	Rio Guaratico, Venezuela
Gymnotus carapo (CA)	2004	MZUSP 76066	GQ862547	GQ862599	GQ862651	Lago Secretaria, Brazil
Gymnotus carapo (CA)	2030	MZUSP 76066	GQ862548	GQ862600	GQ862652	Lago Secretaria, Brazil
Gymnotus carapo (WA)	2006	UF 131129	GQ862549	GQ862601	GQ862653	Rio Amazonas, Peru
Gymnotus carapo (WA)	2007	UF 131129	GQ862550	GQ862602	GQ862654	Rio Amazonas, Peru
Gymnotus cataniapo	2062	UF 174330	GQ862551	GQ862603	GQ862655	Rio Atabapo, Venezuela
Gymnotus cataniapo	2063	UF 174332	GQ862552	GQ862604	GQ862656	Rio Cataniapo,, Venezuela
Gymnotus coatesi	2042	MCP 34471	GQ862553	GQ862605	GQ862657	Lago Tefé, Brazil
Gymnotus coatesi	2043	MCP 34472	GQ862554	GQ862606	GQ862658	Rio Tefé, Brazil
Gymnotus coropinae (GU)	2035	ANSP 179126	GQ862555	GQ862607	GQ862659	Sauriwau River, Guyana
Gymnotus coropinae (GU)	2036	AUM 35848	GQ862556	GQ862608	GQ862660	Sauriwau River, Guyana
Gymnotus coropinae (GU)	2037	ANSP 179127	GQ862557	GQ862609	GQ862661	Mazaruni River, Guyana
Gymnotus coropinae (GU)	2038	ANSP 179127	GQ862558	GQ862610	GQ862662	Mazaruni River, Guyana
Gymnotus coropinae (CA)	2010	MZUSP 75188	GQ862559	GQ862611	GQ862663	Lago Tefé, Brazil
Gymnotus coropinae (CA)	2025	MZUSP 60611	GQ862560	GQ862612	GQ862664	Lago Tefé, Brazil
Gymnotus curupira	2009	MZUSP 75148	GQ862561	GQ862613	GQ862665	Lago Tefé, Brazil
Gymnotus curupira	2021	MZUSP 75146	GQ862562	GQ862614	GQ862666	Lago Tefé, Brazil
Gymnotus cylindricus	2092	ROM 84772	GQ862563	GQ862615	GQ862667	Rio Tortuguero, Costa Rica
Gymnotus cylindricus	2093	ROM 84772	GQ862564	GQ862616	GQ862668	Rio Tortuguero, Costa Rica
Gymnotus cylindricus	2094	ROM 84772	GQ862565	GQ862617	GQ862669	Rio Tortuguero, Costa Rica
Gymnotus javari	2020	UF 122824	GQ862566	GQ862618	GQ862670	Rio Amazonas, Peru
Gymnotus jonasi	2016	MZUSP 103220	GQ862567	GQ862619	GQ862671	Rio Solimões, Brazil
Gymnotus jonasi	2471	UF 131410	GQ862568	GQ862620	GQ862672	Rio Ucayali, Peru
Gymnotus mamiraua	2012	MZUSP 103221	GQ862569	GQ862621	GQ862673	Rio Solimões, Brazil
Gymnotus mamiraua	2013	MCP 29805	GQ862570	GQ862622	GQ862674	Rio Solimões, Brazil
Gymnotus obscurus	2017	MZUSP 75155	GQ862571	GQ862623	GQ862675	Lago Mamirauá, Brazil
Gymnotus obscurus	2018	MZUSP 75157	GQ862572	GQ862624	GQ862676	Lago Mamirauá, Brazil
Gymnotus pantherinus	2039	No voucher	GQ862573	GQ862625	GQ862677	Rio Perequê-Açu, Brazil
Gymnotus pedanopterus	2058	UF 174328	GQ862574	GQ862626	GQ862678	Rio Atabapo, Venezuela
Gymnotus pedanopterus	2059	UF 174328	GQ862575	GQ862627	GQ862679	Rio Atabapo, Venezuela
Gymnotus stenoleucus	2060	UF 174329	GQ862576	GQ862628	GQ862680	Rio Atabapo, Venezuela
Gymnotus stenoleucus	2061	UF 174331	GQ862577	GQ862629	GQ862681	Rio Cataniapo, Venezuela
Gymnotus stenoleucus	2064	UF 174329	GQ862578	GQ862630	GQ862682	Rio Atabapo, Venezuela
Gymnotus tigre	2019	UF 122823	GQ862579	GQ862631	GQ862683	Rio Amazonas, Peru
Gymnotus tigre	2024	UF 122821	GQ862580	GQ862632	GQ862684	Rio Amazonas, Peru
Gymnotus ucamara	1927	UF 126184	GQ862581	GQ862633	GQ862685	Rio Ucayali, Peru
Gymnotus ucamara	1950	UF 126184	GQ862582	GQ862634	GQ862686	Rio Ucayali, Peru
Gymnotus varzea	2014	MZUSP 75163	GQ862583	GQ862635	GQ862687	Rio Solimões, Brazil
Gymnotus varzea	2015	MZUSP 75164	GQ862584	GQ862636	GQ862688	Rio Solimões, Brazil
Hypopomus artedi	2232	ANSP 179505	GQ862585	GQ862637	GQ862689	Mazaruni River, Guyana
Rhamphichthys rostratus	2632	UF 116575	GQ862586	GQ862638	GQ862690	Rio Amazonas, Peru
Sternopygus macrurus	2639	UF 117121	GQ862587	GQ862639	GQ862691	Rio Nanay, Peru

[a] Drainage abbreviations: OR, Orinoco; CA, Central Amazon; WA, Western Amazon; GY, Guyanas.

Table 2
Primers used for amplification and sequencing of the cyt *b*, 16S, and RAG2 genes.

Primer name	Primer sequence (5′–3′)	Source
GLUDG.L	CGAAGCTTGACTTGAARAACCAYCGTTG	Palumbi et al. (1991)
CytbR	CTCCGATCTTCGGATTACAAG	Palumbi et al. (1991)
16Sar	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
16Sbr	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)
RAG2F1	TTTGGRCARAAGGGCTGGCC	Lovejoy and Collette (2001)
RAG2R6	TGRTCCARGCAGAAGTACTTG	Lovejoy and Collette (2001)
RAG2GY-F	ACAGGCATCTTTGGKATTCG	This study
RAG2-GY-R	TCATCCTCCTCATCTTCCTC	This study
GY-RAG2ISP1	GCCATCATTAAGGACATCACAG	This study
GY-RAG2ISP2	CTGAGCATGGACCCAGTGTC	This study

1994) were calculated using the program TreeRot (Sorenson, 1999). For the total evidence analyses, Partitioned Bremer Support (PBS) was calculated to provide an indication of the topological support provided by each data partition for each node (Baker and

DeSalle, 1997). Finally, we explored the effect of different character weighting schemes by inferring transition/transversion ratios from the data and using step matrices to differentially weight these state changes.

We also subjected the dataset to phylogenetic analyses using maximum likelihood and Bayesian methods (Guindon and Gascuel, 2003; Ronquist and Huelsenbeck, 2003). Maximum likelihood phylogenetic methods were implemented in the program PHYML 3.0 (Guindon and Gascuel, 2003; Guindon et al., 2005), under the GTR+I+G model. This model was found to be the best fit for the unpartitioned molecular dataset, according to both hierarchical likelihood ratio tests (LRT) and Akaike Information Criterion (AIC), as implemented in Modeltest v3.7 (Posada and Crandall, 1998). For the likelihood analyses, bootstrapping methods (100 replicates) were used to assess the node support (Felsenstein, 1985b). Bayesian inference was performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The morphological data and each of the three genes were treated as separate data partitions. All partitions had independently estimated (unlinked) parameters. To identify the most appropriate models for each molecular partition, we used MrModeltest 2.3 (Nylander, 2004) and selected among models favored under hierarchical LRT's and AIC. For the cytb and 16S partitions, GTR+I+G was universally found to be the best-fitting model, according to both hierarchical LRT's and AIC; therefore it was used in all Bayesian analyses. For the RAG2 partition, SYM+G was the best-fitting model under hierarchical LRT's, and SYM+I+G under AIC. To reduce the effects of possible overparameterization, particularly since RAG2 is known to evolve at much slower rates than cytb and 16S, and given that the gamma shape parameter (G) and the proportion of invariable sites (I) are strongly correlated (Sullivan and Swofford, 2001), we dropped the I parameter, and chose to use SYM+G for this partition in the Bayesian analyses. For the morphological data partition, a discrete model originally described by (Lewis, 2001) was used, as implemented in MrBayes 3.1.2. We used a version of this model that also allowed for rate heterogeneity across sites. For all parameters in the Bayesian analysis, uniform priors were used, except for branch lengths, for which exponential priors were implemented. Two independent analyses were performed, each composed of 4 Markov chains with default heating values. Markov chains were run for 10 million generations, sampling trees (and parameters) every 1000 generations. Convergence was assessed using a number of methods. The average standard deviation of split frequencies, as calculated in MrBayes, were all well below 0.01 at stationarity. Also implemented in MrBayes, a convergence diagnostic for branch length posterior probabilities, the potential scale reduction factor (PSRF), roughly approached 1 as the runs converged (Gelman and Rubin, 1992). Convergence to stationarity was also assessed by plotting log-likelihood scores and other parameter values in the program Tracer 1.4.1 to ensure that there were no trends in the data post burn-in (Rambaut and Drummond, 2007). Finally, adequacy of mixing was assessed by examining acceptance rates for parameters in MrBayes, and by calculating in Tracer the effective sample sizes (ESS), the number of independent samples from the marginal posterior distribution for each parameter (higher values being indicative of better sampling from the posterior distribution). These values were all well above 100. By these measures convergence was achieved within the first 25% of trees sampled, which were discarded as burn-in, and remaining trees were taken as representative of the posterior probability distribution.

2.5. Divergence time estimates

We estimated ages of *Gymnotus* clades using the divergence of *Brachyhypopomus* n. sp. PAL and *B. diazi* as a calibration point. *B.* n. sp. PAL is distributed west of the Andes in Ecuador, while *B. diazi* is known from Atlantic drainages in northern Venezuela. Both morphological (Sullivan, 1997; Santana and Crampton, unpublished) and mitochondrial DNA analysis (Sullivan, 1997) show that *Brachyhypopomus* species distributed west of the Andes (including *B.* n. sp. PAL) form the sister clade to *B. diazi*. As a result, we assume that the divergence of *B.* n. sp. PAL and *B. diazi* predates the uplift of the Eastern cordillera of the Andes mountain range, which commenced approximately 12.9 mya (Lundberg et al., 1998; Albert et al., 2006). We used a Bayesian method to estimate divergence times under a relaxed molecular clock model with uncorrelated rates among lineages drawn from a lognormal distribution (Drummond et al., 2006), as implemented in BEAST v1.4.8 (Drummond and Rambaut, 2007). The molecular data were partitioned by gene, using the GTR+I+G model. A Yule branching process was implemented with uniform priors. The analysis was run for 10 million generations, with parameters sampled every 1000 generations. The first 1 million generations were discarded as burn-in. Convergence was assessed using the methods implemented for other Bayesian phylogenetic analyses (see Section 2.4). Uncertainty in the timing of the calibration date was incorporated using a normal distribution as a prior in the Bayesian analysis (mean = 12.9 mya, standard deviation = 1).

2.6. Electric waveform recording and analysis

Head to tail EOD waveforms were digitized and recorded from fishes captured in the field using procedures described in Crampton et al. (2008). Using a custom-written Java program, the number of EOD phases were counted as positive or negative deviations from the baseline (zero volts) exceeding 1.5% of the amplitude of the positive phase with the highest amplitude. In adult *Gymnotus*, EODs range from having a single phase (monophasic) to six or more phases. Species with high numbers of phases (four or more) show some degree of intraspecific variability, in some cases ranging from 4 to 6. Thus, a robust coding scheme was determined to be: 1, 2, 3, or 4 + phases. The EOD phase data were optimized as an unordered multistate character on both the total evidence parsimony and Bayesian trees using MacClade 4.0 (Maddison and Maddison, 2001). MacClade was used to trace all most parsimonious character reconstructions on the trees.

3. Results

3.1. Molecular dataset

The cyt *b* dataset consisted of 1106 characters (nucleotide sites), 513 of which were parsimony informative. The 16S dataset, after ambiguously aligned characters had been removed, consisted of 452 characters, 96 of which were parsimony informative. The base composition (AGCT content) of the cytochrome b gene for *Gymnotus* exhibited patterns similar to those found in other fishes, such as low GC content at third positions (e.g., Johns and Avise, 1998). Uncorrected pairwise comparisons for cyt *b* showed the highest sequence divergences between *E. electricus* and non-gymnotid outgroups (approximately 28%). Within Gymnotidae, divergence between *E. electricus* and Gymnotus species was approximately 25%. Within *Gymnotus*, cyt *b* divergences ranged from 19.8% to 0.2%. The RAG2 dataset was composed of 1223 characters, 233 of which were parsimony informative. The highest observed uncorrected pairwise divergence was 11.1% (between *G. curupira* and *B. diazi*). Within Gymnotidae *E. electricus* was approximately 9% divergent from *Gymnotus* species. Within *Gymnotus*, RAG2 divergences between species were low, ranging from 3.8% to 0%.

3.2. Phylogenetic relationships

Fig. 2 shows the single most parsimonious tree obtained from the analysis of all available data and taxonomic units (3256 steps in length, CI = 0.48, RI = 0.81). Weighting transversions at 2 and 5 times the weight of transitions had no effect on this topology. Both decay indices and bootstrap proportions indicated high nodal support for the majority of clades in the tree. Exceptions include the support for relationships between individuals of *G. carapo* and the closely related species *G. arapaima* and *G. ucamara*. The position of *G. pantherinus* as the sister clade of *G. cataniapo*, *G. pedanopterus*, and *G.* sp. cf. *anguillaris* is also not well supported. Parsimony-based direct optimization, which explicitly incorporates insertion–deletion data, provides results that are nearly identical (see Supplementary material).

Fig. 3 shows the results of the Bayesian analysis. The Bayesian, likelihood (not shown) and parsimony trees are nearly identical. Topological differences between these methods of analysis involve relationships that are relatively poorly supported in the individual analyses. Notably, the position of *G. pantherinus* varies across analyses, as do the relationships among the very closely related *G. carapo*, *G. arapaima*, and *G. ucamara*. Separate parsimony analyses of the nuclear and mitochondrial partitions produce topologies that are consistent with total evidence analyses (summarized in

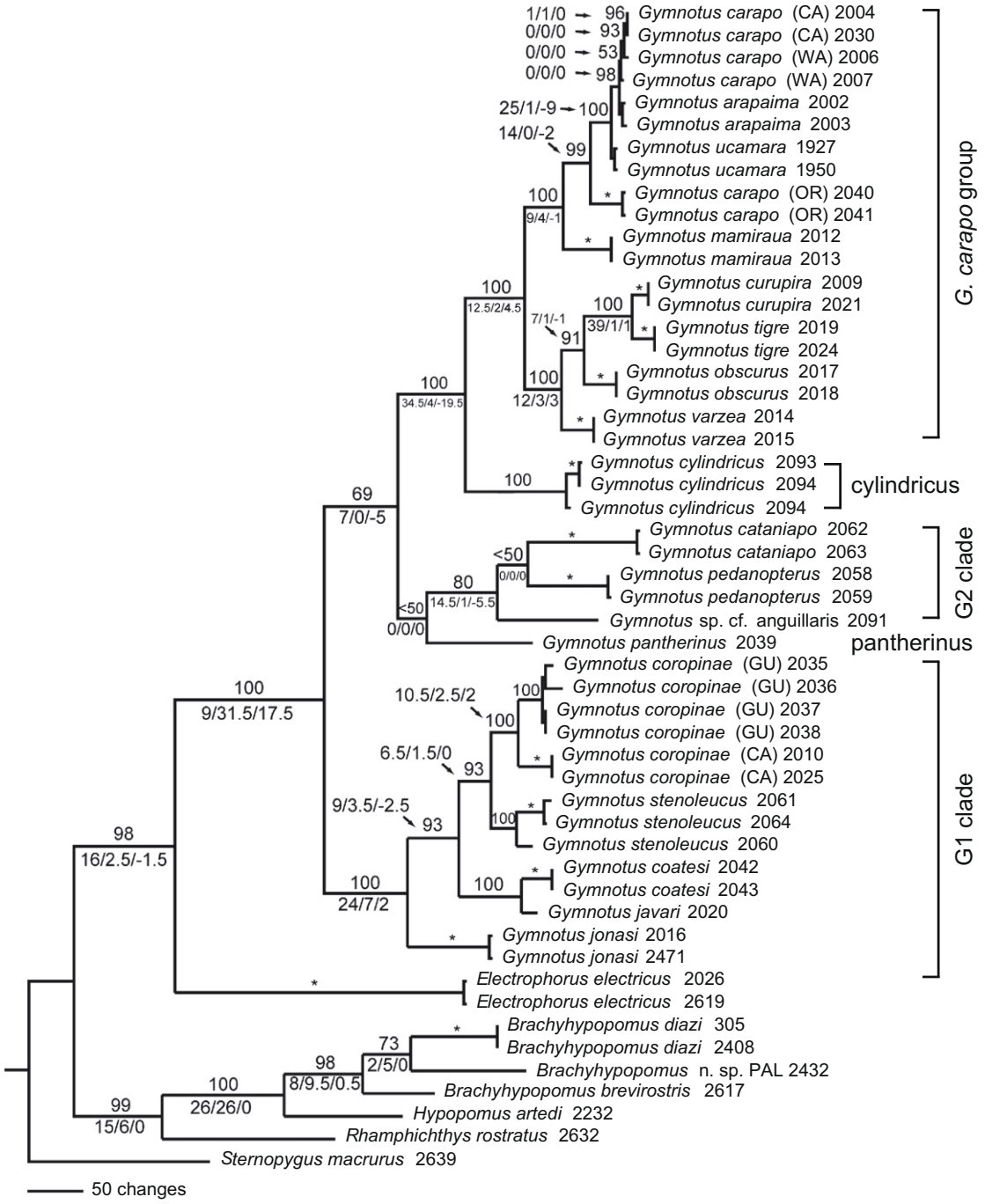

Fig. 2. Single most parsimonious tree of *Gymnotus* phylogenetic relationships, based on combined analysis of nuclear (RAG2), mitochondrial (cytochrome *b* + 16S), and morphological data (2894 characters, 3256 steps, CI = 0.48, RI = 0.81). Numbers above nodes denote bootstrap support values based on 1000 replicates. * Indicates terminal branches supported by 100% bootstrap values. Numbers below nodes or indicated using arrows denote Partitioned Bremer Support values (decay indices) for the mtDNA, RAG2, and morphology partitions respectively.

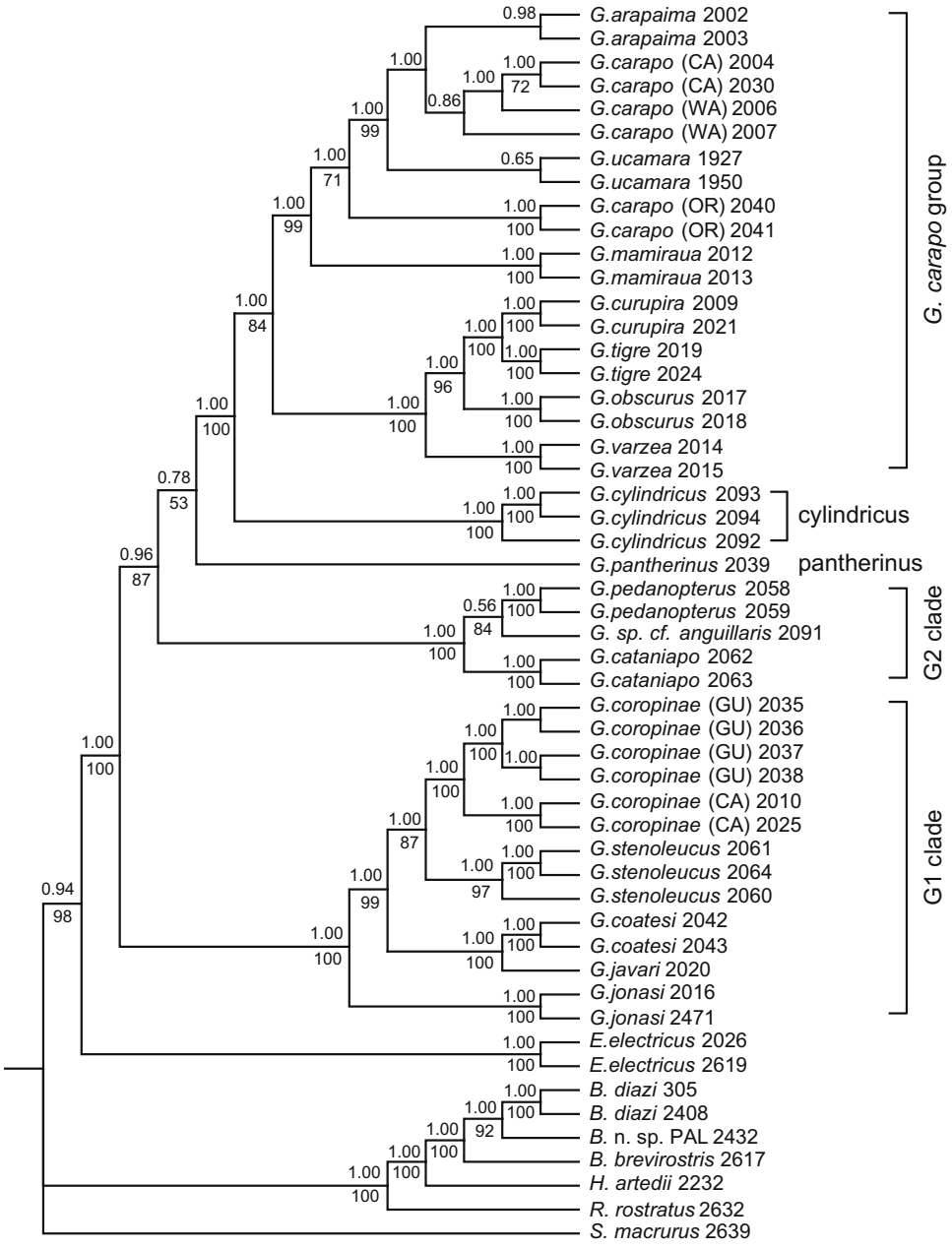

Fig. 3. Bayesian phylogeny for *Gymnotus*, based on a partitioned analysis of nuclear, mitochondrial, and morphological data. Bayesian posterior probabilities are listed above nodes; bootstrap values for maximum likelihood analysis of the molecular dataset are listed below nodes.

Fig. 4). The RAG2 analysis shows a slight decline in resolution compared to other analyses, which was expected given the lower levels of variability and divergence in this gene.

Our results clearly support the monophyly of Gymnotidae (*Gymnotus* + *Electrophorus*), as well as the monophyly of *Gymnotus*. Based on comparisons across our analyses, and with the morphological analysis of Albert et al. (2004), we define five *Gymnotus* lineages (Fig. 5). These are: the *G. carapo* group (eight species), G1 clade (five species), G2 clade (three species), *G. pantherinus*, and *G. cylindricus*. In all analyses (with the exception of morphology), the genus *Gymnotus* is divided into two main clades: G2 and a monophyletic clade containing all other lineages. Within the latter clade, *G. cylindricus* is consistently recovered as the sister group of the *G. carapo* group, which has important implications for biogeographic reconstruction.

3.3. Divergence time estimates

The divergence between Central American *Gymnotus* (*G. cylindricus*) and its sister clade, the South American *G. carapo* group, was inferred to have occurred during the Miocene, approximately 15.17 mya (95% highest posterior density interval = 7.18–24.28). We calculated the age of divergence between *Gymnotus* and *Electrophorus* as 56.6 mya (95% highest posterior density interval = 26.6–91.25).

3.4. Electric waveform evolution

Our optimizations of EOD phase number indicate that the ancestral condition in *Gymnotus* is a multiphasic waveform with four or more phases (Fig. 6). Within *Gymnotus*, two lineages have

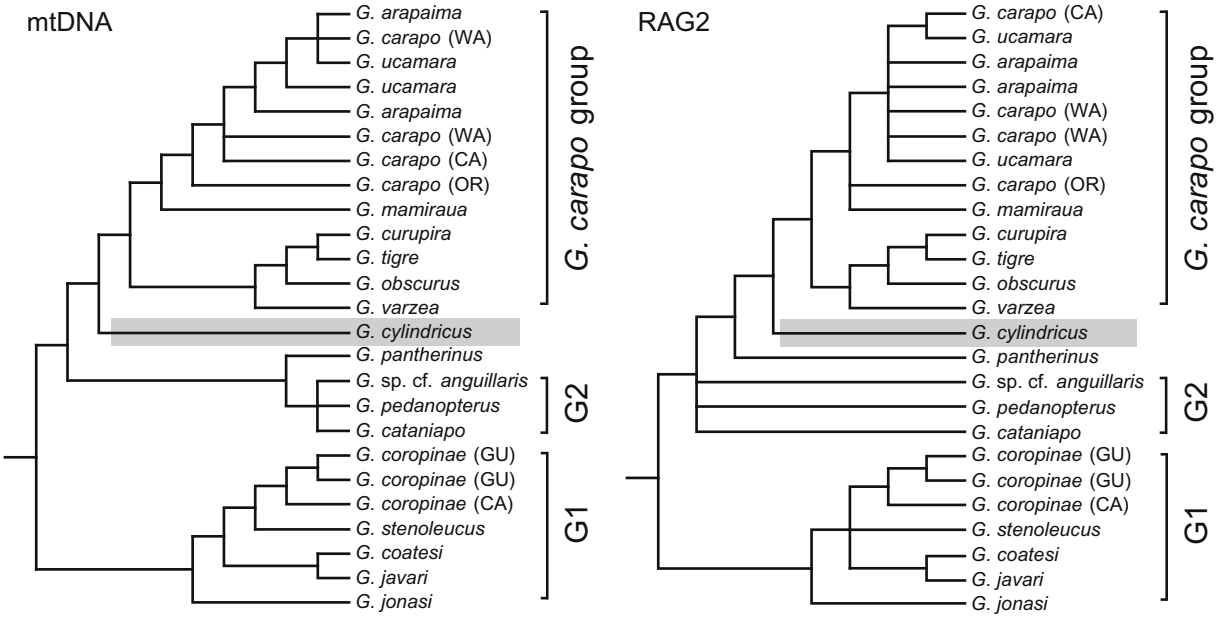

Fig. 4. Results of separate parsimony analyses of mitochondrial and nuclear data. Redundant individuals of each species have been pruned. Grey shading highlights stable phylogenetic position of *G. cylindricus*.

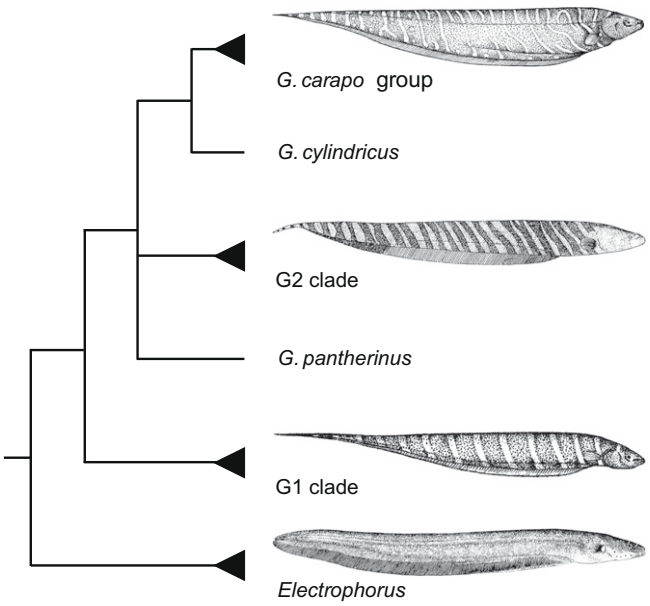

Fig. 5. Conservative consensus of clade relationships for *Gymnotus*, summarizing results across genes and analyses.

independently evolved reduced numbers of EOD phases. The *G. cylindricus* lineage evolved a dramatically reduced number of phases, with *G. cylindricus* having a monophasic EOD. This transition occurred directly from the 4 + multiphasic condition; thus, monophasy in *G. cylindricus* is not a plesiomorphic state shared with *E. electricus*. The second major change occurred in a subclade of the *G. carapo* group composed of *G. curupira*, *G. tigre*, *G. obscurus*, and *G. varzea*. In this lineage the number of EOD phases was reduced to 3 and then to 2 in *G. obscurus*. These state reconstructions are consistent across tree topologies (e.g., parsimony vs. Bayesian) and optimizations (ACCTRAN vs. DELTRAN). EOD conditions in the gymnotid ancestor could not be unambiguously reconstructed.

4. Discussion

4.1. Gymnotus phylogeny

Albert et al. (2004) produced the first comprehensive species-level phylogeny for *Gymnotus*, based on 113 morphological characters. The total evidence and molecular phylogenies presented here confirm some aspects of Albert et al.'s hypothesis, but also conflict with some of the proposed relationships. In both studies, a sister group relationship between *Gymnotus* and *Electrophorus* was well supported, indicating the monophyly of the family Gymnotidae (Ellis, 1913; Triques, 1993; Gayet et al., 1994; Alves-Gomes et al., 1995; Albert and Campos-da-Paz, 1998; Albert, 2001; Albert et al., 2004; Albert and Crampton, 2005). Within *Gymnotus*, three species groups have been proposed based on color pattern, body proportion, and arrangement of laterosensory canals (Fig. 1; Albert and Miller, 1995; Albert, 2001; Albert and Crampton, 2003). These include the *G. carapo* group (16 species), the *G. pantherinus* group (13 species), and the Central American *G. cylindricus* group (two species). The phylogenetic analysis of Albert et al. (2004) supported the monophyly of these groups, which had been proposed earlier (Albert and Miller, 1995) based on morphological similarities rather than formal phylogenetic analysis.

Our study confirms the monophyly of the *G. carapo* group, which receives high support in all analyses that we conducted. Within this clade, our analyses also confirm the monophyly of the '*G. carapo* complex' (Albert et al., 2004), a less inclusive clade composed of *G. carapo*, *G. arapaima*, and *G. ucamara* (and the trans-Andean *G. choco*, not included in the present analysis). However, our analyses do not support the monophyly of the *G. pantherinus* group. In the current study, this proposed taxon was divided into the G1 group (sister clade to the rest of *Gymnotus*), the G2 group, and *G. pantherinus* itself. The phylogenetic position of *G. pantherinus* varied across analyses (including direct optimization), and its sister group relationship was not well supported. The ambiguous positioning of *G. pantherinus* appears to be a phylogenetic phenomenon that extends across datasets—in the morphological analysis of Albert et al. (2004) the node grouping *G. pantherinus* with the rest of the *G. pantherinus* clade was also

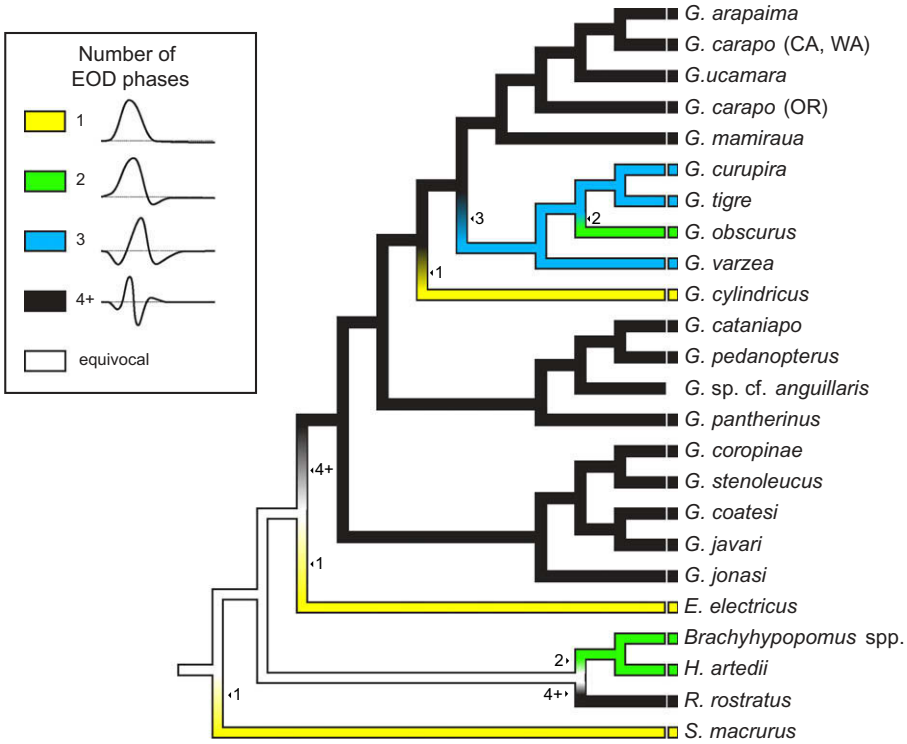

Fig. 6. Optimization of number of phases above and below baseline of electric organ discharge (EOD) for *Gymnotus* species using the parsimony total evidence phylogeny.

weakly supported by decay index and bootstrap values. Future studies incorporating more molecular characters and species will likely help to resolve the relationships between *G. pantherinus* and other *Gymnotus* species. The only previous molecular study (based on analysis of 345 nucleotides of the mitochondrial gene ND2) was conducted on four *Gymnotus* species (*G. pantherinus*, *G. sylvius*, *G. carapo*, and *G. inaequilabiatus*) from the Pantanal region of southeastern Brazil (Fernandes-Matioli and Almeida-Toledo, 2001). This study could not be compared to the analyses here, because only two species were in common.

We were unable to test the monophyly of the *G. cylindricus* group because only a single species from this group was included in our study. However, the phylogenetic position of this Central American lineage within *Gymnotus* is important for biogeographic reconstructions. Our dataset refutes the position of this lineage as the sister clade to all other *Gymnotus*, as proposed by Albert et al. (2004). Analyses based on total evidence, mtDNA, and nuclear data (as well as direct optimization), strongly support *G. cylindricus* as the sister clade to the *G. carapo* group (with bootstrap proportions and posterior probabilities ranging from 99 to 100%). However, partitioned decay indices and separate analyses of morphology (Albert et al., 2004) indicate that morphological data support *G. cylindricus* as the sister group of all other *Gymnotus*. The reason for the conflict between molecules and morphology at this node is unclear. Albert et al. (2004) reported 13 morphological characters that support the monophyly of *Gymnotus* species from South America, to the exclusion of the *G. cylindricus* clade. These characters represent a variety of anatomical structures, including four of pigmentation, one of external meristics, six of cranial osteology, and two from the pectoral fin and girdle. Only two of these characters (shape of the maxilla and dentary) were unique and unreversed in the Albert et al. (2004) analysis, while the others exhibited CI's that ranged from 0.67 to 0.25. If may be that considerable evolutionary lability in these characters is misleading the analyses based on morphology alone; further investigations of the causes for conflict between morphology and molecular data at this node are warranted.

Based on our total evidence analysis, several morphological characters were found to be synapomorphic for *G. cylindricus* and the *G. carapo* group, including: (1) deep body shape in lateral profile (compared to the slender body shape of all other *Gymnotus*); (2) a single dentary tooth row (as opposed to a patch of teeth anteriorly); (3) a broad frontal postorbital process; (4) a broad cleithrum having a curved ventral margin (as opposed to narrow with a straight ventral margin in all other *Gymnotus*); (5) the shape of the fifth rib and its crest; and (6) a moderate depth of the caudal electric organ, consisting of 4–5 rows of electrocytes. Given the strong total evidence support for the clade composed of *G. cylindricus* and the *G. carapo* group, as well as the morphological synapomorphies just mentioned, we believe this phylogenetic arrangement represents the best current hypothesis for the relationships of these taxa.

4.2. Central American biogeography

The topologies of all trees presented in this study (total evidence, nuclear, and mtDNA) show that Central American *Gymnotus* (represented here by *G. cylindricus*) is not the sister group to other *Gymnotus* species, but rather occupies a relatively nested position among South American lineages. Based on molecular estimates of divergence, Central American forms of *Gymnotus* originated sometime during the Miocene, approximately 15 mya. The relatively nested position of *G. cylindricus*, combined with the Miocene age estimate, indicate that Central American *Gymnotus* is more recently derived than Bussing (1985) proposed, and that *Gymnotus* is not part of the 'Old Southern Element'.

This finding is in accord with other studies of origination times for fishes in Central America. Molecular studies of primary freshwater fishes in Central America, including the catfishes *Pimelodella* and *Rhamdia*, knifefishes of the genus *Brachyhypopomus*, and the

characiforms *Roeboides*, *Brycon*, *Bryconamericus*, *Eretmobrycon*, and *Cyphocharax*, have falsified the 'Old Southern Element' hypothesis (Bermingham and Martin, 1998; Martin and Bermingham, 2000; Perdices et al., 2002; Reeves and Bermingham, 2006). Studies of these taxa indicate that colonization of Lower Central America did not occur before approximately 8 mya. Reeves and Bermingham (2006, 249) summarized the molecular phylogenetic evidence as providing "no evidence that primary freshwater fishes colonized Mesoamerica prior to the formation of the Panama landbridge at the end of the Tertiary".

Studies on the evolutionary history of secondary freshwater fishes (taxa with some tolerance for saltwater, sensu Myers, 1938), have provided older estimates of colonization. Divergences between South and Central American lineages have been estimated at 7.7–27.4 mya for synbranchid eels (Perdices et al., 2005), 15–66 mya for cichlids (Martin and Bermingham, 1998; Hulsey et al., 2004; Chakrabarty, 2006; Concheiro Pérez et al., 2007; Rícan et al., 2008), >40–46 mya for *Rivulus* (Murphy and Collier, 1996), and 22–68 mya for poeciliids (Hrbek et al., 2007). Similarly, divergences for several terrestrial clades, including pitvipers, Túngara frogs, salamanders, pseudoscorpions, procyonid mammals, and rainforest trees indicate Miocene or earlier dispersal between South and Central America (Zamudio and Greene, 1997; Zeh et al., 2003; Parra-Olea et al., 2004; Weigt et al., 2005; Erkens et al., 2007; Koepfli et al., 2007; Castoe et al., 2009). These dates are inconsistent with both the 65 mya date of the 'Old Southern Element' scenario and the 3 mya Pliocene emergence of the Isthmus of Panama, and may instead indicate dispersal across marine barriers or via other geographical formations (Iturralde-Vinent and MacPhee, 1999; Pennington and Dick, 2004).

Our age estimate for Central American *Gymnotus* is similar to estimates for secondary freshwater fishes, and suggest that Central American *Gymnotus* might predate the final rise of the Isthmus of Panama. Albert et al. (2004, 2006) proposed that pre-Pliocene oceanic dispersal from South America to Central America may have been facilitated by freshwater outflow from the paleo-Amazonas–Orinoco river. Prior to the Miocene rise of the Merida Andes in northern Venezuela, a large river drained the modern day western Amazon Basin into the proto-Caribbean Sea near the Maracaibo basin (Hoorn et al., 1995). The freshwater plume from this waterway may have been comparable in size to the current plume of the Amazon River, which reaches 6700 km^3 and stretches along the coast of Brazil and the Guianas (Goulding et al., 2003). The plume from the paleo-Amazonas–Orinoco may have facilitated dispersal along the volcanic island arc representing the developing Isthmus of Panama. Recently published paleogeographic models depict the Caribbean Plate as having achieved a relatively modern position by the start of the Neogene (~23 mya), with a volcanic arc rising along its trailing margin in the straits between the North and South American Plates (Doubrovine and Tarduno, 2008; Pindell and Kennan, in press). Therefore a semi-permeable dispersal corridor may have been in place 20 million years was before the formation of a fully terrestrial connection. However, it should be noted that unlike cichlids and poeciliids, all gymnotiforms are considered to be primary freshwater fishes and are intolerant of salt water even at low salinity levels (Myers, 1949). In marine conditions, the gymnotiform electrosensory system is effectively short-circuited, rendering all functions associated with this sensory adaptation useless (Stoddard, 2002a). Thus, dispersal scenarios for *Gymnotus* involving marine or estuarine environments are considerably less likely than for secondary freshwater fishes.

Our molecular divergence estimates, based on a single calibration point, clearly require additional corroboration. Also, while the apparent Miocene divergence of Central American *Gymnotus* may reflect the time of colonization of Central America; alternatively, it could reflect divergences caused by the rise of the Andean Cordillera. Several *Gymnotus* species (aside from *G. cylindricus*) occur in trans-Andean river drainages of South America (west and north of the Andes), but were not included in the present study. It is possible that divergence between trans-Andean and cis-Andean (east of the Andes) lineages occurred approximately 15 mya, but that the trans-Andean clade gave rise to the Central American forms of *Gymnotus* more recently. One of the unsampled trans-Andean species could thus represent the sister group of *G. cylindricus*, and corresponding divergence estimates might be earlier and more in line with Myers's 3 mya hypothesis. For example, in the characiform *Bryconamericus* '*emperador*' group, Central American lineages are closely related to trans-Andean lineages from Colombia (Reeves and Bermingham, 2006). To evaluate this possibility in *Gymnotus*, further molecular phylogenetic and biogeographic studies are needed on the species occurring in Pacific drainages (*G. henni*, *G. choco*, *G. esmeraldas*) and the Magdalena drainage in northern Colombia (*G. ardilai*) in relation to those in Central America and cis-Andean South America.

4.3. South American biogeography

The South American fish fauna is the most diverse freshwater assemblage in the world, and currently comprises some 5600 described species, with many more still to be described (Reis et al., 2003). For some clades of South American fishes, phylogenies can be converted to area cladograms relatively easily, with species/clade boundaries that coincide with major rivers of the continent (e.g., species of the characiform genus *Prochilodus*; Sivasunder et al., 2001). In contrast, the long history of *Gymnotus* in South America has produced a complex pattern, with many relationships (both within and between species) spanning more than a single drainage, often over large distances. At more basal levels in our tree, some evidence for geographic structure among major clades is evident. The *G. carapo* and G1 clades both include many Amazonian taxa, while the G2 clade consists exclusively of taxa whose distributions include drainages of the Guyana Shield. *Gymnotus pantherinus*, a species whose precise phylogenetic position remains unclear, is endemic to Atlantic drainages of southeastern Brazil. Within the major clades, some species, such as *G. carapo* (Albert and Crampton, 2003) and *G. coropinae* (Crampton and Albert, 2003) have distributions that span multiple drainages, indicating a complex history of vicariance, range expansion, and incipient divergence. This pattern reinforces the idea that *Gymnotus* is a relatively ancient group, as indicated by the divergence estimate of 56 mya for the genus. More taxa will be required to fully resolve the biogeographic history of *Gymnotus*, especially from areas underrepresented in the current analysis (trans-Andean South America and south of the Amazon basin).

4.4. Electric signal evolution

The overall trend in EOD phase evolution within *Gymnotus* is the reduction of phases from an ancestral 4 + multiphasic condition. Separate lineages, including *G. cylindricus*, and members of the *G. carapo* species group, have independently evolved reduced numbers of EOD phases (Fig. 6). In previous investigations of *Gymnotus* phylogeny, the *G. cylindricus* lineage was resolved as the sister clade of all other *Gymnotus* species (Albert, 2001; Albert et al., 2004). In that arrangement, the monophasic EOD in *G. cylindricus* represented a plesiomorphic condition shared with *Electrophorus*. In the trees presented here, *G. cylindricus* shows a reversal to monophasy, raising the question of what may have driven this significant change in EOD structure. Numerous abiotic and biotic selective forces may influence the evolutionary design of electric signals (Hopkins and Heiligenberg, 1978; Crampton, 1998a,b; Hopkins, 1999; Stoddard, 1999, 2002b; Crampton, 2006). One impor-

tant type of natural selection on electric signals is the predator-avoidance hypothesis (Stoddard, 1999, 2002a,b; Stoddard and Markham, 2008). This idea posits that multiphasic EODs may have evolved to reduce the conspicuousness of electric signals to electroreceptive predators, including pimelodid catfishes, electric eels, large *Gymnotus*, and river stingrays (Potamotrygonidae). The ampullary electroreceptors of these predators are sensitive to the low frequency electric fields (approximately 0–60 Hz) characteristic of monophasic electric signals. Multiphasic signals, on the other hand have a frequency composition outside the range of detection (Stoddard, 1999; Stoddard and Markham, 2008), and are therefore expected to be relatively cryptic to electroreceptive predators. *G. cylindricus* does not occur sympatrically with the electric eel, large pimelodid catfishes, or river stingrays, which are all restricted to South American drainages. Thus, both biogeography and EOD optimizations suggest that *G. cylindricus* evolved a monophasic discharge in comparatively low predation environments.

Our results for *G. cylindricus* are consistent with the predator-avoidance hypothesis but also require a corollary explanation of why monophasy might evolve in a predatory vacuum. Species in predator-free environments have been shown to reduce or lose adaptations that are involved in avoiding predation (Magurran, 1999; Blumstein, 2002). Maintaining antipredator features may be energetically costly, or costly in terms of mate attraction or foraging behavior (Blumstein, 2002). Thus, in the case of *Gymnotus*, we might predict that monophasic discharges require less energy to produce, or provide some advantage for mate-attraction, hybridization avoidance, or electrolocation performance. Such investigations, along with determination of the EOD characteristics of the other trans-Andean *Gymnotus* species and their position in the phylogenetic tree, are worthy of further study.

Several authors have suggested that the initial evolution of electric signals in gymnotiforms involved a progression from monophasy to multiphasy, based on functional, physiological, and ontogenetic evidence (Stoddard, 1999, 2002a; Kirschbaum and Schugardt, 2002; Crampton and Albert, 2006; Kirschbaum and Schwassmann, 2008). Establishing this initial transition is beyond the scope of the present study, and optimizations of EOD phase at the base of our trees were ambiguous. However, our hypothesized position for the monophasic *G. cylindricus* may have some bearing on the early evolution of EODs in gymnotiforms. In the most recent comprehensive morphological phylogeny, which considers Gymnotidae the sister lineage of all other gymnotiforms, the plesiomorphic EOD condition has been reconstructed as monophasy (Albert and Fink, 1996; Albert and Campos-da-Paz, 1998; Albert, 2001; Crampton and Albert, 2006). Assuming the phylogenetic position of Gymnotidae is accurate, our hypothesis of *G. cylindricus* as a relatively nested member of *Gymnotus* means that an optimization of monophasy as the ancestral state for gymnotidae, and hence gymnotiforms, is less certain. The monophasic signal of *E. electricus* may still represent a plesiomorphic character state for the order. Alternatively, the loss of a multiphasic EOD may have occurred twice during the history of Gymnotidae—in both the *Electrophorus* and *G. cylindricus* lineages. Molecular studies suggest than *Sternopygus* may be the sister taxon of all other gymnotiforms (Alves-Gomes et al., 1995; Alves-Gomes, 1999, 2001). However, the monophasic EOD of *Sternopygus* consists of head positive depolarizations from a negative-offset baseline, and its homology relative to the monophasic EOD of *Electrophorus* is unclear. Accurate determination of the ancestral EOD condition in Gymnotiformes awaits a robust phylogenetic hypothesis for the entire Neotropical electric fish clade.

We examined the evolution of EOD phase number because it constitutes a major source of species-level variation in signal structure and is easily coded as a categorical phylogenetic character. Other waveform characteristics of evolutionary importance in *Gymnotus* include the peak power frequency (PPF), which is typically closely matched to the tuning of some classes of tuberous electroreceptors employed in electrolocation and communication (Hopkins, 1976), as well as the shape, duration, and amplitude of each phase. Crampton et al. (2008) demonstrated that EODs of a community of sympatric *Gymnotus* from the Central Amazon exhibit complete partitioning in a multivariate signal space representing frequency and temporal (shape) parameters of the waveform. Reconstructing the evolution of these waveform features represents fertile ground for further phylogenetic studies of *Gymnotus* behavior and diversification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.09.017.

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