Phylogeny of the weakly electric banded knifefishes: Insights on biogeography and evolution in the Neotropical genus *Gymnotus* (Pisces: Gymnotidae).

by

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfillment of the requirements of the degree of

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Abstract

A total evidence hypothesis of phylogenetic relationships is proposed for the weakly electric banded knifefishes of the genus *Gymnotus* based on data from the mitochondrial cytochrome *b* and 16S genes, the nuclear RAG-2 gene, and 113 morphological characters. Maximum parsimony trees provide partial confirmation of previous findings based on morphological data sets, with the *'carapo'* species group intact. The South American assemblage of *Gymnotus* is shown to be paraphyletic, with Central American species most closely allied to the *'carapo'* species group. This relationship has important implications for biogeography and the evolution of electric signals within the genus. Dating of the node connecting Central American *Gymnotus* to their South American congeners suggests that genetic divergence between the two assemblages took place during the Miocene, prior to the uplift of the Isthmus of Panama.

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Chapter 1: General Introduction

The freshwater ichthyofauna of the Neotropical region comprises one of the most diverse assemblages of freshwater fishes on Earth, consisting of approximately 6000 species, with many still to be described (Vari and Malabarba 1998, Lundberg et al. 2000). Of this diversity, the electric knifefishes of the order Gymnotiformes (~173 known species) represent an order of fishes found exclusively in South and Central America (Albert and Crampton 2005). These fishes are distinct from other Neotropical fish in their ability to produce and detect weak electrical fields using their electric organs (composed of modified muscle cells termed electrocytes) and two types of electroreceptors (Lissman and Machin 1958). The electrical charge created by the electric organ produces an electric field that surrounds the fish. By detecting changes in the shape, frequency, or amplitude of their own electrical fields, the fish are able to use their electrogenerative abilities for electrolocation, whereas the detection of the distortions in the electric fields of other gymnotiforms enables communication within and among species (Carr and Maler 1986). Due to the constraints placed on body morphology as a result of their ability to produce electrical fields, all gymnotiforms retain a rigid knife-like (culteriform) shape and an elongate anal-fin. The undulation of the anal fin provides these fish with their primary means of locomotion, supported in part by the action of the pectoral fins. A single member of the group, *Electrophorus electricus*, the electric eel, has developed the ability to produce large electrical discharges, up to 600V, which are used for both defense and predation and are combined with the use of weaker impulses, which are used for electrolocation (Moller 1995). In all other gymnotiforms the electrical impulses produced

are much smaller (millivolts) and are used for locating prey, navigation, and communication in their nocturnal environments.

In Africa, an independent radiation of electrogenic fishes has occurred within the osteoglossiform families Mormyridae (elephantfishes) and Gymnarchidae. Recent investigations of mormyrid phylogeny have revealed instances of "explosive speciation" in which new species arise rapidly within geographically restricted areas from a single ancestor (Sullivan et al. 2002, Sullivan et al. 2004). It is assumed that the species specificity of the electric signals produced by these fish has in large part contributed to the establishment and maintenance of the diversity observed within the species flock. In contrast, it is thought that the diversity observed in Neotropical knifefish species is the result of the accumulation of species over long periods of geological time and involving continent wide geological processes (Albert et al. 2004), and has been reinforced by the species specificity of their electric signals

Within the South American gymnotiform knifefishes, the closest known relatives of the electric eel are thought to be a geographically widespread group of weakly electric knifefishes from the genus *Gymnotus*. These fish produce weak "pulse" type electrical organ discharges (EODs) that are known to be species specific (McGregor and Westby 1992) and are used for communication (Black-Cleworth 1970) and possibly mate recognition. Electrical signaling could be a critical factor in the diversification of *Gymnotus* species (in addition to other gymnotiforms) as it operates in species recognition and consequently is likely to contribute to prezygotic reproductive isolation. For this reason, it is interesting to examine patterns of evolution of electric organ discharges (EODs) and explore how they may influence the relationships amongst

Gymnotus species. To trace patterns of evolution, a robust phylogeny on which the characters of interest may be traced is required. Patterns of evolution in EODs may be of particular interest when comparing species endemic to the Central and South American landmasses. The waveforms of the electric signals recorded from fish in these locations show significant differences, with Central American species possessing a monophasic waveform that contrasts with the multiphasic waveform typical of South American species.

The South American continent (on which gymnotiform fishes originated and where the majority of extant *Gymnotus* species can be found) has experienced a long and complex geological history involving the emergence of the Andes mountains, the subsequent formation of the Amazon basin, and the collision of the South American plate with Central America resulting in the formation of the Isthmus of Panama (Lundberg et al. 1998). The latter event led to the renowned "Great American Biotic Interchange", during which many organisms, including freshwater taxa, were exchanged between North and South America (Stehli and Webb 1985). Throughout much of the history of South America, only two major geological structures have remained constant, these being the Guiana and Brazilian Shields in the northeastern and southeastern part of the continent respectively. These landmasses lost any possible connections during the Miocene, at which time the Amazon River (which flows in the valley between the two shields) was formed as a result of the uplift of the Central Cordillera of the Andes mountains. Throughout evolutionary history, freshwaters have existed on the shields, while the low-lying areas surrounding them have intermittently been affected by marine incursions of varying degrees (Lundberg et al. 1998). Tectonic and climatic events such as these have

altered river courses and drainage patterns and therefore play a large role in the diversification of aquatic organisms in the Neotropics, including freshwater fishes and by extension, *Gymnotus*. As one of the basal Gymnotiformes (Albert 2001), *Gymnotus* has a long history in South America and as a result a remarkable range that includes drainages between southern Mexico and northern Argentina, including Central America and the Pacific drainages of Peru, Ecuador and Colombia. Its widespread distribution indicates that *Gymnotus* may be particularly useful for tracking earth history events in the Neotropical region.

Molecular phylogenetic analyses and the DNA sequence data associated with them have become a useful tool for the interpretation of taxonomy, biogeography, and evolutionary patterns, in addition to providing information that can benefit conservation efforts (Hillis et al. 1996, Kocher and Stepien 1998, Saitoh et al. 2003). In addition, for taxa lacking an adequate fossil record, molecular data can be used to estimate the timing of speciation events. Associating time scales with phylogenies can facilitate the correlation of speciation events with geological and environmental phenomena. As a preliminary step in the study of the evolutionary history and processes involved in the origin and maintenance of species diversity within the genus *Gymnotus*, this dissertation sought to evaluate phylogenetic relationships of the species using both molecular and morphological data. The phylogeny was then used to investigate South and Central American biogeography, with specific reference to the events surrounding the dispersal of *Gymnotus* to Central America, and to examine the evolution of electric signal waveforms within the group. An understanding of the biogeographical patterns associated with the phylogeny of *Gymnotus* species will provide useful insights into biological

diversification in the Neotropics at the species level, whereas the examination of the phylogenetic pattern of electric signal waveforms will provide information regarding diversification within *Gymnotus*.

Chapter 2: Molecular phylogeny of the weakly electric banded knifefishes: insights on biogeography and the evolution of signal waveforms in the genus *Gymnotus* (Gymnotiformes: Gymnotidae).

1. Introduction

The Neotropical freshwater fish family Gymnotidae comprises the weakly electric naked-backed knifefishes of the genus *Gymnotus* and the electric eel, *Electrophorus electricus* (Albert 2001). Of all gymnotiform taxa, *Gymnotus* species are the most widely distributed and range 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, terre firme streams and varzea (whitewater floodplains), with many inhabiting vegetation such as floating meadows, root masses, or leaf litter (Crampton 1998). Like other gymnotiforms, *Gymnotus* species produce weak, species-specific electrical signals that function in electrolocation (Heiligenberg 1973, Hopkins and Heiligenberg 1978, Lissman and Machin 1958) and communication (Black-Cleworth 1970) in their nocturnal environment. Recent efforts to describe the knifefish fauna of South America have revealed that species diversity is greater than previously thought (Albert et al. 2004), and currently 32 valid species of *Gymnotus* are recognized (Table 1). 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 speciation in the Neotropics.

Until recently, little was known regarding the relationships among the species of *Gymnotus*. This was in large part due to the lack of adequate species descriptions, which have only become available within the last decade (Albert and Crampton 2003, Albert

and Crampton 2001, Albert and Miller 1995, Albert et al. 1999, Crampton et al. 2005, Crampton et al. 2003, Fernandes et al. 2005, Maldonado-Ocampo and Albert 2004). Prior to this surge in description, many *Gymnotus* species were lumped under the title *Gymnotus carapo*, a species that has since been redescribed (Crampton and Albert 2003a). While some authors have attempted to resolve relationships within the order Gymnotiformes based on molecular (Alves-Gomez et al. 1995) and morphological data (Albert 2001), fewer have attempted species level studies (but see Sullivan 1997).

Based on an analysis of 113 phenotypic characters, Albert et al. (2004) proposed the most comprehensive phylogenetic hypothesis of *Gymnotus* species to date. The authors diagnosed three species groups within *Gymnotus*: the 'cylindricus' species group, the 'pantherinus' species group, and the 'carapo' species group (Fig. 1 shows these groups as they appear on the morphological phylogeny of Albert et al. 2004). The 'cylindricus' group is currently known to comprise the two *Gymnotus* species distributed in Central America (*G. cylindricus*, and *G. maculosus*) while both of the 'pantherinus' and 'carapo' groups include species that occur east (Atlantic drainages, OR/GU/WA/EA/PA/SE in Fig.2) and west (Pacific drainages, PS/NW in Fig.2) of the Andes mountain range. Presently, the cylindricus group is considered to be the sister group of a monophyletic lineage consisting of all South American species (pantherinus clade + carapo clade) (Albert et al. 2004).

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 American/Central American biogeography. There is some disagreement in the literature over the timing of the

dispersal of *Gymnotus* into Central America. Based on physiological constraints, Miller (1966) and Myers (1966) considered all primary freshwater fish of South American origin to be the result of recent invasions into Central America, after the establishment of the Isthmus of Panama, which occurred approximately 3.7-2.8 mya (Coates and Obando 1996, Duque-Caro 1990a). In contrast, based on biogeographical distributions and regions of endemicity, Bussing (1985) suggested that Central American forms of *Gymnotus* were part of an invasion that "reached Central America from the south in late Cretaceous or early Tertiary times" (~65 mya), at which time a historical land connection between North and South America is hypothesized to have existed (Marshall et al. 1997, Briggs 1994, Pitman et al. 1993, Rage 1981).

The presence of a land bridge connecting North and South America during the Cretaceous is controversial, and as a result several hypotheses exist as to its exact geographical location and extent. It is thought that this connection consisted either of a proto-island arc in the location of the present day Isthmus of Panama or a landmass that stretched from what is now northern Venezuela to the approximate area of the Yucatan peninsula consisting of land connections between the proto-Antilles and the Aves Ridge (Anderson and Schmidt 1983, Pindell 1985) (Fig.3). The existence of an early Cenozoic land bridge is controversial (Gayet 2001, Gayet et al. 1992), and while supported by fossil evidence of faunal interchanges of mammals between North and South America during the Paleocene (Gingerich 1985), it lacks other forms of geological support. Coastline reconstructions based on numerous paleogeographic references show no connections between the Americas 60 mya (Fig.4). More recently, Iturralde-Vinent and MacPhee (1999) argued that a land mass did at one time exist in the proto-Caribbean sea,

but that the temporal occurrence of the formation was likely during the late Eocene or early Oligocene (35-33 mya). In addition, the paleogeographic reconstructions of these authors suggest that the landform had no connection to the North American continent (Fig.5). The uncertainty over the existence of Early Tertiary land bridges connecting South and North America, in addition to the absence of gymnotiform fishes on the islands of the Antilles and the physiological inability of freshwater organisms (particularly primary freshwater fish) to cross oceanic barriers, are all factors that suggest that *Gymnotus* (and other freshwater species) likely colonized Central America in more recent times during the Pliocene-Pleistocene, at which time the Isthmus of Panama would have provided passage.

Population level studies of some fishes, including the lower Central American forms of the catfish *Pimelodella*, the characid *Roeboides*, and knifefish in the genus *Brachyhypopomus* (family Hypopomidae) have revealed that colonization of Lower Central America (LCA) may have occurred in multiple waves (Bermingham and Martin 1998). The first wave of colonization is proposed to have occurred in the late Miocene (~7-4 mya), the second during the mid-Pliocene (associated with the emergence of the Isthmus of Panama) and the third more recently, possibly in the Pleistocene. In all cases however, it was concluded that the colonization of lower Central America (i.e. Panama and Costa Rica) occurred in more recent times than suggested by Bussing (1985). Further support for a Pleistocene colonization event, following the emergence of the Isthmus of Panama, has been provided by an examination of a genus of parasites (*Sciadicleithrum*) known from the tissues of cichlids distributed across South and Central America, and Mexico (Mendoza-Franco and Vidal-Martínez 2005). More recent studies on the

evolutionary history of the catfish *Rhamdia* (Perdices et al. 2002) and of synbranchid eels (Perdices et al. 2005) indicate earlier divergences between South and Central American lineages (6-8 mya for *Rhamdia* and 7.7-12.4 mya for *Synbranchus*) that agree more with the Bermingham and Martin's (1998) late-Miocene/early Pliocene wave of colonization. Older divergences between Central and South American taxa are also supported by the divergence time of Central American heroine cichlids, which are thought to have an origin in the Miocene between 15-18 mya (Hulsey et al. 2004, Martin and Bermingham 1998). In contrast to the mid-Miocene to Pliocene estimates of the origin of Central American lineages of the previous studies, Murphy and Collier (1996) reported that Central American forms of the guppy *Rivulus* originated 40-46 mya. While some of the aforementioned studies were rigorous in their sampling of Central American taxa, the sampling of taxa from numerous drainages in South America was not often carried out (in many cases the focus of the research was on population level phylogeography concentrating on taxa and areas within Central America, using related South American taxa as outgroups).

The alternative hypotheses regarding the temporal and geographic aspects of fish dispersal into Central America have important phylogenetic implications. Bussing's hypothesis suggests that *G. maculosus* and *G. cylindricus* should appear together as a sister taxon to all South American species, reflecting divergence in approximately the early Tertiary, shortly after the origin of the gymnotiform ancestor (Fig.6A). Alternatively, if Central American *Gymnotus* species were to have dispersed to the region via the Isthmus of Panama during the Pliocene, a close relationship would be expected between them and species isolated to the northwestern corner of the South American

continent (Rio Atrato, Rio Magdalena, and possibly Pacific slope species). These in turn would have close associations with the cis-Andean species that share common ancestors with trans-Andean species (Fig.6B). The current hypothesis of *Gymnotus* relationships based on morphological characters supports the former view that *G. maculosus* and *G. cylindricus* form the sister group to all other species of *Gymnotus* (Albert et al. 2004) and therefore may have colonized Central America during the early Tertiary. A molecular phylogeny of *Gymnotus* is needed to elucidate the position of Central American *Gymnotus* species within gymnotid phylogeny and to provide an alternative method to estimate the date of colonization using molecular clock techniques.

A robust phylogeny of *Gymnotus* species will also be useful for a preliminary exploration of the evolution of electric organ EOD waveforms. Like other weakly electric knifefishes, *Gymnotus* produce species-specific electrical fields. In *Gymnotus* these signals are emitted by modified muscle fiber cells (electrocytes) which are arranged in series and parallel to form an electric organ (Trujillo-Cenóz and Echagüe 1989). Electrocytes generate electrical potentials by exploiting the ionic gradients across membranes in a similar manner to nerve and muscle cells. Recorded signals can be categorized as either mono- or multiphasic based on the shape of the waveform (Fig. 7). The multiphasic category can be further broken down to reflect exactly how many peaks (both positive and negative) appear in the waveform (diphasic, tripahsic etc). The number of phases in a signal waveform results from the morphology, organization, and patterns of innervation of the electrocytes comprising the electric organ (Bennett 1971, Bennett 1961, Macadar et al. 1989). While all South American forms of *Gymnotus* are known to possess multiphasic EODs, Central American species have a monophasic EOD

(Crampton and Albert 2006). The electric eel, the presumed sister group with which *Gymnotus* is assumed to have shared a common ancestor, also possess a monophasic EOD. Monophasic EODs are considered to be plesiomorphic (Stoddard 2002a), and this view has contributed to the placement of *G. cylindricus* and *G. maculosus* as the sister group to all other *Gymnotus* species.

The objectives of this study were to produce a phylogeny of *Gymnotus* species consisting of both nucleotide and morphological data and use it to: 1) test the hypothesis of *Gymnotus* relationships proposed by Albert et al. 2004, particularly with respect to the monophyly of the South American assemblage, 2) use the phylogeny to test hypotheses of the timing/mode of *Gymnotus* dispersal into Central America, and, 3) consider the evolution of electric signal phases in relation to the phylogeny of *Gymnotus* species.

2. Materials and Methods

2.1. Taxon Sampling, DNA extraction and sequencing

Tissues for 17 ingroup and 6 outgroup species were included for analysis (see Table 2 for a complete list of specimens sequenced). Within Table 2, Field/EOD numbers correspond to individual specimens that will be deposited in museums, with museum catalogue numbers obtainable from the Genbank records for accessioned sequence data. Although a comprehensive sample of all *Gymnotus* species could not be obtained, 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 diversity within the genus *Gymnotus*. Whenever possible two individuals of each species were sequenced. In the case of *G. coropinae* and *G. carapo*, multiple geographic variantrs were sequenced, which brought the number of operational taxonomic untis

(OTUs) including outgroup taxa, to 28. As the purpose of this study was not to create a phylogeny for all knifefish, representative outgroup taxa were selected from different families of gymnotiforms. Selection was based upon the availability of tissues, DNA and/or sequence data for each taxon and included individuals from the families Rhamphichthyidae (*Rhamphichthys*), Hypopomidae (*Hypopomus*, *Brachyhypopomus*) and Sternopygidae (*Sternopygus*). Outgroup selection also considered a calibration point to be used when estimating divergence times. *Brachyhypopomus diazi* and *B. palenque*, knifefish species from the family Hypopomidae, are known respectively from drainages in Venezuela and Eastern Colombia and are assumed to have diverged as a result of the vicariant event surrounding the uplift of the Eastern Cordillera of the Andes mountain range approximately 12.9 mya to 11.8 mya (Lundberg et al. 1998). Since this node could be dated, these taxa were selected to be included as part of the outgroup.

Tissues were collected in the field either by colleagues or by the author and specimens were identified by W. Crampton (University of Central Florida), J. Albert (University of Louisiana, Lafayette) and the author. When in the field, fish were located 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 3-4mm mesh size. Muscle tissue was excised and stored in either 95-100% ethanol or a buffered solution consisting of 20% DMSO and 0.25M EDTA at pH 8, saturated with NaCl (Seutin et al. 1991).

Total genomic DNA was isolated from muscle tissue using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's protocols with minor modifications. The polymerase chain reaction (PCR) was used to obtain 1223bp of the nuclear recombinase

activating gene-2 (RAG2), 1106bp of the mitochondrial DNA (mtDNA) gene cytochrome *b* (cyt *b*) and 553bp of the 16S ribosomal mtDNA gene using combinations of the primers listed in Table 3. PCR reactions were carried out in 50ul volumes.

The entire mitochondrial cytochrome *b* gene was amplified using primers in the adjacent glutamine (GLUDG.L) and threonine (CytbR) transfer RNAs (Palumbi et al. 1991). DNA was synthesized in 50 µl volumes consisting of 10X PCR buffer (50mM KCL, 20mM Tris-HCL, pH 8.4), 200 µM of each dNTP, 3mM $MgCl_2$, 0.4 µM of each primer, 1 unit 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). In this case, DNA was synthesized in 50 µl volumes consisting of 10X PCR buffer (50mM KCL, 20mM Tris-HCL, pH 8.4), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 1 unit of Taq DNA Polymerase and 1ul of DNA extract. The amplification of RAG2 was accomplished using the RAG2F1 and RAG2R6 primers of Lovejoy and Collette (2001), in addition to two primers designed specifically for this study RAG2GY-F and RAG2GY-R (Table 3). PCR reaction volumes and concentrations followed those outlined for 16S with the exception that 2-5µl of DNA extract was used.

PCR amplifications were carried out in a Pelletier thermocycler. Thermal cycling conditions for RAG2 followed a touch down protocol of 95°C for 30s 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 90s. For the mitochondrial genes (cyt *b* and 16S) similar standard conditions were used consisting of 95°C for 30s denaturation, 50°-58°C for 60s annealing, 72°C for 60-90s extension. This profile was run through 35 cycles for 16S and

35-40 cycles for cyt *b*. Hold steps of 95°C for 30s – 150s and 72°C for 300s were used at the beginning and end of each protocol respectively.

PCR products were purified using the Montage PCR purification kit (Millipore) with minor modifications. Specifically, the part of the protocol involving the recovery of purified DNA was modified so that after the addition of double distilled water to the filter, a period of ~3 minutes was allowed to elapse prior to the final centrifugation step. This helped to ensure a high enough yield of purified DNA for later sequencing. Samples were then subjected to automated cycle sequencing on either ABI 377 or ABI 3730 Genetic Analyzers, using dye terminators (BigDye version 3.1, Applied Biosystems). Two internal primers, GYRAG2ISP1 and GYRAG2ISP2, (Table 3) were designed and used for RAG2 sequencing based on an initial alignment of *Gymnotus mamiraua*, *G. arapaima*, and *G. carapo*.

2.2. DNA Alignment

Sequences were edited and preliminary alignments accomplished 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 a comparison to 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). An effort was made to exclude those portions of

ambiguous alignments that fell within loop regions. Loop regions of 16S and 12S secondary structures are known to have faster rates of substitution than stems, and often include many insertions/deletions (Broughton et al. 2001, Wang and Lee 2002). While these data can provide accurate phylogenetic reconstructions for recently derived taxa, the genus *Gymnotus* contains many taxa which likely result from more ancient divergence events. In cases where it is difficult to determine positional homology it is often recommended that the highly divergent regions of the gene be excluded from analysis (Olsen and Woese 1993, Swofford et al.1996) and therefore the inclusion of ambiguously aligned loop regions was deemed inappropriate. Table 4 provides an overview of the regions excluded.

2.3. Phylogenetic Analysis

Nucleotide and morphological character data were combined into a single total evidence matrix (a total of 2894 characters for 28 OTUs). Morphological data were all 113 phenotypic characters from the morphological analysis of Albert et al. (2004). Data partitions were defined as nuclear (RAG2), mtDNA (cyt *b* and 16S combined), and morphology. The incongruence length difference test (ILD or partition homogeneity test of PAUP*) was employed to test the combinability of these data partitions (Farris et al. 1996, Swofford 2002). Heuristic searches and 1000 homogeneity replicates were used (10 random taxon additions). In some instances the number of rearrangements tried for each replicate was limited to 10 million in order to avoid issues related to the memory constraints of the computer system.

Trees for the mtDNA, nuclear and total evidence datasets were constructed in PAUP* (Swofford 2002) using the criterion of maximum parsimony and the heuristic

search algorithm with 100 replicates of random addition of taxa, and TBR (Tree Bisection and Reconnection) branch swapping. All trees were rooted using *Sternopygus macrurus*. Each dataset was analyzed: 1) with all characters equally weighted, and, 2) with transversions weighted at 2 and 5 times the weight of transitions. These values were selected to encompass the range of the average transition/transversion ratios derived empirically from the data sets for each gene. Transition/transversion ratios were calculated using the 'state change and stasis' function in the program MacClade (Maddison and Maddison 2001). This function computes the frequency of state changes within the dataset for a given tree or series of trees. In this instance state changes were averaged over a series of 100 randomly generated trees. Weighting of transversions was accomplished using stepmatrices in PAUP*. Gaps were treated as missing data. In an additional analysis, cytochrome *b* third positions were removed from the total evidence analysis due to potential problems caused by base compositional bias (see results). Base compositional bias, the occurrence of the four nucleotides A, G, C, T, in unequal proportions, was examined using the base frequencies function of PAUP* (Swofford 2002). PAUP* was also used to employ a chi-square test of the homogeneity of base frequencies among lineages. Variation in base compositional bias among taxa is known to compromise phylogenetic reconstruction (Collins et al. 1994). In some cases, highly divergent sequences can be erroneously grouped together based on similarities in base compositional bias (Collins et al. 1994, Eyre-Walker 1998).

For each tree, nodal support was calculated using bootstrap support values (Felsenstein 1985) and decay indices (Bremer support values, Bremer 1994). Bootstrap values were calculated in PAUP* using the heuristic search option (1000 replicates, 10

random taxon additions) and decay indices were calculated using the program TreeRot (Sorenson 1999). For both the mtDNA and total evidence trees, Partitioned Bremer Support (PBS) was calculated as an indication of the topological support provided by each data partition for each node (Baker and DeSalle 1997).

After the total evidence tree had been generated and analyzed,for ease of presentation the additional individuals for taxa in which multiple individuals had been included for analysis were pruned from the tree. Similarly, to facilitate comparisons between the morphological, total evidence, mtDNA, and RAG2 trees, trees were produced in which both the excess individuals and outgroup taxa related to estimating divergence dates were pruned from the trees.

2.4. Molecular clocks and Divergence estimates

The concept of the molecular clock, the constant rate of change in amino acid sequences, was first suggested by Zuckerkandl and Pauling (1965). While the concept as applied to entire genomes is highly controversial (Hedges and Kumar 2004, Graur and Martin 2004, Arbogast et al. 2002), some authors have supported the existence of local clocks, in which evolutionary rates for a given gene are relatively constant within lineages (Yoder and Yang 2000). To account for the possible independence of rates among different genes, the cyt *b* and RAG2 datasets were treated separately. The 16S gene was excluded from the molecular clock and divergence estimate analysis due to the relatively small size of the dataset. Small datasets have been shown to provide erroneous estimates of phylogeny (Nei et al. 1998) and may lack the power to reject a molecular clock, which could result in errors in divergence time estimates due to the limited number of characters available to establish rates of evolution (Bromham et al. 2000).

In order to determine whether sequences evolve in a clocklike manner a relative rates test must be used. One method is to employ a log-likelihood ratio test (LRT) (Sanderson 1998). This test is applied to compare the likelihood of the evolutionary model when a molecular clock is enforced (i.e. constant rate across all lineages) to the likelihood of the same model when the rate of evolution is allowed to differ between lineages. Twice the difference between the likelihood values is known to approximate a chi-square (χ^2) distribution with degrees of freedom equal to the number of taxa minus 2 (Muse and Weir 1992). In this case, the LRT was used to test the molecular clock assumption for the cyt *b* and RAG2 datasets and their respective trees. The null hypothesis being tested in this case is rate homogeneity among lineages. In addition to cyt *b* and RAG2, this test was performed on each of the codon position datasets (1^{st}, 2^{nd}, 3^{rd}) for the cytochrome *b* gene as this gene was potentially saturated. Modeltest v.3.6 (Posada and Crandall 1998) was used to select the model of molecular evolution that was most appropriate for each gene (or codon position) based on the raw data. If the likelihood ratio test showed that the rates across lineages were not significantly different ($p > 0.05$), patristic distances were calculated as the sum of the branch lengths connecting taxa and/or clades. In cases where the divergence between different clades was being analyzed, the distance between clades was obtained by calculating the average distance (branch length) between the members of the clade of interest (e.g. Central American clade) and each member of its sister clade (e.g. each member of the carapo clade). Raw patristic distances were transformed into percent divergence values by dividing each distance by the number of base pairs in the original dataset for each gene.

Divergence times were then estimated using the divergence of *Brachyhypopomus palenque* and *Brachyhypopomus diazi* as a calibration point. *B. sp.* "*palenque*" is geographically distributed west of the Andes in Colombia, while *B. diazi* is known from northern Venezuela. Based on a morphological and mitochondrial DNA analysis, Sullivan (1997) showed that *Brachyhypopomus* species distributed west of the Andes (namely *B. occidentalis* and *B. sp.* "*palenque*") form the sister clade to *B. diazi*. As a result, it is assumed that the divergence of these taxa corresponds to the uplift of the Eastern cordillera of the Andes mountain range, which began between 12.9 and 11.8 mya (Lundberg 1998).

To account for rates which were deemed non-clocklike, the Non-Parametric Rate Smoothing (NPRS) technique of Sanderson (1997) was employed using the program TreeEdit (Rambaut and Charleston 2002, http://evolve.zoo.ox.ac.uk/software/TreeEdit). This method of estimating divergence dates employs a least squares smoothing of the local estimates of substitution rates, which relaxes the assumption of a molecular clock. The result is an ultrametric tree in which the branch lengths leading to all taxa are equally distant from the root of the tree. The relative times displayed in this tree were converted to absolute ages using the aforementioned *B. diazi-B. palenque* divergence for calibration. For comparison, this technique was also applied to those datasets in which clocklike rates were found.

Thirdly, ages were also estimated solely on the basis of uncorrected pairwise comparisons of cytochrome *b* sequences using a calibration for 'teleost' mitochondrial DNA of 1.2% divergence per million years. This rate is derived from comparisons of

sequence divergence in marine fishes that were separated by the establishment of the Isthmus of Panama during the Pliocene (Bermingham et al. 1997).

Saturation of nucleotide substitution in a given gene can result in errors in branch length reconstructions and thus lead to misinterpretation of divergence estimates (Swofford et al. 1996). To examine the potential effects of saturation, a saturation plot was constructed for cytochrome *b* by plotting the number of third position transitions for all pairwise species comparisons against uncorrected pairwise sequence divergence (Griffiths 1997). The third positions of amino acid codons are subject to a high rate/degree of substitution as a result of the degenerate nature of the genetic code (Li 1997), and therefore were deemed a suitable indicator of potential saturation.

2.5. Character Optimization

Characters relating to electric organ discharge, specifically the numbers of phases above and below the baseline in recorded signals were optimized on the total evidence phylogeny in MacClade 4.0 (Maddison and Maddison 2001) using both the ACCTRAN and DELTRAN optimization techniques. The former technique accelerates the transformation of characters, thereby favoring the reversal or secondary loss of characters. The latter technique delays the transformation of characters, thereby favoring either the convergence of characters, or parallelisms. In addition, signals were traced taking into consideration only the basic waveform shape, either monophasic or multiphasic, without distinguishing between the different categories of multiphasic signals.

3. Results

3.1. Characterization of sequence data

The cytochrome *b* dataset consisted of 1106 characters (nucleotide sites), 513 of which were parsimony informative (i.e. sites at which there are at least two types of nucleotides, each of which are represented in at least two different sequences). The base composition (AGCT content) of the cytochrome b gene for *Gymnotus* (Table 5) exhibited patterns similar to those found in other fishes, such as low G content predominantly within third positions (Canatore et al. 1994, Johns and Avise 1998, Lydeard and Roe 1997, Meyer 1993). However, the results of the chi-square test of homogeneity of base composition across taxa suggest that base frequencies within the gene are not homogenous across taxa ($p < 0.001$, Table 5). Further analysis by codon position revealed that the heterogeneity of base composition observed in the cytochrome *b* gene and mtDNA data partitions could be explained solely by third positions (Table 5). In addition, the saturation plot (Fig. 8) indicated that third position transitions became saturated beyond ~15-16% sequence divergence, which could be problematic when reconstructing nodes deep in the phylogeny. Based on these results, a new total evidence tree was constructed using a dataset in which cytochrome *b* third positions were excluded from phylogenetic analysis (see results section 3.2).

Uncorrected pairwise comparisons of sequence divergence for cytochrome b (Table 6) revealed that among all taxa the greatest divergence (26.8%) existed between *Electrophorus electricus* and *Brachyhypopomus palenque*. Within the Gymnotidae, the greatest divergence was observed between *E. electricus* and new species *G. sp.* aff. *anguillaris*. (25.9%) followed closely by *E. electricus* × *G. cylindricus* (25.6%). Within *Gymnotus*, *G. sp.* 2091 and *G. coatesi* were most divergent (19.8%). The smallest

divergence observed existed between *G. arapaima* and *G. ucamara* (0.2%). Within the genus, the Central American form of *Gymnotus*, *G. cylindricus*, was equally most distant from *G. jonasi*, *G. javari*, and *G. coropinae* (WA) (18.7%) and least distant from *G. varzea* (11.3%). Comparisons of sequence divergence between *G. cylindricus* and the G1 clade ranged from 18.1-18.7%, while comparisons to the '*carapo*' clade were smaller ranging from 11.3-12.9%. Sequence divergence between *G. cylindricus* and the G2 clade ranged from 15.1-18.1%.

The 16S dataset in which ambiguously aligned characters had been removed consisted of 452 characters, 96 of which were deemed parsimony informative. When combined with the cytochrome b data to form the mtDNA dataset, this represents 1558 characters of which 609 were parsimony informative.

The RAG2 dataset was composed of 1223 characters, 233 of which were parsimony informative. Analysis of nucleotide frequencies revealed that comparisons across taxa were homogeneous (Table 5). Uncorrected pairwise comparisons of sequence divergence for RAG2 (Table 7) showed *G. curupira* and *B. diazi* to be most divergent (11.1%). Within Gymnotidae *E. electricus* was most divergent from *G. jonasi* (9.9%). Comparing pairwise sequence divergence within *Gymnotus* revealed a marked decline in divergence compared to the divergences exhibited at higher taxonomic levels for this gene. Within the genus, the largest divergence observed occurred between *G. coropinae* GU and *G. tigre* (3.8%). In contrast the smallest distance observed within the genus was between *G. carapo WA*, *G. ucamara*, and *G. arapaima*. All comparisons of pairwise distances between these three taxa yielded divergences of 0.00%, indicating that the RAG2 sequences were identical for all three species. However, examination of the

aligned sequences, revealed a single base pair change across the 1223 nucleotides comprising the RAG2 dataset. When considering distances between the Central American form and other *Gymnotus* species it was found that *G. cylindricus* was equally most distant from *G. javari*, *G. coatesi* and *G. coropinae* GU (3.3%) and least distant from *G. arapaima* (1.4%). Comparisons of uncorrected pairwise sequence divergence between the Central American clade and the other clades of *Gymnotus* produced a similar pattern to that observed for the cyt *b* dataset. Divergence between *G. cylindricus* and the G1 clade was highest (3.0-3.3%), followed by the G2 clade (2.6-2.9%), and the smallest pairwise sequence divergences were observed between *G. cylindricus* and members of the carapo clade (1.4 -2.0%). The distances observed between *G. cylindricus* and other forms of *Gymnotus* are consistent with the majority of the groupings seen in the trees produced by the phylogenetic analysis.

3.2. Trees, support values, and general biogeographic patterns

Total Evidence

A total of 2781 molecular characters and 113 morphological characters were used for the total evidence analysis. This included 1101 variable characters, 954 of which were parsimony informative.

The partition homogeneity test comparing all partitions (mitochondrial DNA, nuclear DNA, and morphology) indicated incongruence (p=0.001). However, pairwise comparisons of nuclear and mitochondrial datasets however indicated that the molecular partitions were congruent ($p > 0.05$). A comparison among mitochondrial partitions (cyt *b* and 16S) also showed congruence ($p > 0.05$). Pairwise comparisons of morphology with each molecular partition (mtDNA and nuclear) were incongruent ($p < 0.05$), suggesting

that the majority of incongruence among all datasets can be explained by morphology alone. The incongruence of morphology with molecular data was also reflected in negative partitioned Bremer support values for the morphology partition in the total evidence phylogeny (see below). The combination of incongruent data partitions has been observed to contribute to greater phylogenetic accuracy. A study of slow-loris phylogeny by Yoder et al. (2001) indicated that an inverse relationship might exist between the incongruence of data partitions and phylogenetic accuracy; therefore it was considered acceptable to proceed with the combination of the morphological and molecular data.

Figure 9 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 tree topology; however, tree length changed from 3256 to 4067 steps in each case. Figure 10 shows a summary of species relationships – it is the single most parsimonious tree from Fig. 9 with additional individuals of each species pruned.

Based on the total evidence analysis, *Gymnotus* is separated into two principal clades. Notably, the Central American clade, represented here by *Gymnotus cylindricus*, is not basal to all other *Gymnotus* species, but rather is the sister group of a clade consisting of species recognized as part of the *carapo* species group of Albert et al. (2004), which are found predominantly in the western Amazon basin. This relationship was well supported, with a bootstrap support value of 99% and a decay index of 19 (Fig. 9). A breakdown of the partitioned Bremer support values reveals that this node is highly supported by the mtDNA and RAG2 datasets at values of 34.5 and 4 respectively, while the morphological dataset contributes no support as indicated by the negative value of the

PBS for this partition. The pantherinus group of Albert et al. (2004) appears in this study as a paraphyletic group in the total evidence tree. Here we refer to the two clades formed by this paraphyletic grouping as G1 and G2 (Fig. 9).

Nodal support was generally high (>90% bootstrap) for the majority of clades (Fig. 9). Exceptions include the placement of *G. pantherinus*, which in the total evidence tree appears as the sister group to a clade consisting of *G. pedanopterus* and *G. cataniapo*. This relationship was support by bootstrap support values of less than 50%. In addition, the sister relationship of the G2 clade to the *cylindricus + carapo* clade showed lower support values than the majority of the other nodes in the total evidence tree with a bootstrap value of less than 70%.

Total Evidence – Cyt b Third Positions Removed

Removal of the third positions of the cytochrome *b* gene resulted in a total evidence dataset composed of 2526 characters, 613 of which were parsimony informative. Phylogenetic analysis resulted in a single most parsimonious tree of 1544 steps (CI=0.59; RI=0.86; RC=0.51). Compared to the total evidence tree in which all cytochrome *b* characters were included, excluding third positions produced one important change in tree topology. Here, the G2 clade, composed of *G. pantherinus*, *G. pedanopterus*, *G. cataniapo* and *G. sp.* aff. *anguillaris*, was positioned at the base of the G1 clade (Fig. 11). This particular relationship is most similar to the "pantherinus" clade delineated in the morphological analysis of Albert et al. (2004). In the tree created with cytochrome *b* 3rd positions removed, *G. pantherinus* is the most basal member of the "pantherinus clade" (supported by 59% bootstrap and a decay index of 0). *G. cataniapo*, *G. pedanopterus* and *G. sp.* aff. *anguillaris*, together are the sister group to the G1 clade

of the initial total evidence analysis (supported by 69% bootstrap and decay index of 0). The position of the Central American species, *G. cylindricus*, has not changed, but in this tree represents the base of the carapo clade (a relationship that is supported by 73% bootstrap and a decay index of 19).

mtDNA

Independent analysis of the mitochondrial dataset resulted in 4 most parsimonious trees, each consisting of 2411 steps (CI = 0.42, RI = 0.79). A strict consensus of these trees (2425 steps, CI = 0.42, RI = 0.79) shows a similar topology to that of the total evidence tree (Fig.12). Both the G1 and G2 clades are present, although, in the latter clade, the relationships between *G. cataniapo*, G. *pedanopterus* and *G. sp.* 2091 are unresolved, creating a polytomy. *G. cylindricus*, the Central American form, was again found to be the sister group of the carapo clade.

RAG2

Independent analysis of the nuclear gene RAG2 produced 8 most parsimonious trees, the strict consensus of which is shown in Figure 13 (489 steps; CI = 0.77; RI = 0.92). While the majority of nodes deep in the tree are well resolved, relationships among species within the major clades are less clear, including portions of the G1, G2, and carapo clades. Within the carapo clade this is evident in the polytomy involving *G. ucamara*, *G. carapo* from the western Amazon, and *G. arapaima*). The Central American clade again appears nested within the phylogeny, creating a paraphyletic South American assemblage. In contrast to both the mtDNA and total evidence trees, the presumed sister group of *Gymnotus* is not the electric eel. In this tree *Electrophorus* is the sister taxon of

the clade comprising members of the Rhamphichthyidae and Hypopomidae. The latter relationship has little support, having been recovered in less than 50% of trees during bootstrap analysis.

Morphology

Independent analysis of the morphological data based on the subset of taxa presented in this study resulted in 150 trees of 305 steps each. A strict consensus of these trees (Fig.14) was topologically different from that of Albert et al. (2004) as a result of the smaller subset of taxa used in this analysis. In this tree, *G. pantherinus* is the sister group of the *carapo* clade, which includes *G. cataniapo* in an unresolved relationship with *G. tigre*, *G. curupira*, and *G. varzea* + *G. obscurus*. *G. pedanopterus* appears as the sister lineage to *G. stenoleucus*. *G. jonasi*, while still a part of the basal lineage of the G1 clade from the total evidence tree, is associated with *G. coropinae* from the western Amazon in the morphological tree. The two geographical forms of *G. coropinae* in this case are paraphyletic. The majority of the clades observed in the morphological tree have very little support from either bootstrap or decay indices, however one of the most well supported relationships is that of the Central American clade (*G. cylindricus*) as the sister group to all other *Gymnotus* species (with a bootstrap value of100% and a decay index of 10). The largest difference in the interpretation of the morphological and total evidence trees presented here is the relationship of *G. cylindricus* to the other forms of *Gymnotus*. In the morphological tree *G. cylindricus* is the sister taxon of all South American forms of *Gymnotus*, as opposed to occupying a more derived position on the tree (Fig.15).

Summary

In all trees, with the exception of those based solely on the morphological data, Central American forms of *Gymnotus*, as represented by *G. cylindricus*, are found to be nested within *Gymnotus* phylogeny, and do not represent the earliest divergence in the *Gymnotus* phylogeny. Other general biogeographic patterns include close connections between species occurring in the western and eastern Amazon (*G. coatesi* and *G. javari*). *G. carapo*, while shown here to occupy a derived position in the tree, is also one of the most widespread species, occurring in many drainages in South America. *G. pantherinus*, a species known from the Atlantic drainages of southeastern Brazil and Uruguay is here shown to be closely related to G. *pedanopterus*, *G. cataniapo* from the Orinoco basin, and the new unidentified species from Guyana (*G. sp.* aff. *anguillaris*). However, little support is given to this relationship in the total evidence tree. The nuclear RAG2 tree, lends greater support (74% bootstrap) to a sister relationship between *G. pantherinus* and the *cylindricus+carapo* clade. Exclusion of cyt *b* third positions from the total evidence analysis produced a phylogeny in which the major clades more closely resemble those found in the morphological analysis, with *G. pantherinus* being the sister group to a clade consisting of *G. pedanopterus*, G. *cataniapo*, *G. sp.* aff. *anguillaris*, *G. jonasi*, *G. javari*, *G. coatesi*, *G. stenoleucus* and *G. coropinae*.

3.3. Dating

Cytochrome b

Tests of rate homogeneity for the *Gymnotus* cytochrome *b* dataset and phylogeny resulted in the rejection of the molecular clock ($\chi^2 = 119.6$, d.f. = 50, $p < 0.001$). When broken down by codon position, rate homogeneity was rejected only for first positions (χ^2

= 89.6, d.f. = 50, $p < 0.001$), but accepted for both second ($\chi^2 = 60.4$, d.f. = 50) and third positions ($\chi^2 = 47.8$, d.f. = 50). The patristic distance (branch length divergences) between *Brachyhypopomus diazi* and *B. sp.* 'palenque' (Table 8) established a rate of divergence of 1.16% per million years for the entire cytochrome *b* gene. When rates were examined by codon position, third positions showed the highest rate (3.10% divergence per million years), while first and second positions were considerably slower (0.36%/my and 0.04%/my respectively).

Table 9 shows the results of all three methods of age estimation for both the Central American – South American split (*G. cylindricus* – '*carapo*' clade) as well as the origin of the genus *Gymnotus* (*Electrophorus electricus* – *Gymnotus*). Estimates of divergence based on patristic distances (branch length distances between species and clades) from the cytochrome *b* data place the divergence of Central American taxa from their South American congeners between 11.7 and 27.2 million years ago (mya). Non-parametric rate smoothing produced slightly older estimates, ranging from 12.9 mya to 30.3 mya depending on the data partition. Examining uncorrected pairwise sequence divergence and applying the standard rate calibration for the fish cytochrome *b* gene (1.2% sequence divergence per million years) produced an age estimate for the Central American-South America split of 10.1 mya. Age estimates for the origin of the genus *Gymnotus* based on the full cytochrome *b* data partition ranged from 20.9 mya to 35.1 mya depending on the dating method.

RAG2

Using the RAG2 tree and dataset, a test for rate heterogeneity using the hierarchical likelihood ratio test failed to reject the null hypothesis of rate homogeneity among lineages ($\chi^2 = 52.5$, d.f. = 50) and as a result it was accepted that the RAG2 sequence data evolved in a clock-like manner.

The rate of divergence for the patristic distance data (Table 10), as established by the *B. diazi – B. sp.* 'palenque' split at 12.9 mya, was 0.17% per million years. This rate produced an age estimate of 10.2 mya for the divergence of Central and South American forms of *Gymnotus* (Table 9). Estimating the divergence of *G. cylindricus* from its South American counterparts using the NPRS method, resulted in an older age estimate of 21.6 mya (Table 9). Using the RAG2 data to assess the age of origin for the genus *Gymnotus* produced age estimates of 69.6 mya (patristic distance method) and 67.1 mya (NPRS method) (Table 9).

When comparing the estimates produced by cytochrome *b* and RAG2 data, the majority gave age estimates for the divergence between the Central American clade (*G. cylindricus*) and the South American clade ('*carapo*' clade) that fall within the Miocene epoch (23.8-5.3 mya). When comparing the age estimates between the two datasets for the origin of the *Gymnotus* clade, there was a larger discrepancy, with cytochrome *b* giving some more recent dates, and RAG2 producing estimates that suggest an origin during the late Cretaceous. This is likely the result of the difficulty that certain mitochondrial genes have in reconstructing older divergences that occur near the base of the tree (Farias et al. 2001, Meyer 1994).

3.4. Character evolution

Optimization of waveform shape on the total evidence tree revealed that monophasic signals in *Gymnotus* likely evolved from a multiphasic ancestor. Figure 16 shows that the monophasic signal of *G. cylindricus* is a reversal from multiphasy to monophasy. Upon breaking down waveform shape evolution based on the number of phases in the electric signal, this result was reiterated when using ACCTRAN optimization (Fig. 17). In this case, the EOD shape of the hypothetical ancestor to the *G. cylindricus* and '*carapo*' clades is shown specifically to be triphasic. However, when reconstructed using DELTRAN optimization (Fig. 18), it was shown that the EOD of this ancestor could equivocally be biphasic, triphasic, or multiphasic.

4. Discussion

4.1. Phylogenetic relationships of Gymnotus species and comparison with previous studies

There are few species-level phylogenetic studies for any gymnotiform taxa. In the case of *Gymnotus* this is likely a result of the failure to recognize the full extent of species diversity within the group until recently. In addition to the comprehensive morphological analysis of Albert et al. (2004), a single molecular study (based on the analysis of 345 nucleotides of the mitochondrial gene ND2) of *Gymnotus* relationships was conducted for four species from the pantanal region of south eastern Brazil, including *G. pantherinus*, *G. sylvius*, *G. carapo*, *G. inaequilabiatus* (Fernandes-Matioli and Almeida-Toledo 2001). The results of the latter phylogenetic analysis suggested that *G. carapo* is a derived species in the *Gymnotus* phylogeny, with *G. pantherinus* occupying a basal position in comparison, which agrees with the results presented here and with those of Albert et al. (2004). The greatest contrast between Albert et al.'s (2004)

morphological analysis and the total evidence analysis presented here is: 1) the relatively nested position of *G. cylindricus*, and 2) the paraphyletic nature of their "pantherinus" clade or "group B" (here recognized as two clades, G1 and G2, but see Fig.11 wherein the pantherinus is restored by the exclusion of cytochrome *b* third positions).

Gymnotus morphological characters used by Albert et al. (2004) that are synapomorphic (i.e. shared characters derived from a common ancestor) for the clade consisting of *G. cylindricus* and the 'carapo' clade include: 1) deep body profile/depth (compared to the slender body depth of all other *Gymnotus*; 2) a single dentary tooth row (as opposed to a patch of teeth); 3) a broad frontal postorbital process; 4) a broad cleithrum (a supporting element of the pectoral fin) 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 electric organ depth, consisting of 4 rows of electrocytes. The sister relationship between the Central American clade and the 'carapo' clade is not completely unexpected as trans-Andean forms of *Gymnotus* (specifically *G. esmeraldas*, *G. henni*, and *G. choco*) are thought to be the sister taxa of species from the Western Amazon belonging to the 'carapo' clade.

The paraphyletic nature of Albert et al's. (2004) "pantherinus" clade (here reperesented as the G1 and G2 clades) is one of the major disagreements between the total evidence and the morphological analyses. However, that portion of the total evidence tree had very little support (from either bootstrap support values or decay indices), especially when compared with some of the other nodes. Also, there are no morphological characters that can be used to unquestionably argue the position of the G2 clade as the sister group to the Central America+'*carapo*' clade., whereas several

morphological characters do support the monophyly of the "pantherinus" clade. These charcters include (but are not limited to) body depth, head length, the shape of the mesocoracoid and cleithrum (supporting elements of the pectoral fin), scale counts, length of the body cavity, shape of the fifth rib, and structure of the electric organ. In particular, fishes of the 'pantherinus' clade (which would include *G. pantherinus*, *G. pedanopterus*, *G. cataniapo* and all those species recognized here as part of the G1 clade) are known to lack the presence of a clear patch at the caudal end of the anal fin (unlike those fish belonging to the carapo clade whose anal fin is darkly pigmented for its entire length) and have a single laterosensory pore on the preopercle (as opposed to two in members of the carapo clade). The latter two characters have often been used to distinguish between the '*pantherinus*' and '*carapo*' clades, even before a formal phylogeny for *Gymnotus* was proposed (Albert and Miller 1995).

While the total evidence position of the G2 clade may reflect a true relationship in disagreement with morphology, it is also possible that this relationship is the result of the saturation of the cytochrome *b* gene. The fact that removal of cytochrome *b* third positions from the total evidence dataset produced a tree in which the position of the G2 clade changed to become part of the G1 clade (Fig.11) may support the idea that the cytochrome *b* gene is saturated, at least at 3rd positions (Fig.8). This change in position restores the 'pantherinus' clade of Albert et al. (2004), and is therefore more congruent with morphological characters. The paraphyly of the "pantherinus" clade might also be explained by long branch attraction (LBA) between the members of the G2 clade (*G. pantherinus*, *G. cataniapo*, *G. pedanopterus* and *Gymnotus sp.* aff. *anguillaris*) and the *cylindricus* + *carapo* clade. LBA is a phenomenon in which the long branches in a

phylogenetic tree are erroneously linked together due to the high degree of convergence or parallelism among their character states (Felsenstein 1978, Hendy and Penny 1989) and is typically characterized in trees by long branches connected by short internodes (Felsenstein 1978, Huelsenbeck 1997). The relatively short internode (27 steps) leading to the long branches of the G2 clade suggests that LBA may be affecting the position of species within the clade. The internode connecting the G2 clade to the *cylindricus* + *carapo* clade has a moderate length (70 steps), which may suggest that LBA is not affecting the positions of these lineages on the tree. The inclusion of more species belonging to each of the *pantherinus* and *carapo* clades should help to divide any long branches and therefore reduce the possibility of LBA within the tree.

In contrast to both the total evidence and mtDNA trees, the nuclear RAG2 tree suggests that *G. pantherinus* alone forms the sister group to the *cylindricus* + *carapo* clade. A closer relationship between *G. cylindricus* and *G. pantherinus* could potentially be inferred based on colour pattern alone, as the two species represent two of the several species (*G. maculosus*, *G. bahianus*, *G. pantherinus*, *G. esmeraldas*, *G. onca*) which do not possess the definitive banding pattern characteristic of all other *Gymnotus* species. *G. pantherinus* (southeastern Brazil) most closely resembles *G. esmeraldas* (Pacific slope Ecuador) and *G. henni* in colour pattern, in that they all lack the alternating dark and light pigment bands on the majority of the body and have a rather mottled appearance with patches of dark pigment on a light background. Including *G. esmeraldas* in the molecular phylogeny could provide better resolution of the true position of *G. pantherinus*, however the two species are dissimilar for many morphological characters outside of colour pattern (Albert et al. 2004) and in this instance the colour pattern character is more likely

the result of convergence. Future studies incorporating more molecular characters and species will likely help to resolve the relationships between *G. pantherinus* and other *Gymnotus* species.

The differing positions of *E. electricus* between the mtDNA and total evidence analyses and the RAG2 analysis could have important implications for the phylogeny of Gymnotiformes. It is currently accepted based on morphological analyses that the electric eel is the sister group to *Gymnotus* and this relationship has been used to argue the placement of the electric eel within the family Gymnotidae (Ellis 1913, Albert 2001). The conflicting position of *E. electricus* between the cytochrome *b* and RAG2 trees could be the result of the low divergence values observed between the electric eel and the outgroup *Hypopomus artedi*. The sequence divergence between these two species for the RAG2 gene is approximately 9.2%, whereas the average genetic divergence between the electric eel and the *Gymnotus* clade is 9.4%. While this finding could be the result of convergence in the RAG2 gene between *E. electricus* and *H. artedi*, it could also reflect faster rates of evolution in the *Electrophorus* lineage than in the *Gymnotus* lineage. The latter view is supported by the long branch leading to *Electrophorus* in the total evidence tree (>200 changes). Alternatively, the changing position of the electric eel could reflect the differences between nuclear and mitochondrial datasets. A better understanding of the position of the electric eel is needed in order to properly establish the true sister group to *Gymnotus*. This would entail a revision of gymnotiform phylogeny, particularly at the molecular level with reference to multiple nuclear and mitochondrial genes.

4.2. Historical Biogeography

4.2.1 Central American – South American Biogeography

The rise of the Isthmus of Panama has had profound effects on the intercontinental exchange of organisms. The focus of the biogeographic and evolutionary events surrounding the development of the Isthmus of Panama has concentrated largely on terrestrial mammals, (Marshall 1988, Marshall et al. 1979, Marshall et al. 1997, Webb 1991) and geminate species pairs of marine fishes (Bermingham et al. 1997) and invertebrates (Beu 2001, Knowlton et al. 1993). While authors such as Myers (1966) and Bussing (1985) highlighted the importance of the Central American land bridge to the dispersal of freshwater fish species, detailed analyses of their phylogenetic relationships to South American taxa have only more recently become available (Bermingham and Martin 1998, Perdices et al. 2002, Perdices et al. 2005, Martin and Bermingham 1998).

The topologies of all trees presented in this study (total evidence, nuclear, and mtDNA) show that the Central American species of *Gymnotus* are not the sister group to all other forms of *Gymnotus* (i.e. South American forms), but rather occupy a more derived position on the tree. Also, based on estimates of divergence derived from the cytochrome *b* gene, Central American forms of *Gymnotus* (represented here by *G. cylindricus*) are presumed to have an origin sometime during the Miocene, approximately 16.7-10.1 mya. This Miocene estimate was further supported by dating estimates based on the nuclear RAG2 gene, which dated the split between South and Central American taxa to approximately 21.6-10.2 mya. This is a good indication that the Central American gymnotid fauna may be more recently derived than proposed by Bussing (1985) and that the timing of the invasion of Central America by a gymnotid ancestor is more recent than proposed by the placement of *Gymnotus* in Bussing's "Old Southern Element".However,

the dates based on the molecular divergence of the South and Central American taxa suggest that *Gymnotus* is also older than predicted Miller (1966) and Myers (1966), and therefore older than the earliest emergence of the Isthmus of Panama. If the estimate reflects the true timing of these events, then *Gymnotus* must have invaded Central America prior to the completion of a terrestrial land bridge connection between North and South America, indicating that some oceanic dispersal may have taken place.

Unlike cichlids, which are secondary freshwater fishes (some members being tolerant of varying degrees of salinity), all gymnotiforms are considered to be primary freshwater fishes and are thus thought to be intolerant of salt water, even at low salinity levels (Myers 1949). This physiological intolerance to salt water is demonstrated by the fact that not a single species of *Gymnotus* is known to occupy estuarine habitats. This is likely also a result of the sensitivity of the electric organ of these species to environments with high salinity. In seawater, the electrosensory system would effectively be short-circuited, rendering all functions associated with this sensory adaptation useless (Stoddard 2002a). As electric knifefish rely on their electrical currents for navigation, foraging, and communication, it is difficult to understand how they may have survived in a marine or estuarine environment. There are examples of fish species from a number of families dispersing through marine or brackish waters, including some primary freshwater fishes (Darlington 1957). The dispersal of primary freshwater fish across marine barriers is infrequent, but is thought to have led to range extensions in some species such as the peamouth chub, *Mylocheilus caurinus*. An ostariophysan (like *Gymnotus*) and a member of the family Cyprinidae, this species is thought to have dispersed from the coast of mainland British Columbia to reach rivers and lakes on

eastern Vancouver Island (Clark and McInerney 1974). In the process the fish are thought to have crossed the Straight of Georgia, a body of saltwater 48km wide whose salinity is reduced in comparison to the open ocean due to the large number of rivers (the Fraser River in particular) discharging freshwater into the region.

The pre-Pleistocene age estimates obtained here for the Central American *Gymnotus* species lend support to a third hypothesis concerning the timing of Central American colonization by *Gymnotus*, which is that oceanic dispersal from South America to Central America may have been facilitated by the freshwater outflow of the palaeo-Amazonas-Orinoco river during the Miocene (~14 mya) (Albert et al. 2006, Albert et al. 2004). During the Miocene, prior to the rise of the Merida Andes in northern Venezuela, the drainage of many rivers in the low-lying portions of northwestern South America occurred from south to north (Hoorn et al. 1995) forming a large river which flowed from an area within the modern day western Amazon Basin into the proto-Caribbean Sea at a point approximating the locality of what is now the Maracaibo basin (Fig. 19). Albert et al. (2004) suggest that the outflow from this waterway would have caused a large influx of freshwater into the area, much like the Amazon River's current outflow which results in a freshwater plume 6700 km^3 which is then distributed by the Southern Equatorial current along the east coast of Brazil and the Guianas (Goulding et al. 2003).The freshwater outflow of rivers emptying into the proto-Caribbean sea during the mid-Miocene could have been distributed westward in a similar manner by the Circumtropical current which promoted the movement of ocean waters from the Atlantic to the Pacific (Droxler et al.1998, Mullins et al. 1987), toward the developing Central American isthmus. While the Isthmus of Panama was incomplete until the Pliocene (~3.5 mya) it is

thought to have consisted of a volcanic arc ~16 mya, with fully developed volcanic islands being established ~12 mya (Coates et al. 2003). Based on the presence of certain foraminiferan assemblages on the Atlantic side of the isthmus which were absent on the Caribbean side, Duque-Caro (1990b) went so far as to suggest that middle Miocene tectonic events, which resulted in the partial subaerial emergence of the isthmus, was extensive enough to develop a circulation barrier between the Atlantic and Pacific oceans (at least 10 million years prior to the Pliocene closure of the Panamanian seaway). An alternative to the proto-Orinoco origin of the Central American lineage, but that may have involved a similar process, is the colonization of Miocene volcanic island(s) by a gymnotid ancestor occurring in what is now the area of the Atrato and Magdalena river basins in northwestern South America. Rivers draining northwestern Colombia would have compounded the freshwater effects of the palaeo-Orinoco drainage on the southern Caribbean Sea, and could have provided the necessary dispersal routes.

While the Miocene divergence of Central American *Gymnotus* may reflect the time period of the colonization of Central America, it may alternatively reflect divergences based on tectonic events related to the rise of the Andean Cordillera. Here it has been assumed that the divergence of *B. palenque* and *B. diazi* was a consequence of the uplift of the Eastern Cordillera 12.9-11.8 mya. This vicariance event has also played a role in the diversification of *Gymnotus*, and several trans-Andean species are known (Albert et al. 2004). It remains a possibility that divergence occurred between trans-Andean forms and their cis-Andean counterparts 12.9 mya, and that the trans-Andean clade then gave rise to the Central American forms of *Gymnotus* via a more recent colonization of the area. Isolation of trans-Andean lineages in northwestern South

America after the uplift of the Eastern Cordillera, followed by more recent dispersal over the completed Isthmus of Panama (~3.1-2.9 mya) could explain the divergence estimates obtained for Central American *Gymnotus*. In order to completely exclude this possibility with regards to *Gymnotus* phylogeny, further molecular phylogenetic and biogeographic studies are needed on the *Gymnotus* species occurring in Pacific drainages (*G. henni*, *G. choco*, *G. esmeraldas*) and the Magdalena drainage in northern Colombia (*G. ardilai*), as well as Panama, in relation to those in both Central America and cis-Andean South America. If further analysis were to discover that any of these species are the sister taxon of the Central American clade (and not the sister taxa of members of the '*carapo*' clade as suggested by the morphological analysis of Albert et al. 2004), a more recent date of divergence between South and Central American forms would be inferred (as the latter divergence would post-date the divergence of trans-Andean forms 12.9 mya). Currently, morphological characters indicate that *G. choco* and *G. henni*+*G. esmeraldas* are the sister taxa of *G. ucamara* and *G. tigre* respectively. The morphological study of Albert et al. (2004), while inferring that Central American gymnotids diverged at the base of the *Gymnotus* tree, suggested that Central American colonization occurred prior to the Pleistocene rise of the Panamanian Isthmus, which agrees with the results obtained by this study.

Although the potential Miocene age of the colonization of Central America by *Gymnotus* is inconsistent with the hypotheses of both Bussing (1985), and Miller (1966) and Myers (1966), it is congruent with age estimates based on the divergence of the cytochrome *b* gene of Mesoamerican cichlids (Martin and Bermingham 1998, Perez et al. 2007). Central American cichlids are thought to have diverged from their South

American congeners between 15 and 24 mya, having crossed the oceanic barrier between South and Central America. However, a more recent study suggests that these cichlids may have colonized Central America during the Late Cretaceous (55-66mya) (Chakrabarty 2006).

Other taxa with which *Gymnotus* may share a common biogeographical history include swamp eels of the genus *Synbranchus* (Perdices et al. 2005) and túngara frogs belonging to the species *Physalaemus pustulosus* (Weigt et al. 2005). Phylogenetic analysis of swamp eels supports two distinct invasions of Central America by *Synbranchus*, one occurring 22.0-27.4 mya and the other more recently at 7.7-12.4 mya. The latter date, which is more in agreement with the results obtained here, represents the divergence between *Synbranchus* in northern South America and Lower/Middle Central America (Perdices et al. 2005). The authors suggest that the split may have been the result of the establishment of the modern Orinoco during the late Miocene (7-8 mya). Túngara frogs are hypothesized to have entered lower Middle America during the Miocene, approximately 9 mya, possibly via raft dispersal (Weigt et al. 2005). Central American forms of the bushmaster (*Lachesis muta*) are also thought to have diverged from their South American counterparts during the Miocene, approximately 18-6 mya, prior to the completion of the Isthmus (Zamudio and Greene 1997).

Interestingly, the Middle to Late Miocene estimates of species divergence obtained between many of these South and Central American species, fall within a period of time (14.2 – 10.2 mya) during which global sea levels reached some of their lowest levels during the Cenozoic (Haq et al. 1987). At 10.2 mya sea levels experienced a particularly large drop, to the lowest levels of the Miocene. While there is no evidence

suggesting that the decline in sea level during the Late Middle to Late Miocene may have resulted in terrestrial links between North and South America, these events, in combination with the beginnings of tectonic building in the area, would have at least increased subaerial exposure of whatever landmasses were in existence (volcanic islands), possibly to a point where the distances between river mouths and/or freshwater plumes on the South American continent and the emerging landforms in the area of what is now Central America were reduced enough to facilitate the dispersal of certain freshwater organisms.

4.2.2. South American Biogeography

The South American freshwater fish fauna is the most diverse in the world and currently comprises some 6000 species, with many still to be described (Reis et al. 2003). Several hypotheses concerning the evolution of the highly diverse South American fauna have been postulated, with particular emphasis on the diversification of terrestrial vertebrates within the Amazon basin (reviewed in Haffer 1997). Some groups of South American fishes have shown definitive patterns in terms of area cladograms (trees in which geographic areas are substituted for species names), typically with species/clade boundaries coinciding with the major river basins of the continent (e.g. species of the characiform genus *Prochilodus*, Sivasunder 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. The latter phenomenon is exemplified by the relationship between *G. pantherinus* (a species from southeastern Brazil) and the clade including *G. pedanopterus* and *G. cataniapo*, (both of which are found in the Orinoco drainage). While some of the

diversity of the Amazon is often attributed to events occurring during the Pleistocene (refuge hypothesis) or late Miocene (~5 mya, museum hypothesis), the preliminary estimates of the ages for the speciation events that have resulted in the current diversity observed in *Gymnotus* date to early and middle Miocene, with only the most recent clades being established during the late Miocene/early Pleistocene. This agrees with the view that much of the fauna of South America, and particularly the Amazon, were to a large extent modern during the late Miocene, with many of the families known today already well-established (Gayet and Meunier 1998, Lundberg et al. 1998).

The placement of *G. pantherinus* in the total evidence versus the nuclear RAG2 trees presents two interesting biogeographical hypotheses. In the total evidence tree, *G. pantherinus*, which is endemic to the Atlantic drainages of southeastern Brazil and Uruguay, is shown have a close relationship to a clade consisting of species found exclusively in the Guiana shield region of South America. The shield terraines of South America may have served important roles in the evolution of many South American faunas, as they are two of the few landmasses to have remained above sea level throughout much of the geological history of the continent (Lundberg et al. 1998). In more recent times these two landmasses have been separated from one another by the Amazon river. It is thought that the Amazon, and other large South American rivers, may play important roles in isolating certain faunas by acting as barriers between populations (Gascon et al. 2000 and references therein). In Brazil, the width of the Amazon ranges between 1-6 kms during the dry season and in some places can reach over 60 kms during the rainy season (Mertes et al. 1996). The large width and depth (averaging 10-20 m mid-channel during the dry season) of the river have been postulated as a barrier to gene flow

(Gascon et al. 2000). While the Amazon more than likely acts as a dispersal route for aquatic species, it is possible that this potential barrier, which began forming during the middle to late Miocene as the result of Andean uplift (Hoorn 1994), is the cause for the divergence between *G. pantherinus* and the Guiana shield species. There is, however, a single record of *G. pantherinus* from coastal streams in Guiana (Westby 1988). It is not yet known whether the populations of *G. pantherinus* in Guiana and southeastern Brazil are the result of dispersal across the mouth of the Amazon or via the vicariant separation of a formerly contiguous population (perhaps as a result of the formation of the Amazon). Dating of the node connecting *G. pantherinus* to *G. pedanopterus*+*G. cataniapo*+*G. sp.* aff. *anguillaris* using the patristic distances method places the split at 15.8 mya for the cytochrome *b* gene, while the non-parametric rate smoothing (NPRS) method of Sanderson (1999) dates this node as 18 mya for the cyt *b* tree. Both of these dates indicate that the divergence of these taxa predates the establishment of the modern Amazon River drainage. It is possible that *G. pantherinus* may be connected to *G. pedanopterus*+*G. cataniapo* through species in the intervening areas between southeastern Brazil and the Guiana shield that either we were unable to include in this study, or that have gone extinct. However, this hypothesis is not well supported due to the lack of bootstrap or Bremer support for the relationship of *G. pantherinus* to the other members of the G2 clade (*G. pedanopterus*+*G. cataniapo*+*G. sp.* aff. *anguillaris*).

Examination of the position of *G. pantherinus* in the nuclear RAG2 tree, reveals its potential sister relationship to the *cylindricus*+*carapo* clade, a relationship that is supported to a greater degree than in either the total evidence or mtDNA trees. This relationship could indicate a close link between species from the Atlantic drainages of

Brazil and the western Amazon. There are two pathways through which this type of relationship might be possible: 1) connections through the mouth of the Amazon, or, 2) connections to the Amazon through the Parana/Paraguay system, which has also shared connections to Atlantic drainages through river capture in the headwaters of rivers in this region (particularly between the São Francisco and Paraguay drainages) as evidenced by the presence of *G. sylvius* and *G. inaequilabiatus* in both systems. This pattern could therefore involve sister group relationships between *G. pantherinus* and species from the Parana/Paraguay system, likely *G. pantanensis* or *G. paraguensis*. While the drainage basins of each of these areas are now clearly separated, fossil evidence from potamotrygonid stingrays indicates that there may have been a period during the Miocene when fluvial connections existed between the Parana and paleo-Amazonas-Orinoco systems (Brito and Deynat 2004). The structural divide which now separates the two drainages (Chapare Buttress) is thought to have arisen in the early Miocene (approximately 30-20 mya years ago) as a result of contact between the western edge of the Brazilian shield and the Andean thrust front (Lundberg et al. 1998). The node connecting *G. pantherinus* to the *G. cylindricus* + *carapo* clades in the RAG2 tree is dated at 17.4 mya and 29 mya using the patristic distance and NPRS methods respectively for the RAG2 gene, suggesting that the aforementioned vicariant event separating the Parana and paleo-Amazon-Orinoco may have influenced the divergence observed between the two groups. However, this scenario would be dependent on a sister relationship between *G. pantherinus* and members of the genus from the Parana/Paraguay system. The morphological analysis of Albert et al. (2004) shows an unresolved relationship between these species, however, one Parana/Paraguay species (*G.*

pantanensis) is shown to be part of the same species group as *G. pantherinus*. While a Miocene date for this event is confirmed in other groups of freshwater fish, including the loricariid catfishes of the genus *Hypostomus* (Montoya-Burgos 2003), present drainage patterns for the Parana and Paraguay systems are thought to have established themselves at an earlier date during the Oligocene, approximately 30 mya (Lundberg et al. 1998).

Despite the uncertainty surrounding the relationship of *G. pantherinus* to other species within the genus, it is perhaps more certain that this species represents one of the oldest lineages of *Gymnotus*, as evidenced by the high sequence divergence observed between it and the other members of the genus. The isolation of *G. pantherinus* in the coastal drainages of Southeastern Brazil likely coincides with the erosion of the eastern margin of southeastern Brazil, which can cause the introduction of species from upland rivers into those along the coast (Ribiero 2006). It is therefore difficult to associate the timing of the divergence of *G. pantherinus* to geological events, as erosional events take place continuously over time.

Diversification within each the G1 clade and the '*carapo*' clade, both of which are composed predominantly of Western Amazon species, may have begun approximately during the late Miocene (11.7 – 6.7 mya, for G1, 9.3 – 8.2 mya for the '*carapo*' clade, with the ranges encompassing values for both the NPRS and patristic distance measures, for each of the cyt *b* and RAG2 genes). These dates agree with the geological evidence that suggests that the establishment of modern várzea (whitewater floodplain) habitats occurred approximately 12 mya (Hoorn et al. 1995). Many of the species in each of the G1 and G2 clades make use of varzea habitats, which have likely helped to promote and maintain species diversity within the genus.

While some biogeographic patterns/information relating to South American waterways can be extracted from the phylogeny presented here, the overall arrangement is one in which no definitive pattern of relationships truly exists between drainage basins and species. Sister relationships between species and even within species (such as *G. carapo* and *G. coropinae*) exist across drainage divides, a clear indication of the ancient nature of the genus *Gymnotus* and of the family Gymnotidae. The importance of the Amazon Basin to the diversification of *Gymnotus* species is likely two-fold. It has provided the opportunity for speciation within the basin (as evidenced by the paraphyletic nature of *G. carapo*, and its relationships with *G. ucamara* and *G. arapaima*), and it has provided an area in which species from many lineages may accumulate (as evidenced by the presence of western Amazonian species in the two main clades of the *Gymnotus* phylogeny).

4.2.3. Biogeography, Dating, and rates of evolution in Gymnotus

While the dates presented here appear conclusive, particularly for the divergence of Central American and South American species, the possibility remains that the evolutionary rates within *Gymnotus* lineages are higher than for other freshwater fishes for which more recent dates have been estimated for the same biogeographic events. For example, Pliocene estimates of divergence between Central and South American lineages have been established for several groups including neotropical rattlesnakes (Wüster et al. 2005), parrots (Eberhard and Bermingham 2004), and most importantly freshwater fishes (Bermingham and Martin 1998, Perdices et al. 2002).

The highly complex biogeography of *Gymnotus* is likely influenced by other factors, including habitat use and their ability to use electric signals for species

recognition. *Gymnotus* shows widespread use of habitats in South America, including terre firme streams and the floodplains of whitewater and blackwater rivers. Electric signaling would have enabled the widespread distribution of fishes from several lineages, while maintaining reproductive barriers.

4.3. Electric signal evolution in Gymnotus

The ability to receive and process signals in the environment is crucial to the survival of any species. While many fish are known to possess receptors that enable them to detect electrical currents in their respective environments (Bullock 1982), others, including mormyrid elephantfishes, malapterid catfishes, electric stargazers (*Astroscopus* and *Uranoscopus*) and some elasmobranches (skates and rays in the genera *Raja*, *Torpedo*, and *Narcine*), have independently evolved the capacity to produce electrical fields. Gymnotiform fishes, including all members of *Gymnotus* are capable of both detecting and producing species-specific electrical signals which can be used for electrolocation and communication. In *Gymnotus* these signals are emitted by electrocytes, which are embryonically derived from hypaxial muscle cells and are arranged in series and in parallel to form an electric organ (Bennett 1971). Electrocytes generate electrical potentials by exploiting ionic gradients across cell membranes in a similar manner to nerve and muscle cells. Species specific signals result from the combined interaction of several physiological factors including the pattern of innervation of the electrocytes, the distribution of ion channels and number of excitable membranes in the electrocytes, in addition to the number of electrocytes that comprise the electric organ (reviewed in Bass 1986). The electrical discharge of the electric organ (EOD) can be detected using electrodes, amplified, and recorded to an oscilloscope. Recorded

signals can be categorized as monophasic or multiphasic depending on the shape of the electrical waveform. The fish are also equipped with two types of electrical receptors (modified from lateral line pores), ampullary and tuberous, which are used to detect the electrical fields of other organisms/objects and changes in their own electrical fields respectively (Bullock 1982, Castelló et al. 2000, Zakon 1986). .

The evolution of electric signals appears, for the most part, as a progression from monophasic to multiphasic signals. There are several lines of evidence supporting the assumption that monophasic electric signals are plesiomorphic (ancestral). First, in all cartilaginous fishes in which the ability to produce electrical signals is observed, the electric organ is a form in which electrocytes are innervated only at their anterior/posterior end causing the electrical current generated by the action potential of the neuron to propagate in a single direction (Alves-Gomes 2001, Bennett 1971). As a result of this 'simple' design, the shape of the electric signal waveform is monophasic. Secondly, during the ontogeny of many species whose signal waveforms have more than a single phase (including members of *Gymnotus*), the signal progresses from monophasic to multiphasic as the organisms increase in size and the adult electric organ develops (Crampton and Hopkins 2005, Franchina 1997, Kirschbaum 1977). Strong support for monophasic signals as the ancestral state in electric fishes can also be derived from the molecular phylogeny of mormyroid fishes (Gymnarchidae + Mormyridae). The mormyroid phylogeny clearly shows that *Gymnarchus niloticus,* whose electric signal is monophasic, is the sister group of all other mormyroids (Sullivan et al. 2000, Alves-Gomes and Hopkins 1997). These observations have influenced the phylogenetic hypotheses not only for *Gymnotus*, but for all gymnotiform fish, as several species which

possess monophasic signals, *Electrophorus electricus*, *Gymnotus cylindricus* and *G. maculosus*, are thought to be basal members of the gymnotiform tree (Albert 2001, Albert and Campos-da-Paz 1998, Albert and Fink 1996; but see Alves-Gomez et al. 1995 in which it is suggested that either the Sternopygidae or the Gymnotidae could represent the sister group to all other gymnotiform fishes). The phylogenetic analysis presented here reveals that *G. cylindricus* from Central America occupies a more derived position in the *Gymnotus* tree and that its monophasic EOD is therefore likely to be a reversal to the ancestral EOD state.

In order to understand what evolutionary pressures may have influenced the reversal to (or retention of) a monophasic EOD in *G. cylindricus*, it is necessary to consider those factors that have potentially promoted the evolution of multiphasic EODs. Most prominent in the literature today is the predator avoidance hypothesis (Stoddard 1999, Stoddard 2002a, Stoddard 2002b), which is the result of a theory in animal communication which suggests that signals used for communication should reflect a compromise between the generation of a signal which can be effectively detected by receivers (note that in the case of electric fish such as gymnotiforms and mormyriforms, signalers are also receivers of their own signals for the purposes of electrolocation), while remaining cryptic to potential predators (Bradbury and Vehrencamp 1998). Stoddard's hypothesis suggests that biphasic and multiphasic EODs have evolved as the result of the sensitivity of electroreceptive predators, such as the electric eel (Westby 1988) and pimelodid catfish. The detection of electrical signals by predators has also been observed in Africa, where the sharptooth catfish, *Clarias gariepinus*, is known to exploit the electric signals of male mormyrids of the genus *Marcusenius* for the purposes of

predation (Hanika and Kramer 2000, Hanika and Kramer 1999). In South America, Gymnotiformes are known to comprise a large portion of the diet for many large catfishes (Duque and Winemiller 2003) and these predators are thought to be capable of detecting low frequency EODs in the range of 8-30 Hertz (Stoddard 2002). In contrast the multiphasic EODs of most gymnotiforms often result in signals which have higher frequencies that are outside the range of detection (>30 Hertz) (Stoddard 2002).

Gymnotus cylindricus and *G. maculosus* do not occur sympatrically with either the electric eel or large pimelodid catfish, whose distributions are restricted to South American drainages. The main predators of *Gymnotus* in Central America are likely catfish from the genus *Rhamdia*. Little is known regarding the sensitivity of the ampullary electrical receptors of the fish in this genus (or even the family Pimelodidae), however, many catfish are assumed to be able to detect signals in the 0-30 Hz range (Peters and Buwalda 1972). The monophasic signals of *G. cylindricus* are recorded as having frequencies <10Hz, which places the signals within the range of frequencies that the ampullary receptors of catfish are known to detect. The persistence of monophasic signals in Central American gymnotids may indicate that the ampullary receptors of their predators are adapted to receive signals that are outside of the range of those produced by *Gymnotus*, and therefore outside of the generally accepted range for catfish. Alternatively, predation pressure from catfish may in general be lower in Central America than in South America due to the reduction in the number of potential electrosensitive predators.

While predation pressure in Central America may be equal to that of South America, the regenerative abilities observed in gymnotiform fishes likely allow many

adult individuals to avoid predation. Predation attempts may be less successful due to the smaller size of the predators found in Central American watersheds. The main predators of *Gymnotus* in Central America are likely catfish from the genus *Rhamdia*, the largest of which may reach 50 cm in length (Gomes et al. 2000). Predation attempts on knifefish do not always result in the mortality of the individual as they are capable of regenerating the caudal portions of their bodies, up to 1/3 of their body length (Baillet-Derbain 1969). This ability could function to satiate predators without removing individual knifefish from the breeding population. In South America, the abundance of knifefish has led to the evolution of at least two species (*Magosternarchus raptor* and *M. duccis*) that are adapted to feed solely on the tails of conspecifics and other knifefishes (Lundberg et al. 1996). A similar situation may exist in Central America in which the catfish predators may prey on knifefish tails as opposed to the entire fish, and as a result, further reduce predation pressure on Central American gymnotids. This scenario is perhaps more plausible when considering the discrepancies in the size and the number of species of large predatory catfishes between South and Central America.

It has been suggested that the presence of predators may drive diversification in some species (as observed with guppies, Langerhans et al. 2004) and in some cases may affect signals involved in communication (Cummings et al. 2003). In relation, the evolution of organisms in the absence of major predators has been observed to be accompanied by the reduction, or loss, of adaptations that are involved in avoiding predation (Magurran 1999). This may also apply to *G. cylindricus* and the loss of multiphasic signal, however, it might then be expected that other traits associated with predator avoidance (such as nocturnal behaviour) would also be lost, and in this case,

they are retained. The loss of multiphasic signals in *G. cylindricus* could imply that the maintenance of these types of signals is more energetically costly than for monophasic signals. However, as of yet, there has been little research concerning the energetic costs of electric organ discharges among gymnotiforms (Hopkins 1999), much less among species of *Gymnotus* or comparing amongst different signal types.

There are few South American electric fish which have retained/reversed to monophasic EODs. While *G. obscurus* possesses an EOD which verges on being monophasic, the signal waveform still retains a small secondary negative peak, making it a somewhat intermediate form between the monophasic signal of the electric signal and truly biphasic species (such as many of the species from the family Hypopomidae). The only species of electric fish known to date from cis-Andean South America that possesses a truly monophasic signal (outside of the electric eel), is a member of the family Hypopomidae, *Brachyhypopomus* sp. ("*monophasus*"). It has been suggested that this species developed a monophasic signal as a form of Batesian mimicry, in which it mimics the resting EOD of the sympatric electric eel, thereby avoiding predation (Stoddard 1999). It is possible that the ancestor of the Central American species of *Gymnotus* evolved its signal in a similar manner, and due to reduced predation pressure in Central America, the lineage has been able to maintain the monophasic waveform. However, the character optimizations presented here (Figs. 16, 17, 18) show that the ancestor of *G. cylindricus* was likely multiphasic, and therefore the monophasic signal observed in the latter species is due to the loss of any additional phases the ancestor may have possessed.

If sternopygids can be confirmed as the base of the gymnotiform tree, as shown by Alves-Gomes et al. (1995), then the loss of a multiphasic EOD would have occurred twice during the history of gymnotids in each of the *Electrophorus* and *G. cylindricus* lineages. Alternatively, if gymnotids are the basal lineage within the gymnotiform phylogeny (as suggested by the morphological analysis of Albert 2001), it is possible that the monophasic signal of *E. electricus* simply represents the retention of the primitive character state. In either case (loss of multiphasic vs. retention of monophasic) the cause is likely the electric eel's ability to defend itself against predators using high amplitude electrical impulses (up to 600V).

Although the predominant mechanism of the change from mono- to multiphasic signals may relate to the electrical receptivity of their predators, there are also other aspects of evolutionary and behavioural biology that have likely contributed to the change. One important behavioural aspect would be sexual selection. If multiphasic signals have been the result of an evolutionary attempt to avoid predation, there would likely have been a corresponding shift in the preferences of signal receivers (females and males) for the more complicated signals (Endler 1992). This would entail morphological or physiological changes at the level of the electroreceptors, particularly the tuberous receptors which are tuned to detect the high frequency signals of other electric fishes. Unlike the obvious sexual differences observed in the electric signal waveforms of hypopomid knifefishes (males that produce biphasic EODs exhibit a reduction in the amplitude of the second, head-negative, phase of the waveform which is also longer in duration than that observed in females and is associated with morphological differences in the electrocytes of males and sex differences in sodium and potassium currents,

Hopkins et al. 1990, Stoddard 2006, Stoddard et al. 1999) there is no evidence that the signals of male and female *Gymnotus* differ. This may suggest that sexual selection in this family can only be observed in the signal during the breeding season or specifically during mating encounters, or that the difference exists on so fine a scale that it goes unobserved in recorded signals.

Chapter 3: Conclusion

The phylogeny of the genus *Gymnotus* presented here, resolves some relationships that have not been observed in previous studies of the group. In particular the more derived position of the Central American *G. cylindricus* and its estimated divergence time during the middle to late Miocene results in new interpretations of biogeography and electric signal evolution for both *Gymnotus* and other gymnotiforms.

The complex evolution of the geological landscape in the Neotropics has contributed to the development of a vast diversity of organisms that has yet to be fully understood. However, geological phenomena alone do not always explain the relationships among species, as is demonstrated in the *Gymnotus* phylogeny. The molecular phylogenetic analysis confirms previous hypotheses that unlike their African electrogenic counterparts the mormyroids, evolution within the South American knifefish fauna has been long and complex, with species accumulating over many millions of years.

Within gymnotiform fishes this diversity is further complicated as a result of their electrosensory system. The species recognition capabilities of the electrosensory system of these fish likely provides a pre-mating isolation barrier which in turn helps to maintain the cohesiveness of populations separated by large distances (as exemplified in this study by *G. coropinae* from the western Amazon and *G. coropinae* from the Guyana/Orinoco region).

In general the biogeographic relationships of *Gymnotus* in the present study are not very telling about the tectonic history of South America. Many relationships span multiple basins and sister relationships occur between species from non-adjacent drainages. The latter may be resolved by future studies which include more species,

particularly from rivers occurring south of the Amazon in western and eastern Brazil, Bolivia and Argentina. However, the varied relationships between fishes in different drainage systems also hint at the interconnectedness of river basins over the long history of the South American continent.

Future studies on the biogeography of *Gymnotus* should also focus on population level genetics. It is likely that the group is characterized by widespread morphologically cryptic species, which may show high levels of molecular divergence. As a result, applying geography to an understanding of population level relationships may be more comparable to other Neotropical fishes, particularly within cis-Andean basins.

As the number of studies addressing the colonization of Central America by South American organisms continues to rise, it is becoming increasingly evident that a portion of the colonization events may have occurred prior to the emergence of the Isthmus of Panama and may therefore have involved some form of oceanic dispersal. Interestingly, many of the organisms for which oceanic dispersal has been invoked are typically tied to freshwater in some way (as it is the medium in which they live or breed). While the idea of oceanic dispersal is certainly not a new one, having been suggested on several occasions by Darlington (1937, 1957) and most recently reviewed by de Queiroz (2005), it has not always been fully considered in the past, particularly with respect to the colonization of Central America (as seen in Miller (1966), Myers (1966) and Bussing (1985)). The combined effects of emerging landmasses (as a result of tectonic activity and lower sea levels) and the possible influx of freshwater into the region of the Panama straits may have provided these organisms with the geographical and ecological means necessary for dispersal. However, the possibility that Central American fish are the

descendants of an ancestral stock that was isolated in the coastal regions of northwestern South America as a result of Andean tectonic activity and/or marine incursions during the Miocene cannot be discounted. In light of the intolerance of electric fishes to changes in salinity, the latter scenario may in fact be more plausible than suggestions of oceanic dispersal.

The importance of the Miocene period to the diversification of Amazonian faunas is now being recognized (Hardman and Lundberg 2006). Further research into the biological and geological history of South and Central America should give a better understanding of the processes that have helped shape the diversity of the region. In order to better understand these processes as they relate to gymnotiform fishes and the Neotropical ichthyofauna in general, more studies are needed at both the species and population levels. Specifically, an in-depth phylogeographic study of *G. carapo* would shed light on the relation of cis-Andean drainage basins, as it is the most widespread species of *Gymnotus*. Adding species that have not been included here, as well as undertaking phylogeographical studies of different populations within a number of species would allow a more detailed analysis of the biogeography of both cis- and trans-Andean South America.

Tables

Table 1. *Gymnotus* species formally recognized in scientific literature.

Species	Citation
G. anguillaris	Hoedeman (1962)
*G. arapaima**	Albert and Crampton (2001)
G. ardilai	Maldonado-Ocampo and Albert (2004)
G. bahianus	Campos-da-Paz and Costa (1996)
*G. carapo**	Linnaeus (1758)
*G. cataniapo**	Mago-Leccia (1994)
G. choco	Albert and Crampton (2003)
*G. coatesi**	LaMonte (1935), Crampton and Albert (2004)
G. coropinae	Hoedeman (1962), Crampton and Albert (2003b)
*G. curupira**	Crampton et al. (2005)
*G. cylindricus**	LaMonte (1935)
G. diamantinensis	Campos-da-Paz (2002)
G. esmeraldas	Albert and Crampton (2003)
G. henni	Albert and Crampton (2003)
G. inaequilabiatus	Valenciennes (1842)
*G. javari**	Albert and Crampton (2003)
*G. jonasi**	Albert and Crampton (2001)
G. maculosus	Albert and Miller (1995)
*G. mamiraua**	Albert and Crampton (2001)
G. melanopleura	Albert and Crampton (2001)
*G. obscurus**	Crampton et al. (2005)
G. onca	Albert and Crampton (2001)
G. panamensis	Albert and Crampton (2003)
G. pantanal	Fernandes et al. (2005)
*G. pantherinus**	Steindachner (1908)
G. paraguensis	Albert and Crampton (2003)
*G. pedanopterus**	Mago-Leccia (1994)
*G. stenoleucus**	Mago-Leccia (1994)
G. sylvius	Albert and Fernandes-Matioli (1999)
*G. tigre**	Albert and Crampton (2003)
*G. ucamara**	Crampton et al. (2003)
*G. varzea**	Crampton et al. (2005)

*Species included in this analysis

Table 2. List of specimens included for study detailing tissue lots, field collection numbers, and collection locality.

Genus	Species	Tissue/DNA Lot*	Field/EOD Number/ Museum Number	Locality
Brachyhypopomus	sp. "palenque"	2432	T28	Rio Palenque, Ecuador
Brachyhypopomus	*brevirostris*	A18	01.270301	Tefé, Estado Amazonas, Brazil
Brachyhypopomus	*diazi*	305	NRL-305	Rio Los Marias, Venezuela
Brachyhypopomus	*diazi*	2408	SCW-1197	Móron, Estado Carabobo, Venezuela
Electrophorus	*electricus*	A17	01.220301	Tefé, Estado Amazonas, Brazil
Electrophorus	*electricus*	2026	02.090301	Tefé, Estado Amazonas, Brazil
Gymnotus	*arapaima*	2002	01.170401	Tefé, Estado Amazonas, Brazil
Gymnotus	*arapaima*	2003	03.010799	Tefé, Estado Amazonas, Brazil
Gymnotus	*carapo* (OR)	2040	NR02.020403	Rio Guaratico, Estado Apure ,Venezuela
Gymnotus	*carapo* (OR)	2041	08.020403	Rio Guaratico, Estado Apure ,Venezuela
Gymnotus	*carapo* (CA)	2004	02.300699	Tefé, Estado Amazonas, Brazil
Gymnotus	*carapo* (CA)	2030	03.300699	Tefé, Estado Amazonas, Brazil
Gymnotus	*carapo* (WA)	2006	03.270502	Iquitos, Peru
Gymnotus	*carapo* (WA)	2007	06.270502	Iquitos, Peru
Gymnotus	*cataniapo*	2062	07.150304	Puerto Ayacucho, Estado Bolivar, Venezuela
Gymnotus	*cataniapo*	2063	01.180304	Puerto Ayacucho, Estado Bolivar, Venezuela
Gymnotus	*coatesi*	2042	01.250293	Tefé, Estado Amazonas, Brazil
Gymnotus	*coatesi*	2043	02.250293	Tefé, Estado Amazonas, Brazil
Gymnotus	*coropinae* (GU)	2035	ANSP 179126 (2240)	Sauriwau River, Takutu-Branco Drainage, Rupununi, Guyana
Gymnotus	*coropinae* (GU)	2036	AUM 35848 (2421)	Sauriwau River, Takutu-Branco Drainage, Rupununi, Guyana

Table 2 continued…

Genus	Species	Tissue/DNA Lot	Field/EOD Number/ Museum Number	Locality
Gymnotus	*coropinae* (GU)	2037	ANSP 179127 (2270)	Essequibo Drainage, Cuyuni-Mazaruni Region, Guyana
Gymnotus	*coropinae* (GU)	2038	ANSP 179127 (2273)	Essequibo Drainage, Cuyuni-Mazaruni Region, Guyana
Gymnotus	*coropinae* (CA)	2010	03.200201	Tefé, Estado Amazonas, Brazil
Gymnotus	*coropinae* (CA)	2025	04.040300	Tefé, Estado Amazonas, Brazil
Gymnotus	*curupira*	2009	07.130300	Tefé, Estado Amazonas, Brazil
Gymnotus	*curupira*	2021	02.040300	Tefé, Estado Amazonas, Brazil
Gymnotus	*cylindricus*	2092	KOW04-3/H8038	Río Tortuguero, Costa Rica
Gymnotus	*cylindricus*	2093	KOW04-3/H8039	Rio Tortuguero, Costa Rica
Gymnotus	*cylindricus*	2094	KOW04-3/H8040	Rio Tortuguero, Costa Rica
Gymnotus	*javari*	2020	05.270502	Iquitos, Peru
Gymnotus	*jonasi*	2016	04.080301	Tefé, Estado Amazonas, Brazil
Gymnotus	*jonasi*	2471	N/A	Peru
Gymnotus	*mamiraua*	2012	06.080301	Tefé, Estado Amazonas, Brazil
Gymnotus	*mamiraua*	2013	07.250699	Tefé, Estado Amazonas, Brazil
Gymnotus	*obscurus*	2017	03.170401	Tefé, Estado Amazonas, Brazil
Gymnotus	*obscurus*	2018	07.170401	Tefé, Estado Amazonas, Brazil
Gymnotus	*pantherinus*	2039	01.06022003	Rio Perequê-Açu, Estado Rio de Janeiro, Brazil
Gymnotus	*pedanopterus*	2058	04.150304	Pueblito Magua, Estado Amazonas, Venezuela
Gymnotus	*pedanopterus*	2059	09.150304	Pueblito Magua, Estado Amazonas, Venezuela
Gymnotus	*sp.* aff. *anguillaris*	2091	V045/AUM 36616	Guyana

Table 2 continued.

Genus	Species	Tissue/DNA Lot	Field/EOD Number/ Museum Number	Locality
Gymnotus	*stenoleucus*	2060	11.150304	San Fernando de Atabapo, Estado Amazonas, Venezuela
Gymnotus	*stenoleucus*	2061	03.180304	Puerto Ayacucho, Estado Bolivar, Venezuela
Gymnotus	*stenoleucus*	2064	24.150304	San Fernando de Atabapo, Estado Amazonas, Venezuela
Gymnotus	*tigre*	2019	04.270502	Iquitos, Peru
Gymnotus	*tigre*	2024	02.270502	Iquitos, Peru
Gymnotus	*ucamara*	1927	T104	Peru
Gymnotus	*ucamara*	1950	T174	Peru
Gymnotus	*varzea*	2014	02.170401	Tefé, Estado Amazonas, Brazil
Gymnotus	*varzea*	2015	04.170401	Tefé, Estado Amazonas, Brazil
Hypopomus	*artedi*	2232	ANSP 179505	Mazaruni River, Cuyuni-Mazaruni, Guyana
Rhamphichthys	*rostratus*	A06	02.290301	Tefé, Estado Amazonas, Brazil
Sternopygus	*macrurus*	A20	01.260301	Tefé, Estado Amazonas, Brazil

Table 3. Primers used for amplification and sequencing of the cyt *b*, 16S and RAG2 genes.

Primer Name	Primer sequence (5′ to 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 1996
RAG2R6	TGRTCCARGCAGAAGTACTTG	Lovejoy and Collette 1996
RAG2GY-F	ACAGGCATCTTTGGKATTCG	This study
RAG2-GY-R	TCATCCTCCTCATCTTCCTC	This study
GY-RAG2ISP1	GCCATCATTAAGGACATCACAG	This study
GY-RAG2ISP2	CTGAGCATGGACCCAGTGTC	This study

Table 4. 16S clustalX alignment. Shaded sections represent ambiguously aligned portions of loop structures excluded from phylogenetic analysis.

Species	Sequence
	10 20 30 40 50
	. . . . .
Sternopygus macrurus	GAGGTCCTGCCTGCCCAGTGACTATTAGTTTAACGGCCGCGGTATTTTGA
Rhamphichthys	.....G.....
Hypopomus artedi	.....C.....-CACT.....A.
Brachy. brevirostris	.....C.....-CACT.....A.
Brachyhypopomus diazi	.....C.....-CACT.....A.
Brachy. 'palenque'	.....C.....-CCCT...C.....A.
Electrophorus electricus	.....---A..-..A.....
Gymnotus arapaima	.....-.A.....CC...
Gymnotus carapo (WA)	.....-.A.....CC...
Gymnotus carapo (GO)	.....-.A.....CC...
Gymnotus cataniapo	.....G.....A.-C.....CC...
Gymnotus coatesi	.....G.....G.AC....C.....CC...
Gymnotus coropinae (GO)	.....G.....G.AC....C.....CC...
Gymnotus coropinae (WA)	.....G.....G.AC....C.....CC...
Gymnotus curupira	.....-.A.....CC...
Gymnotus cylindricus	.....-.A.....CC...
Gymnotus javari	.....G.....G.AC....C.....CC...
Gymnotus jonasi	.....A.A.....C.....CC...
Gymnotus mamiraua	.....-.A.....CC...
Gymnotus obscurus	.....A.-.....CC...
Gymnotus pantherinus	.....-.AC.A.....CC...
Gymnotus pedanopterus	.....-.A.....CC...
Gymnotus sp.aff. anguillaris	.....-.AC.A.....CC...
Gymnotus stenoleucus	.....G.....G.AC....C.....CC...
Gymnotus tigre	.....-.A.....CC...
Gymnotus ucamara	.....-.A.....CC...
Gymnotus varzea	.....-.A.....CC...
	60 70 80 90 100
	. . . . .
Sternopygus macrurus	CCGTGCAAAGGTAGCGCAATCACTTGTCTTTTAAATGAAGACCTGTATGA
Rhamphichthys	.....C.....G.....
Hypopomus artedi	.....C.....G.....
Brachy. brevirostris	.....
Brachyhypopomus diazi	.....C.....
Brachy. 'palenque'	.....C.....
Electrophorus electricus	.....C.CC....T.AGG.G.....
Gymnotus arapaima	.....G.....A.G.....
Gymnotus carapo (WA)	.....G.....A.G.....
Gymnotus carapo (GO)	.....G.....A.G.....
Gymnotus cataniapo	.....G.....A.G.....
Gymnotus coatesi	.....G.....C.....AGG.....
Gymnotus coropinae (GO)	.....G.....C.....AGG.....
Gymnotus coropinae (WA)	.....G.....C.....AGG.....
Gymnotus curupira	.....G.....A.....
Gymnotus cylindricus	.....G.....A.G.....
Gymnotus javari	.....G.....C.....AGG.....
Gymnotus jonasi	.....G.....C.....AG.....
Gymnotus mamiraua	.....G.....A.G.....
Gymnotus obscurus	.....G.....A.G.....
Gymnotus pantherinus	.....G.....A.G.....
Gymnotus pedanopterus	.....A.G.....
Gymnotus sp. aff. anguillaris	.....A.....C.....
Gymnotus stenoleucus	.....G.....C.....AGG.....
Gymnotus tigre	.....G.....A.....
Gymnotus ucamara	.....G.....A.G.....
Gymnotus varzea	.....G.....A.G.....

Table 4 continued.

Species	Sequence
	110 120 130 140 150
	. . . . .
Sternopygus macrurus	ATGGCAAAACGAGGGCTTAACTGTCTCCCCTTTCCAGTCAATGAAATTGA
Rhamphichthys	.A.....T.....A...A.....G.....
Hypopomus artedi	......CC.....T.....T....A.....G.....
Brachy. brevirostris	......T.....CT.....A.....G.....
Brachyhypopomus diazi	......T.....T.....A.....G.....
Brachy. 'palenque'	......T.....T.....A.....G.....
Electrophorus electricus	.....T.G....A...CC.....T....A.A...G.T.....
Gymnotus arapaima	.....T..A.A.....G.....
Gymnotus carapo (WA)	.....T..A.A.....G.....
Gymnotus carapo (GO)	.....T..A.A.....G.....
Gymnotus cataniapo	.....T..A.A.....G.....
Gymnotus coatesi	.....A.....T..A.A.....G.....
Gymnotus coropinae (GO)	.....T..A.A.....G.....
Gymnotus coropinae (WA)	.....G.....T..A.A.....G.....
Gymnotus curupira	.....T..A.A.....G.....
Gymnotus cylindricus	.....T..A.A.....
Gymnotus javari	.....A.....G.....T..A.A.....G.....
Gymnotus jonasi	.....G.....T..A.A.....G.....
Gymnotus mamiraua	.....T..A.A.....G.....
Gymnotus obscurus	.....T..A.A.....G.....
Gymnotus pantherinus	.....T....A.....G.....
Gymnotus pedanopterus	.....T..A.A.....G.....
Gymnotus sp. aff. anguillaris	.....G.....T..A.A.....G.....
Gymnotus stenoleucus	.....T..A.A.....G.....
Gymnotus tigre	.....T..A.A.....G.....
Gymnotus ucamara	.....T..A.A.....G.....
Gymnotus varzea	.....T..A.A.....G.....
	160 170 180 190 200
	. . . . .
Sternopygus macrurus	TCTACCCGTGCAGAAGCGGGTATAAAAATACAAGACGAGAAGACCCTTTG
Rhamphichthys	...G.....AC...TT.....
Hypopomus artedi	...G.....CAT.....T.....
Brachy. brevirostris	...G.....AC.....T.....
Brachyhypopomus diazi	...G.....AC.....T.....
Brachy. 'palenque'	...G.....A.C...G...T.....
Electrophorus electricus	.....A....TC.CC.....
Gymnotus arapaima	C..G.....T.....C...C.....
Gymnotus carapo (WA)	C..G.....T.....C...C.....
Gymnotus carapo (GO)	C..G.....T....AC...C.....
Gymnotus cataniapo	C..G.....T.....C...T.....
Gymnotus coatesi	C..G.....T.....C.C..T.....
Gymnotus coropinae (GO)	C..G.....T.....C.C..T.G.....
Gymnotus coropinae (WA)	C..G.....T.....C.C..T.T.....
Gymnotus curupira	C..G.....T....AC...T.....
Gymnotus cylindricus	C..G.....T....AC...T.....
Gymnotus javari	C..G.....T.....C.C..TG.....
Gymnotus jonasi	C..G.....T.....C.C..T.....
Gymnotus mamiraua	C..G.....T.....C...T.....
Gymnotus obscurus	C..G.....T.....C...T.....
Gymnotus pantherinus	C..G.....T....AC..G.T.....
Gymnotus pedanopterus	C..G.....T.....C...C.....A..
Gymnotus sp. aff. anguillaris	C..GT.....T....AC...T.....
Gymnotus stenoleucus	C..G.....T.....C.C..TT.....
Gymnotus tigre	C..G.....T.....C...T.....
Gymnotus ucamara	C..G.....T.....C...C.....
Gymnotus varzea	C..G.....T....AC...T.....

Table 4 continued.

Species	Sequence
	210 220 230 240 250
	. . . . .
Sternopygus macrurus	GAGCTTAAGACCTAAACCACCTATGTTAATA-ATCTACAAACTAGTTTAA
Rhamphichthys	.....AC..G...A.....-.A.C.--T.T-.ACC.GG
Hypopomus artedi	.....A.C.....A....A.....-GG..C--.C.-.ACCG.G
Brachy. brevirostris	.....TA...G...A.....-GG..C--.C.A.C.CAGC
Brachyhypopomus diazi	.....TA...G...A...C.....AGC.CC--CCAACCC-AGC
Brachy. 'palenque'	.....TA...G...A...C.....-.A..T--.TTA.ACAAGT
Electrophorus electricus	.....TTA..GT..T...CA.....-.GT.--C--A--C....
Gymnotus arapaima	.....A...G...A.....-GC.--.TTTA.A.-AGC
Gymnotus carapo (WA)	.....A...G...A.....-GC.--.TTTA--AA..G
Gymnotus carapo (GO)	.....A...G...A.....-.C.-----.T.TAAA..G
Gymnotus cataniapo	.....AC.....A.....-..T..--CC.C.CC.A.-
Gymnotus coatesi	.....A..AC..G.....C.C..C....-GC.---T.A..ACC..G
Gymnotus coropinae (GO)	.....A..AC..G.....C.C..C...G-G...T--.GTA.CCCAG.
Gymnotus coropinae (WA)	.....A..AC..G.....C.C..C...G-G...T--.GT-.ACCC.G
Gymnotus curupira	.....A...GT..A.....-..-.T--.TT..TC.A.-
Gymnotus cylindricus	.....TA...G...A.....-G....--TT.ATA.A...
Gymnotus javari	.....A..AC..G.....C.C..C....-GC..T--..T-.ACC..G
Gymnotus jonasi	.....A..G-.....CGC.....-GCT.G--TA..AC.C.G
Gymnotus mamiraua	.....AC..G...A.....-.C....--TTTA...AG--
Gymnotus obscurus	.....A...G...A.....-..T..---C..-ACC...
Gymnotus pantherinus	.....AC..G...AT.....-GC...--...ACC.AG--
Gymnotus pedanopterus	.....AC..G...A.C....C....-..-.T--.CA-CACC...
Gymnotus sp.aff. anguillaris	.....AC..GT..A.....-..T.G--TCAC.CC.A.-
Gymnotus stenoleucus	.....A..AC..G.....C.C..C...G-G...T--..TA.CCCAG-
Gymnotus tigre	.....A...GT..A.....-..-.T--.TT-TA.C...
Gymnotus ucamara	.....A...G...A.....-GC.--.TTTA.A.-AGC
Gymnotus varzea	.....A...G...A.....ATCT-.--CCTATC.AG.-
	260 270 280 290 300
	. . . . .
Sternopygus macrurus	ACTAAA------TAGCATTGGCCCAA---GTCTTCGGTTGGGGTGACCGC
Rhamphichthys	CT....CT-AAA...A.C.....CC---.....C.....
Hypopomus artedi	...T..ACTCAA...ACC.....CC---.....A.....C...GAT
Brachy. brevirostris	C.....CTC-AA...TCA.....C---A.....C...AAT
Brachyhypopomus diazi	TT....CTC-AA...TTA.....C---A.....C...AAT
Brachy. 'palenque'	CT....CTC-AA....TG.....GT---A.....C...AAT
Electrophorus electricus	..C..GTA------.AT.C..A..T.C---A.....C....A.
Gymnotus arapaima	CT-...CT---G..A..C....TC.---.....C....A.
Gymnotus carapo (WA)	C.....CT---G..A..C....TC.---.....C....A.
Gymnotus carapo (GO)	G.....CT---G..A..C.....C.---.....C....A.
Gymnotus cataniapo	.T....CT---G..A..C....AT.---.....C....A.
Gymnotus coatesi	.....CA---AC....C.....T.....C....A.
Gymnotus coropinae (GO)	CT-...CA---AC.A.....-C.---.T.....C....A.
Gymnotus coropinae (WA)	.....---CAAC.A.....T.....C....A.
Gymnotus curupira	.T....CT---G..A..C..A..T..---.....C....A.
Gymnotus cylindricus	.....C---TG..A..C....TC.---.....C....A.
Gymnotus javari	.....---CAAC....C.....T.....C....A.
Gymnotus jonasi	.....CA---AC.AT.C....T-C.---.....C....A.
Gymnotus mamiraua	.....CT---G..A..C.....TC.---.....C....A.
Gymnotus obscurus	.T....CT---G..A..C.....T..---.....C....A.
Gymnotus pantherinus	.....CT---A..C..C.....C.---.....C....A.
Gymnotus pedanopterus	.T.....---CTA..A..C.....TC---.....C....A.
Gymnotus sp. aff. anguillaris	.T....CT---A..A..C..A...CCCCC.....C....A.
Gymnotus stenoleucus	.....CA---AC.A.CC.....-C.---.T.....C....A.
Gymnotus tigre	.T....---CTG..A..C..A..T..---.....C....A.
Gymnotus ucamara	CT-...CT---G..A..C.....TC.---.....C....A.
Gymnotus varzea	TT-...CT---G..A..C.....T..---A.....C....A.

Table 4 continued.

Species	Sequence
	310 320 330 340 350
Sternopygus macrurus	GGGGGAAAACAAAGCCCCCATGTGG-AACGGGGACAGCCCCCTAAAA-CC
Rhamphichthys	.....C....-.GT.A...T.TTA..T..C..-..
Hypopomus artedi	..A.A....T......T...C.CA.-C.T..AA-TT--TT.......-T.
Brachy. brevirostris	..A.A.....T...C.C..C.CG..ACTTC----...A....-T.
Brachyhypopomus diazi	..A.....T...C.C..T.TG..-.TAC--....A....-T.
Brachy. 'palenque'	..A.....T...C....-T.T..-...T--A...C....AT.
Electrophorus electricus	.....T.....AA.-..A.ATAGAC---.TTCT...-..
Gymnotus arapaima	..A.T.....A..T...C.C..-.TA..-..AT--A.....-..
Gymnotus carapo (WA)	..A.T.....A..T...C.C..-.TA..-..AT--A.....-..
Gymnotus carapo (GO)	..A.T.....A..T...C.A..-.TA...A.T.T..T---.....-..
Gymnotus cataniapo	..A.T....T...A..T.....C..-.TA...C.T.---.....-..
Gymnotus coatesi	..A.T....T...A.....CA.-.CA...A.T.C..T---.....-..
Gymnotus coropinae (GO)	..A.T....T...A.....CA.-.CA..-..AT--A.....-..
Gymnotus coropinae (WA)	..A.T....T...A.....CA.-.CA...A-AT--A.....-..
Gymnotus curupira	..A.T.....A..T.....A..-.TA..-..AT--AT.....-..
Gymnotus cylindricus	..A.T....T...A..T.....A..-.TA...A.A.--AT.....-T.
Gymnotus javari	..A.T....T...A.....CA.-.CA...A-AT--A.....-..
Gymnotus jonasi	..A.T....TG..A..T.....CA.-.CA...A.T.C..T---.....-..
Gymnotus mamiraua	..A.T.G.....T...C.C..-.TA...A.T.---.....-..
Gymnotus obscurus	..A.T.....A..T.....C..-.TA...A.T.T..T---.....-..
Gymnotus pantherinus	..A.C.....A..T.....A..-.TA...A.T.---.....-..
Gymnotus pedanopterus	..A.T....T...A..T.....C..-.TTA..ACAC--AT.....-..
Gymnotus sp. aff. anguillaris	..A.T.....A..T.....C..-.TA..-.CAC--A.....-..
Gymnotus stenoleucus	..A.T....T...A.....CA.-.CA...A.T.---.....-..
Gymnotus tigre	..A.T.....A..T.....A..-.TA...A-AT--AT.....-..
Gymnotus ucamara	..A.T.....A..T...C.C..-.TA..-..AT--A.....-..
Gymnotus varzea	..A.T.....A..T.....A..-.TA..-..AT--AT.....-..
	360 370 380 390 400
Sternopygus macrurus	AAGAGAGACATCTCTAAGCCACAGAACATCTGACCAAAAA-GATCCGGCT
Rhamphichthys	.....CC.....T.....T.....G...-.....C
Hypopomus artedi	.....TA..CC...A..A..T.....TC.....TT.C.-.....C
Brachy. brevirostris	.....CA..CC.....AT.T.....C.....T.-.....
Brachyhypopomus diazi	.....CA..CC.....AT.T.....C.....T..C.-.....
Brachy. 'palenque'	.....CA..CC.....AT.T.....C.....T..T.-.....C
Electrophorus electricus	T...A.....T.....T.G.....T...-.-.....TT.
Gymnotus arapaima	.....C.....C.T.-.....C
Gymnotus carapo (WA)	.....C.....C.T.-.....C
Gymnotus carapo (GO)	.....C...T.....T.....T.-.....C
Gymnotus cataniapo	....A.....CT..C.....TT.T.C.....C
Gymnotus coatesi	....A.A....T.....T.....T.T.T.-.....C
Gymnotus coropinae (GO)	....A.A....T.....T.....T.T.C.-.....C
Gymnotus coropinae (WA)	....A.A....T.....T.....T.T.C.-.....C
Gymnotus curupira	.....C...T.....T.T.-.....C
Gymnotus cylindricus	G.....C..AT.....T.....T.T.-.....C
Gymnotus javari	....A.A....T.....T.....T.T.T.-.....C
Gymnotus jonasi	....A.....T.....T.....T.C.C.-.....C
Gymnotus mamiraua	.....C.....T.T.-.....C
Gymnotus obscurus	.....C...T.....T.....T.T.-.....C
Gymnotus pantherinus	.....C.....T.T-.....C
Gymnotus pedanopterus	.....C...C.....C.T.-.....C
Gymnotus sp. aff. anguillaris	.....C.....C.T.-.....C
Gymnotus stenoleucus	....A.A....T.....T.....T.T.T.-.....C
Gymnotus tigre	.....C...T.....T.T.-.....C
Gymnotus ucamara	.....C.....C.T.-.....C
Gymnotus varzea	.....A.....C...T.....T.....T.T.T.-.....C

Table 4 continued.

Species	Sequence
	410 420 430 440 450
	. . . . .
Sternopygus macrurus	ACTAAAGCCGATTAACGGACCAAGTTACCCTAGGGATAACAGCGCAATCC
Rhamphichthys	CT.-.-.....C....A.....
Hypopomus artedi	C.----.....C....A.....
Brachy. brevirostris	C..CG--.....C.....
Brachyhypopomus diazi	C..CG--.....C.....
Brachy. 'palenque'	C.CCG--.....C....A.....
Electrophorus electricus	TTAT.---...CC.G..A.....A.....
Gymnotus arapaima	T.CCG-.....C....A.....A.....
Gymnotus carapo (WA)	T.CCG-.....C....A.....A.....
Gymnotus carapo (GO)	T.CCG-.....C....A.....A.....
Gymnotus cataniapo	TT..G-.....C.G.....
Gymnotus coatesi	TTCCG-.....C....A.....C.....
Gymnotus coropinae (GO)	CTCCG-.....C....A.....
Gymnotus coropinae (WA)	CTCCG-.....C....A.....
Gymnotus curupira	CTCCG-.....C....A.....A.....
Gymnotus cylindricus	T.CCG-.....C....A.....A.....
Gymnotus javari	TTCCG-.....C....A.....
Gymnotus jonasi	T..TG-.....C....A.....C.....
Gymnotus mamiraua	T.CCG-.....C....A.....A.....
Gymnotus obscurus	CTCCG-.....C....A.....A.....
Gymnotus pantherinus	T.CCG-.....C....A.....
Gymnotus pedanopterus	TT.C.-.....C....A.....
Gymnotus sp. aff. anguillaris	TTCC.-.....C....A.....
Gymnotus stenoleucus	CTCCG-.....C....A.....
Gymnotus tigre	CTCCG-.....C....A.....A.....
Gymnotus ucamara	T.CCG-.....C....A.....A.....
Gymnotus varzea	CT.C.-.....C....A.....A.....
	460 470 480 490 500
	. . . . .
Sternopygus macrurus	CCTCTCAGAGTCCCTATCGACGAGGGGGTTTACGACCTCGATGTTGGATC
Rhamphichthys	....CG.....A.....
Hypopomus artedi	...TCT.....A.....
Brachy. brevirostris	...T.....T.....
Brachyhypopomus diazi	...TCT.....T.....A.....
Brachy. 'palenque'	...TCT.....T.....A.....
Electrophorus electricus	T..TC.....A.....
Gymnotus arapaima	...TC.....A.....
Gymnotus carapo (WA)	...TC.....A.....
Gymnotus carapo (GO)	...TC.....A.....
Gymnotus cataniapo	...TC.....A.....
Gymnotus coatesi	....C.....A.....A.....
Gymnotus coropinae (GO)	....C.....A.....A.....
Gymnotus coropinae (WA)	....C.....A.....A.....
Gymnotus curupira	...TC.....A..A.....
Gymnotus cylindricus	...TC.....A.....
Gymnotus javari	....C.....A.....A.....
Gymnotus jonasi	....C.....A.....A.....
Gymnotus mamiraua	...TC.....A.....
Gymnotus obscurus	...TC.....A.....
Gymnotus pantherinus	...TC.....A.....
Gymnotus pedanopterus	...TC.....A.....
Gymnotus sp. aff.anguillaris	...TC.....A.....
Gymnotus stenoleucus	....C.....A.....A.....
Gymnotus tigre	...TC.....A..A.....
Gymnotus ucamara	...TC.....A.....
Gymnotus varzea	...TC.....A.....

Table 4 continued.

Species	Sequence
	510 520 530 540
	. . . .
Sternopygus macrurus	AGGACATCCTAATGGTGCAGCCGCTATTAAGGG????????????????
Rhamphichthys	......C.....?????????????
Hypopomus artedi	.....TTCGT??????????
Brachy. brevirostris	.....TT?????????????
Brachyhypopomus diazi	.....TT?????????????
Brachy. 'palenque'	.....TT?????????????
Electrophorus electricus	.....T.....TTCGTTTGTTCAACG
Gymnotus arapaima	.....TTCGTT?????????
Gymnotus carapo (WA)	.....TTCGTT?????????
Gymnotus carapo (GO)	.....TTCGTT?????????
Gymnotus cataniapo	.....T.....TTCG???????????
Gymnotus coatesi	.....T.....TTCGT??????????
Gymnotus coropinae (GO)	.....T.....T.....TTCGT??????????
Gymnotus coropinae (WA)	.....T.....TTCGT??????????
Gymnotus curupira	.....TTCGTT?????????
Gymnotus cylindricus	.....TTC????????????
Gymnotus javari	.....T.....TTCGT??????????
Gymnotus jonasi	.....T.....TTCGTT?????????
Gymnotus mamiraua	.....TTCGTT?????????
Gymnotus obscurus	.....TTCGTT?????????
Gymnotus pantherinus	.....TTCGTTT????????
Gymnotus pedanopterus	.....TTCGT??????????
Gymnotus sp. aff. anguillaris	.....TT?????????????
Gymnotus stenoleucus	.....T.....TTCGT??????????
Gymnotus tigre	.....TTCGTT?????????
Gymnotus ucamara	.....TTCGTT?????????
Gymnotus varzea	.....T.....TTCGTT?????????

Table 5. Mean nucleotide frequencies for each gene and p-values for chi-square test of base frequency homogeneity across taxa.

Gene	A	C	G	T	χ^2 value	p-value
Cyt *b*	0.271	0.308	0.133	0.288	218.91	<0.001*
Cyt *b* 1st positions	0.263	0.267	0.235	0.236	55.96	1.00
Cyt *b* 2nd positions	0.202	0.257	0.126	0.415	4.15	1.00
Cyt *b* 3rd positions	0.347	0.402	0.038	0.212	499.18	<0.001*
16S	0.298	0.258	0.227	0.217	34.03	1.00
RAG-2	0.239	0.275	0.265	0.221	9.34	1.00
mtDNA	0.280	0.292	0.163	0.265	207.89	<0.01*

* indicates significant values

Table 6. Uncorrected pairwise sequence divergence for the cytochrome *b* data partition. Shaded areas indicate distances between the Central American species *G. cylindricus* and other taxa.

Species	Distance									
	S. macrurus	*R. rostratus*	*H. artedi*	*B. brevirostris*	*B. diazi*	*B. palenque*	*E. electricus*	*G. jonasi*	*G. javari*	*G. coatesi*
S. macrurus	-									
R. rostratus	0.213	-								
H. artedi	0.205	0.211	-							
B. brevirostris	0.196	0.203	0.164	-						
B. diazi	0.202	0.203	0.160	0.126	-					
B. palenque	0.196	0.221	0.193	0.148	0.144	-				
E. electricus	0.257	0.278	0.283	0.281	0.282	0.286	-			
G. jonasi	0.215	0.246	0.246	0.248	0.241	0.259	0.259	-		
G. javari	0.199	0.221	0.234	0.224	0.217	0.226	0.253	0.122	-	
G. coatesi	0.201	0.219	0.234	0.224	0.217	0.226	0.254	0.124	0.023	-
G. stenoleucus	0.208	0.216	0.240	0.228	0.222	0.227	0.246	0.110	0.097	0.097
G. coropinae (WA)	0.202	0.235	0.228	0.230	0.224	0.239	0.259	0.107	0.105	0.103
G. coropinae GU	0.211	0.237	0.231	0.235	0.215	0.244	0.246	0.110	0.103	0.101
G. pantherinus	0.193	0.234	0.224	0.213	0.206	0.233	0.255	0.173	0.189	0.190
G. sp. aff. anguillaris	0.195	0.229	0.221	0.210	0.203	0.239	0.248	0.191	0.194	0.198
G. pedanopterus	0.202	0.231	0.246	0.232	0.231	0.232	0.248	0.180	0.172	0.171
G. cataniapo	0.201	0.248	0.242	0.259	0.235	0.245	0.256	0.186	0.194	0.185
G. cylindricus	0.204	0.243	0.235	0.236	0.217	0.237	0.246	0.187	0.187	0.186
G. varzea	0.205	0.232	0.236	0.231	0.217	0.237	0.256	0.171	0.183	0.180
G. obscurus	0.204	0.229	0.239	0.233	0.229	0.238	0.250	0.169	0.186	0.185
G. tigre	0.200	0.231	0.236	0.228	0.224	0.235	0.245	0.177	0.184	0.181
G. curupira	0.209	0.237	0.231	0.220	0.225	0.230	0.250	0.178	0.183	0.182
G. mamiraua	0.187	0.213	0.212	0.217	0.208	0.228	0.253	0.173	0.176	0.181
G. carapo (GO)	0.196	0.230	0.229	0.220	0.213	0.241	0.251	0.179	0.179	0.186
G. ucamara	0.203	0.232	0.247	0.231	0.231	0.233	0.251	0.175	0.188	0.189
G. carapo Amazon	0.199	0.221	0.234	0.224	0.217	0.226	0.252	0.175	0.188	0.189
G. arapaima	0.198	0.238	0.243	0.238	0.223	0.233	0.248	0.175	0.189	0.190

Table 6 continued.

Species	Uncorrected pairwise sequence divergence									
	G. stenoleucus	*G. coropinae (WA)*	*G. coropinae GU*	*G. pantherinus*	*G. sp.*	*G. pedanopterus*	*G. cataniapo*	*G. cylindricus*	*G. varzea*	*G. obscurus*
G. stenoleucus	-									
G. coropinae (WA)	0.065	-								
G. coropinae GU	0.067	0.042	-							
G. pantherinus	0.168	0.171	0.167	-						
G. sp. aff. *anguillaris*	0.193	0.188	0.187	0.178	-					
G. pedanopterus	0.166	0.170	0.167	0.139	0.138	-				
G. cataniapo	0.175	0.167	0.165	0.154	0.162	0.154	-			
G. cylindricus	0.184	0.187	0.181	0.151	0.181	0.156	0.176	-		
G. varzea	0.172	0.174	0.171	0.146	0.167	0.148	0.165	0.113	-	
G. obscurus	0.171	0.171	0.170	0.151	0.156	0.142	0.167	0.124	0.049	-
G. tigre	0.183	0.178	0.177	0.160	0.160	0.142	0.168	0.131	0.062	0.050
G. curupira	0.181	0.177	0.176	0.157	0.162	0.142	0.168	0.129	0.062	0.050
G. mamiraua	0.175	0.183	0.176	0.150	0.157	0.153	0.166	0.124	0.081	0.088
G. carapo (GO)	0.183	0.187	0.184	0.143	0.166	0.153	0.166	0.118	0.079	0.084
G. ucamara	0.180	0.185	0.180	0.140	0.159	0.148	0.167	0.117	0.078	0.078
G. carapo Amazon	0.181	0.185	0.179	0.141	0.160	0.148	0.167	0.115	0.079	0.079
G. arapaima	0.180	0.185	0.180	0.141	0.159	0.148	0.167	0.115	0.077	0.077

	G. tigre	*G. curupira*	*G. mamiraua*	*G. carapo (GO)*	*G. ucamara*	*G. carapo Amazon*	*G. arapaima*
G. tigre	-						
G. curupira	0.005	-					
G. mamiraua	0.107	0.107	-				
G. carapo (GO)	0.095	0.094	0.057	-			
G. ucamara	0.099	0.099	0.054	0.039	-		
G. carapo Amazon	0.100	0.100	0.054	0.038	0.005	-	
G. arapaima	0.098	0.098	0.053	0.038	0.002	0.004	-

Table 7. Uncorrected pairwise sequence divergence for the RAG2 data partition. Shaded areas indicate distances between the Central American species *G. cylindricus* and other taxa.

Species	Uncorrected pairwise sequence divergence									
	S. macrurus	*R. rostratus*	*H. artedi*	*B. brevirostris*	*B. diazi*	*B. palenque*	*E. electricus*	*G. jonasi*	*G. javari*	*G. coatesi*
S. macrurus	-									
R. rostratus	0.090	-								
H. artedi	0.093	0.089	-							
B. brevirostris	0.097	0.092	0.037	-						
B. diazi	0.096	0.094	0.037	0.035	-					
B. palenque	0.093	0.090	0.033	0.031	0.023	-				
E. electricus	0.093	0.103	0.092	0.099	0.101	0.097	-			
G. jonasi	0.086	0.096	0.102	0.101	0.106	0.104	0.092	-		
G. javari	0.091	0.100	0.102	0.106	0.105	0.104	0.091	0.012	-	
G. coatesi	0.091	0.100	0.103	0.106	0.105	0.104	0.092	0.012	0.002	-
G. stenoleucus	0.091	0.100	0.102	0.106	0.105	0.104	0.093	0.013	0.006	0.006
G. coropinae (WA)	0.086	0.101	0.106	0.105	0.109	0.109	0.095	0.011	0.003	0.003
G. coropinae GU	0.083	0.097	0.102	0.104	0.108	0.105	0.096	0.013	0.005	0.000
G. pantherinus	0.081	0.096	0.103	0.104	0.108	0.105	0.097	0.031	0.031	0.031
G. sp. aff. anguillaris	0.081	0.095	0.103	0.104	0.108	0.105	0.096	0.026	0.027	0.027
G. pedanopterus	0.094	0.103	0.107	0.108	0.109	0.105	0.095	0.023	0.025	0.025
G. cataniapo	0.091	0.099	0.103	0.106	0.105	0.104	0.095	0.026	0.028	0.028
G. cylindricus	0.083	0.097	0.104	0.106	0.110	0.107	0.097	0.031	0.033	0.033
G. varzea	0.085	0.094	0.105	0.108	0.109	0.107	0.099	0.029	0.033	0.033
G. obscurus	0.092	0.101	0.104	0.107	0.106	0.106	0.092	0.031	0.034	0.034
G. tigre	0.093	0.102	0.105	0.108	0.107	0.103	0.094	0.034	0.038	0.038
G. curupira	0.090	0.101	0.106	0.107	0.111	0.110	0.097	0.033	0.036	0.036
G. mamiraua	0.084	0.095	0.102	0.101	0.106	0.106	0.092	0.031	0.035	0.035
G. carapo (GO)	0.083	0.097	0.103	0.105	0.109	0.106	0.097	0.028	0.028	0.034
G. ucamara	0.094	0.101	0.105	0.108	0.107	0.103	0.092	0.029	0.033	0.033
G. carapo Amazon	0.091	0.100	0.103	0.106	0.105	0.104	0.091	0.029	0.033	0.033
G. arapaima	0.091	0.098	0.102	0.105	0.104	0.100	0.090	0.029	0.033	0.033

Table 7 continued.

Species	Uncorrected pairwise sequence divergence									
	G. stenoleucus	*G. coropinae (WA)*	*G. coropinae GU*	*G. pantherinus*	*G. sp.*	*G. pedanopterus*	*G. cataniapo*	*G. cylindricus*	*G. varzea*	*G. obscurus*
G. stenoleucus	-									
G. coropinae (WA)	0.004	-								
G. coropinae GU	0.006	0.002	-							
G. pantherinus	0.030	0.028	0.032	-						
G. sp. aff. *anguillaris*	0.026	0.024	0.028	0.021	-					
G. pedanopterus	0.025	0.023	0.026	0.024	0.015	-				
G. cataniapo	0.027	0.024	0.028	0.026	0.015	0.026	-			
G. cylindricus	0.032	0.030	0.033	0.029	0.026	0.028	0.026	-		
G. varzea	0.031	0.030	0.033	0.030	0.025	0.026	0.028	0.017	-	
G. obscurus	0.033	0.031	0.035	0.029	0.026	0.027	0.029	0.018	0.011	-
G. tigre	0.036	0.035	0.038	0.032	0.029	0.030	0.032	0.021	0.009	0.007
G. curupira	0.035	0.034	0.037	0.032	0.027	0.028	0.029	0.020	0.008	0.006
G. mamiraua	0.034	0.032	0.036	0.029	0.026	0.029	0.029	0.017	0.015	0.014
G. carapo (GO)	0.032	0.030	0.034	0.027	0.024	0.026	0.027	0.015	0.012	0.011
G. ucamara	0.031	0.030	0.034	0.027	0.024	0.027	0.027	0.015	0.013	0.012
G. carapo Amazon	0.031	0.030	0.033	0.027	0.024	0.027	0.026	0.015	0.013	0.012
G. arapaima	0.031	0.030	0.033	0.026	0.023	0.026	0.026	0.014	0.012	0.011

	G. tigre	*G. curupira*	*G. mamiraua*	*G. carapo (GO)*	*G. ucamara*	*G. carapo Amazon*	*G. arapaima*
G. tigre	-						
G. curupira	0.003	-					
G. mamiraua	0.017	0.017	-				
G. carapo (GO)	0.014	0.013	0.005	-			
G. ucamara	0.015	0.015	0.005	0.003	-		
G. carapo Amazon	0.015	0.015	0.005	0.003	0.000	-	
G. arapaima	0.015	0.014	0.004	0.002	0.000	0.000	-

Table 8. Average patristic distances (tree-based divergence) among *Gymnotus* species derived from the cytochrome *b* dataset using ACCTRAN optimization. Shaded portions indicate distances used to estimate divergence dates between the *Electrophorus-Gymnotus* clades and the *G. cylindricus-G. carapo* clades (i.e. the divergence between Central and South American clades).

	Species	Patristic Distance (branch length divergence)										
		1	2	3	4	5	6	7	8	9	10	11
1	*Electrophorus electricus*	--										
2	*Gymnotus jonasi*	317.5	--									
3	*Gymnotus javari*	357.0	146.5	--								
4	*Gymnotus coatesi*	356.0	145.5	25.0	--							
5	*Gymnotus stenoleucus*	357.0	146.5	122.0	121.0	--						
6	*Gymnotus coropinae* (WA)	366.0	155.5	131.0	130.0	79.0	--					
7	*Gymnotus coropinae* (GO)	366.5	156.0	131.5	130.5	79.5	50.5	--				
8	*Gymnotus pantherinus*	359.0	242.5	282.0	281.0	282.0	291.0	291.5	--			
9	*Gymnotus sp. aff. anguillaris*	461.0	344.5	384.0	383.0	384.0	393.0	393.5	242.0	--		
10	*Gymnotus pedanopterus*	411.5	295.0	334.5	333.5	334.5	343.5	344.0	192.5	152.5	--	
11	*Gymnotus cataniapo*	395.5	279.0	318.5	317.5	318.5	327.5	328.0	176.5	186.5	137.0	--
12	*Gymnotus cylindricus*	368.7	252.2	291.7	290.7	291.7	300.7	301.2	193.7	295.7	246.2	230.2
13	*Gymnotus varzea*	386.0	269.5	309.0	308.0	309.0	318.0	318.5	211.0	313.0	263.5	247.5
14	*Gymnotus obscurus*	405.0	295.0	328.0	327.0	328.0	337.0	337.5	230.0	332.0	282.5	266.5
15	*Gymnotus tigre*	420.0	303.5	343.0	342.0	343.0	352.0	352.5	245.0	347.0	297.5	281.5
16	*Gymnotus curupira*	420.0	303.5	343.0	342.0	343.0	352.0	352.5	245.0	347.0	297.5	281.5
17	*Gymnotus mamiraua*	399.0	282.5	322.0	321.0	322.0	331.0	331.5	224.0	326.0	276.5	260.5
18	*Gymnotus carapo* (GO)	406.0	289.5	329.0	328.0	329.0	338.0	338.5	231.0	333.0	283.5	267.5
19	*Gymnotus ucamara*	406.5	290.0	329.5	328.5	329.5	338.5	339.0	231.5	333.5	284.0	268.0
20	*Gymnotus carapo* (WA)	404.8	288.3	327.8	326.8	327.8	336.8	337.3	229.8	331.8	282.3	266.3
21	*Gymnotus arapaima*	405.5	289.0	328.5	327.5	328.5	337.5	338.0	230.5	332.5	283.0	267.0

		12	13	14	15	16	17	18	19	20	21
12	*Gymnotus cylindricus*	--									
13	*Gymnotus varzea*	140.7	--								
14	*Gymnotus obscurus*	159.7	55.0	--							
15	*Gymnotus tigre*	174.7	70.0	55.0	--						
16	*Gymnotus curupira*	174.7	70.0	55.0	6.0	--					
17	*Gymnotus mamiraua*	153.7	93.0	112.0	127.0	127.0	--				
18	*Gymnotus carapo* (GO)	160.7	100.0	119.0	134.0	134.0	65.0	--			
19	*Gymnotus ucamara*	161.2	100.5	119.5	134.5	134.5	65.5	44.5	--		
20	*Gymnotus carapo* (WA)	159.4	98.8	117.8	132.8	132.8	63.8	42.8	5.5	--	
21	*Gymnotus arapaima*	160.2	99.5	118.5	133.5	133.5	64.5	43.5	2.5	4.8	--
	B. palenque-B. diazi	166.0									
	B.palenque-B.brevirostris	164.0									

Table 9. Divergence date estimates (dates of origins) for the cylindricus-carapo clade and *Electrophorus-Gymnotus* clades. (assuming *Brachyhypopomus palenque-Brachyhypopomus diazi* diverged ~12.9 mya)

Node	Data Partition	Average patristic divergence (%)	Age based on patristic distance (mya)	Age based on NPRS (mya)	Age based on pairwise sequence divergence and standard fish calibration[1] (mya)
cylindricus-carapo	Cyt *b*	14.5	12.5	16.7	10.1
(CA-SA split)	Cyt *b* 1st	6.1	17.2	30.3	N/A
	Cyt *b* 2nd	1.1	27.2	12.9	N/A
	Cyt *b* 3rd	36.3	11.7	15.5	N/A
	RAG2	1.7	10.2	21.6	N/A
Electrophorus-	Cyt *b*	35.1	30.2	30.1	20.9
Gymnotus	Cyt *b* 1st	21.4	59.9	48.6	N/A
	Cyt *b* 2nd	6.6	158.1	68.8	N/A
	Cyt *b* 3rd	77.4	25.0	27.9	N/A
	RAG2	11.7	69.6	67.1	N/A

[1] Based on teleost evolutionary rate of 1.2% divergence per million years (Bermingham et al.1997).

Table 10. Average patristic distances (tree-based divergence) among *Gymnotus* species derived from the RAG2 dataset using ACCTRAN optimization. Shaded portions indicate distances used to estimate divergence dates between the *Electrophorus-Gymnotus* clades and the *G. cylindricus-G. carapo* clades (i.e. the divergence between Central and South American clades).

	Species	Patristic Distance (branch length divergence)										
		1	2	3	4	5	6	7	8	9	10	11
1	*Electrophorus electricus*	--										
2	*Gymnotus jonasi*	135.0	--									
3	*Gymnotus javari*	134.0	15.0	--								
4	*Gymnotus coatesi*	135.0	16	3	--							
5	*Gymnotus stenoleucus*	135.0	16	7	8	--						
6	*Gymnotus coropinae* (WA)	132.0	13	4	5	5	--					
7	*Gymnotus coropinae* (GO)	134.5	15.5	6.5	7.5	7.5	2.5	--				
8	*Gymnotus pantherinus*	145.0	46	45	46	46	43	45.5	--			
9	*Gymnotus sp. aff. anguillaris*	133.0	34	33	34	34	31	33.5	30	--		
10	*Gymnotus pedanopterus*	133.0	34	33	34	34	31	33.5	30	18	--	
11	*Gymnotus cataniapo*	133.0	37	36	37	37	34	38	33	21	21	--
12	*Gymnotus cylindricus*	150.0	51	50	51	51	48	50.5	35	35	35	38
13	*Gymnotus varzea*	152.0	53	52	53	53	50	52.5	37	37	37	40
14	*Gymnotus obscurus*	157.0	58	57	58	58	55	57.5	42	42	42	45
15	*Gymnotus tigre*	155.0	56	55	56	56	53	55.5	40	40	40	43
16	*Gymnotus curupira*	154.0	55	54	55	55	52	54.5	39	39	39	42
17	*Gymnotus mamiraua*	150.0	51	50	51	51	48	50.5	35	35	35	38
18	*Gymnotus carapo* (GO)	152.0	49	48	49	49	46	48.5	33	33	33	36
19	*Gymnotus ucamara*	147.5	48.5	47.5	48.5	48.5	45.5	48	32.5	32.5	32.5	35.5
20	*Gymnotus carapo* (WA)	147.5	48.5	47.5	48.5	48.5	45.5	48	32.5	32.5	32.5	35.5
21	*Gymnotus arapaima*	147.0	48	47	48	48	45	47.5	32	32	32	35

		12	13	14	15	16	17	18	19	20	21
12	*Gymnotus cylindricus*	--									
13	*Gymnotus varzea*	22.0	--								
14	*Gymnotus obscurus*	27.0	13	--							
15	*Gymnotus tigre*	25.0	11	8	--						
16	*Gymnotus curupira*	24.0	10	7	3	--					
17	*Gymnotus mamiraua*	20.0	18	23	21	20	--				
18	*Gymnotus carapo* (GO)	18.0	16	21	19	18	6	--			
19	*Gymnotus ucamara*	17.5	15.5	20.5	18.5	17.5	5.5	3.5	--		
20	*Gymnotus carapo* (WA)	17.5	15.5	20.5	18.5	17.5	5.5	3.5	0.5	--	
21	*Gymnotus arapaima*	17.0	15	20	18	17	5	3	0.5	0.5	--
	B. palenque-B. diazi	26.5									

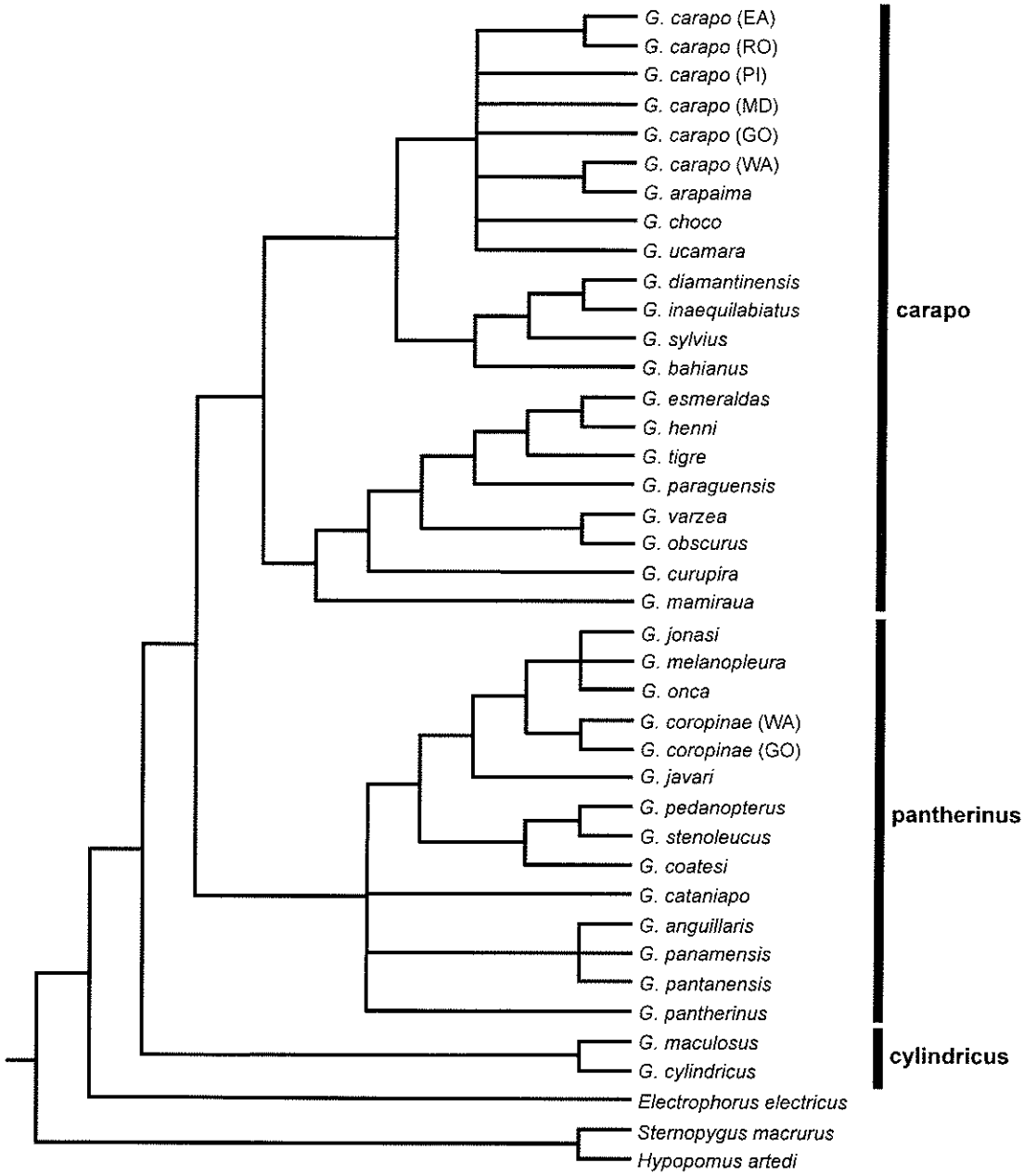

Figure 1. Phylogenetic hypothesis of Gymnotus relationships based on the morphological analysis of Albert et al. (2004).

Figure 2. Map of Middle and South America depicting nine drainage areas (MA=Middle America, PS=Pacific Slope, NW=Northwest, OR=Orinoco, GU=Guianas, WA=Western Amazon, CA=Central Amazon, EA=Eastern Amazon, PA=Paraguay/Parana, SE=Southeastern, NE=Northeastern).

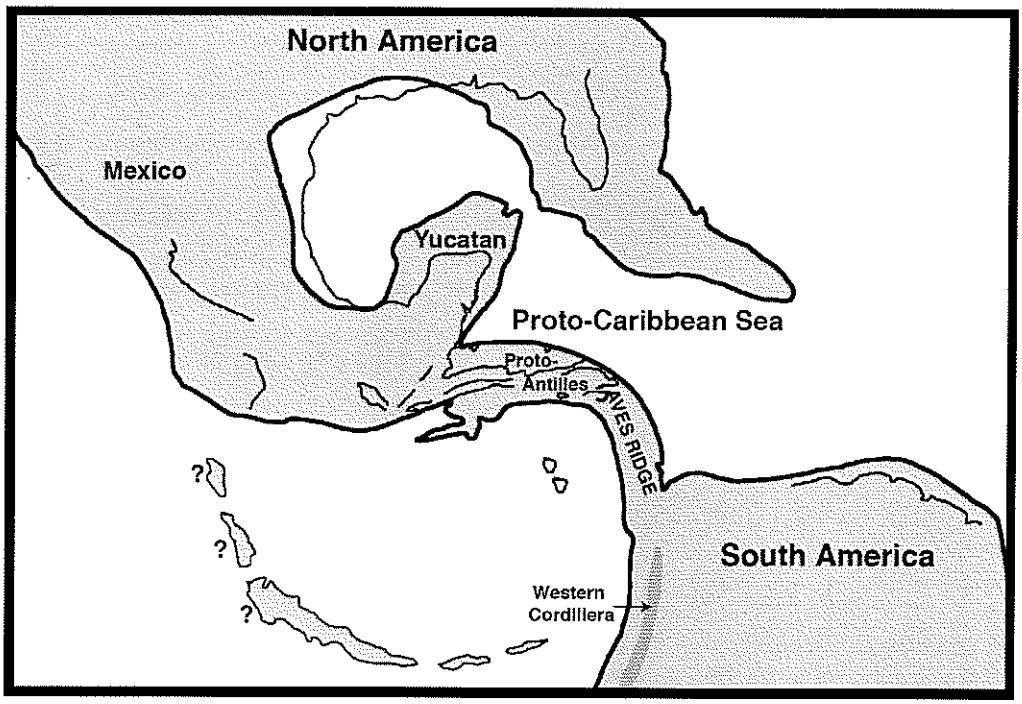

Figure 3. Paleogeographic map depicting the positions of postulated land bridges connecting the North and South American continents during the Late Cretaceous-Early Tertiary (~65mya) (adapted from Gayet et al. 1992 and Pindell et al. 1988).

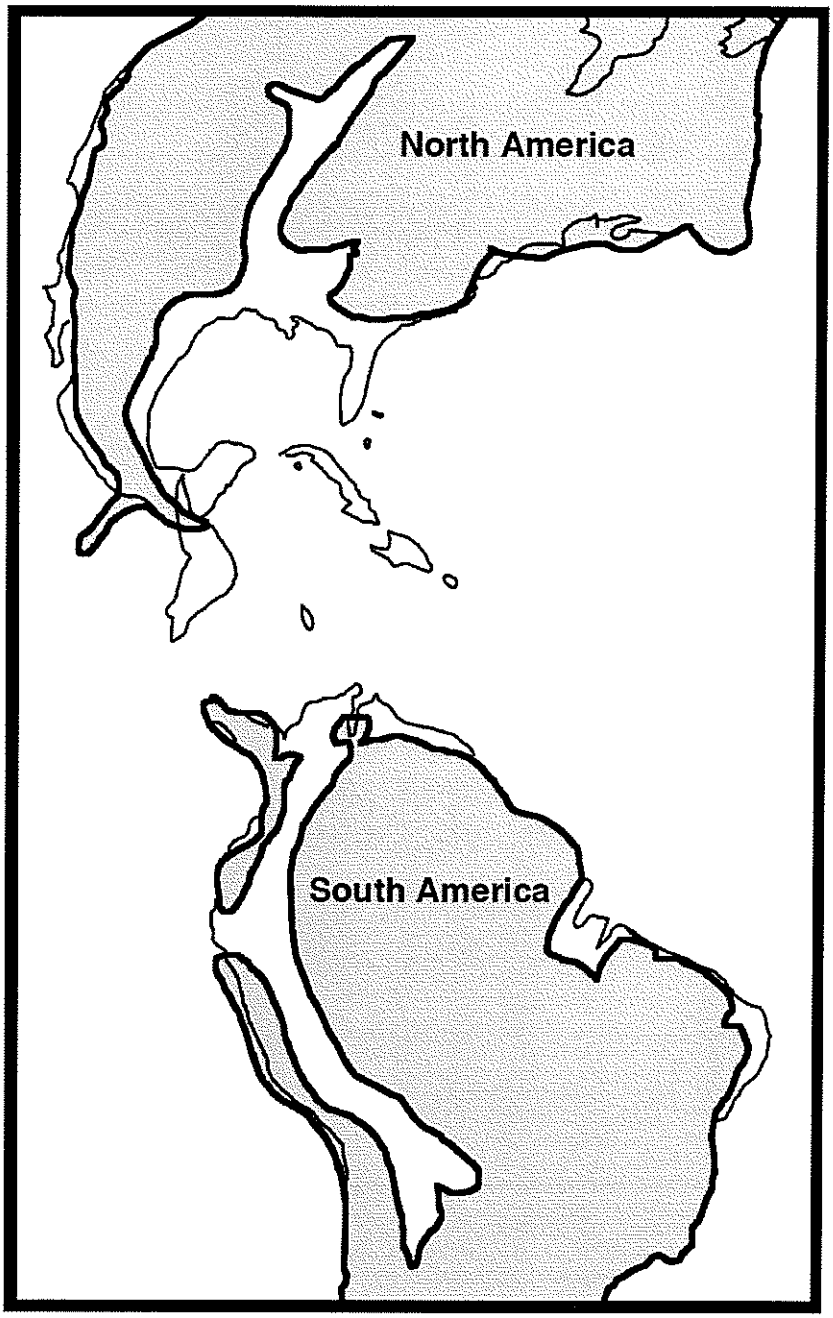

Figure 4. Paleogeographic map showing the absence of land bridges during the Early Tertiary (Paeleocene, 60 mya) (adapted from Smith et al. 1994). Gray areas represent the limits of coastlines.

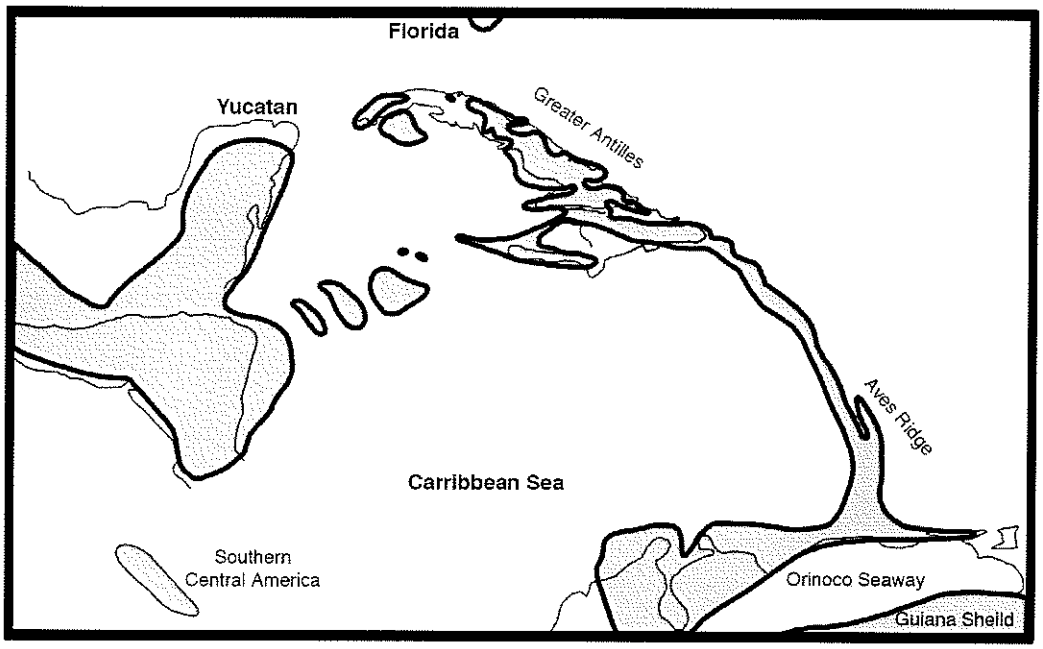

Figure 5. Paleogeographic map depicting quasi-land bridge connections comprising proto-Carribean land masses during the Eocene (35 mya) (adapted from Iturralde-Vinent and MacPhee 1999).

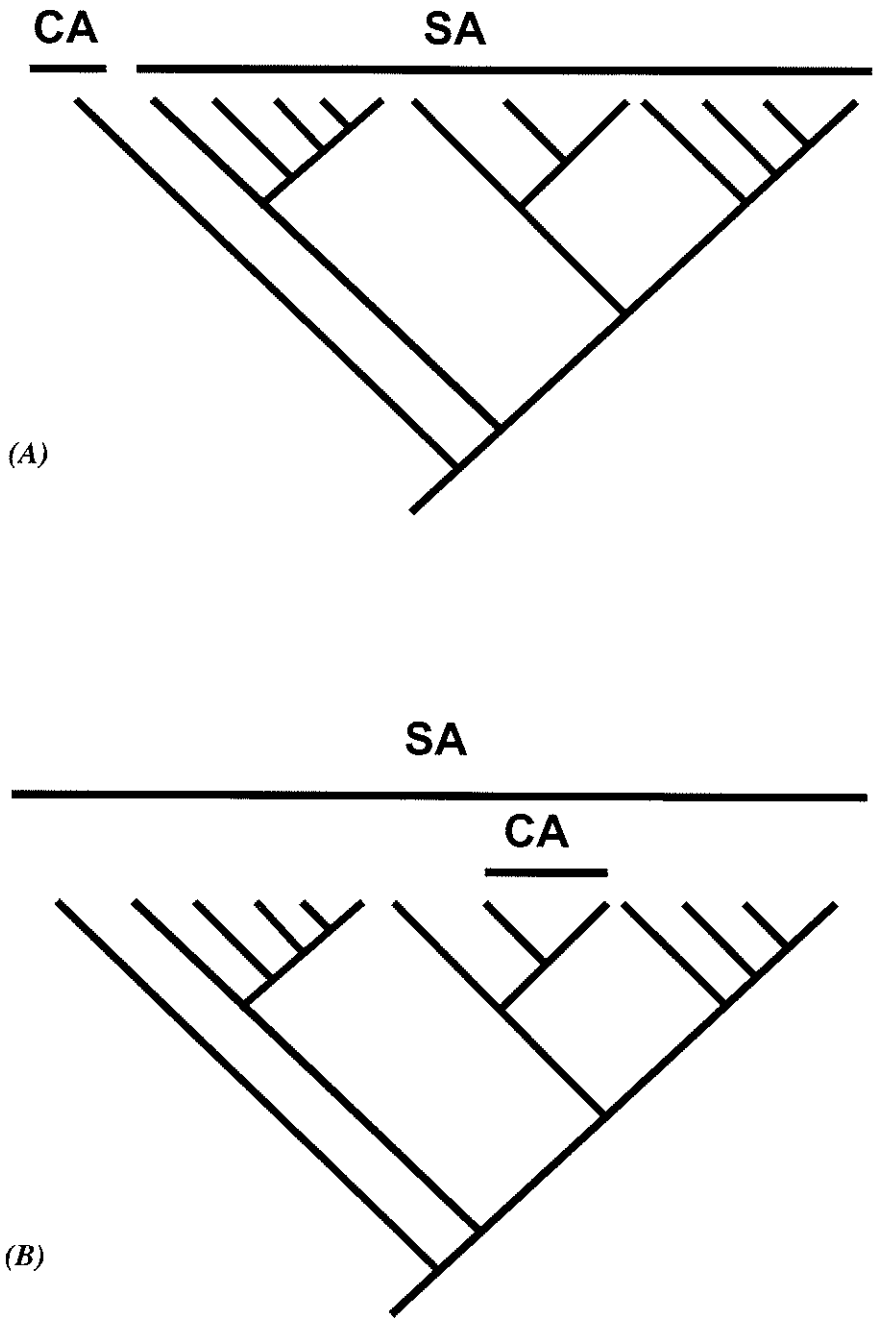

Figure 6. Expected tree topologies for *Gymnotus* based upon (**A**) the Old Southern/LateCreataceous-Early Tertiary hypothesis of Bussing (1985) and (**B**) the Pliocene/Pleistocene hypothesis of Miller (1966) and Meyers (1966). SA=South America lineages, CA=Central American lineages.

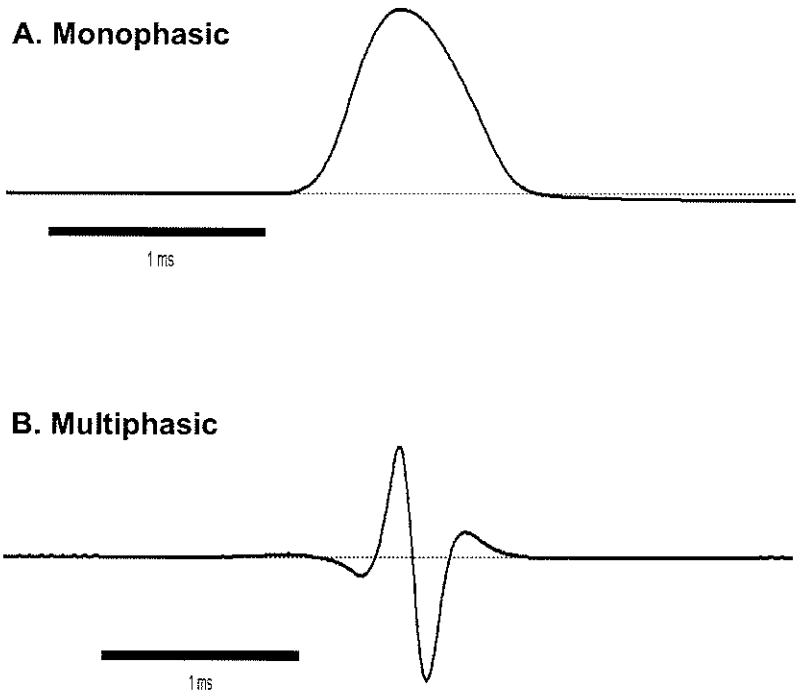

Figure 7. Shape of monophasic and multiphasic electric organ discharges as exhibited respectively by Central American (*G. cylindricus*) and some South American forms of *Gymnotus*.

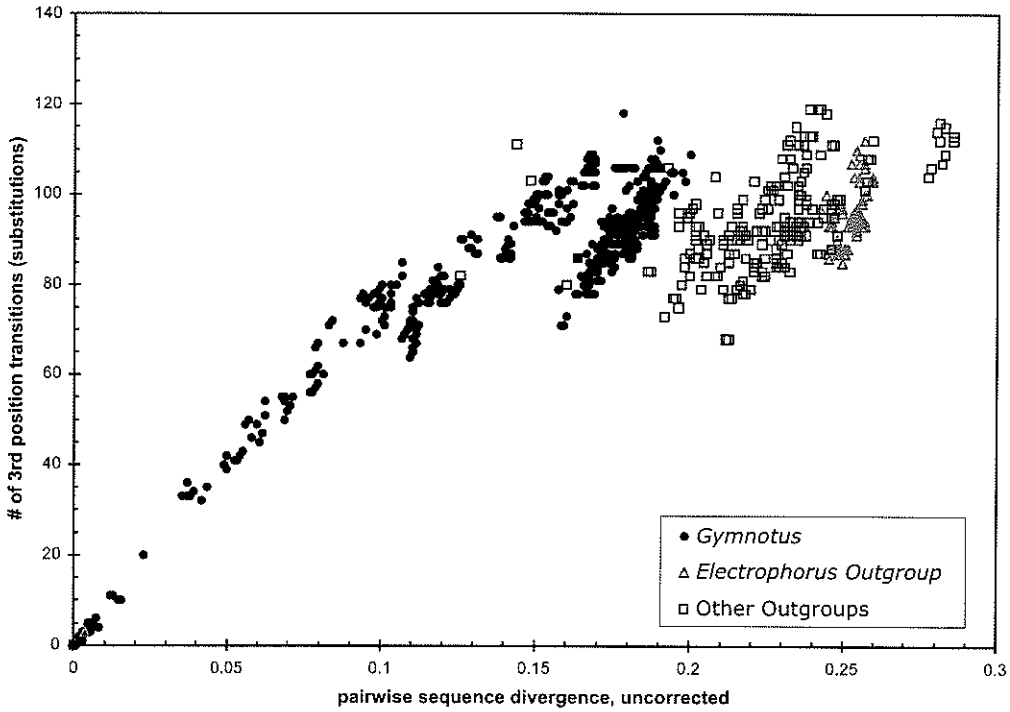

Figure 8. Saturation plot of cytochrome *b* third position transitions versus pairwise sequence divergence for all pairwise comparisons amongst all *Gymnotus* species (black dots), and between all *Gymnotus* species and the outgroups.

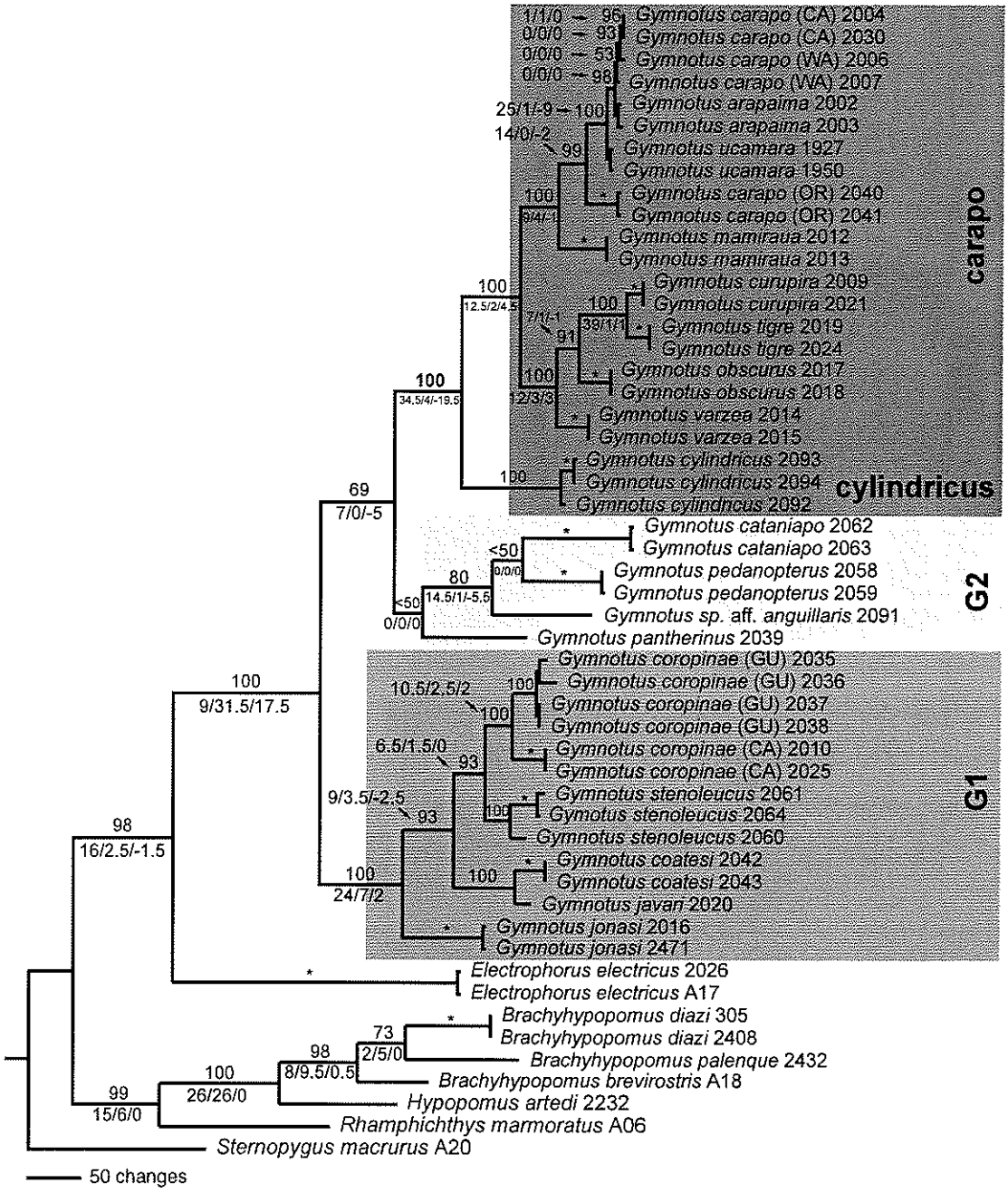

Figure 9. Single most parsimonious tree demonstrating *Gymnotus* phylogenetic relationships based on the combined analysis of nuclear (RAG2), mitochondrial (cytochrome *b* + 16S), and morphological data (2894 characters, 3256 steps, CI=0.48, RI=0.81). Numbers above nodes denote bootstrap support values 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. Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon, OR = Orinoco, GU = Guyana.

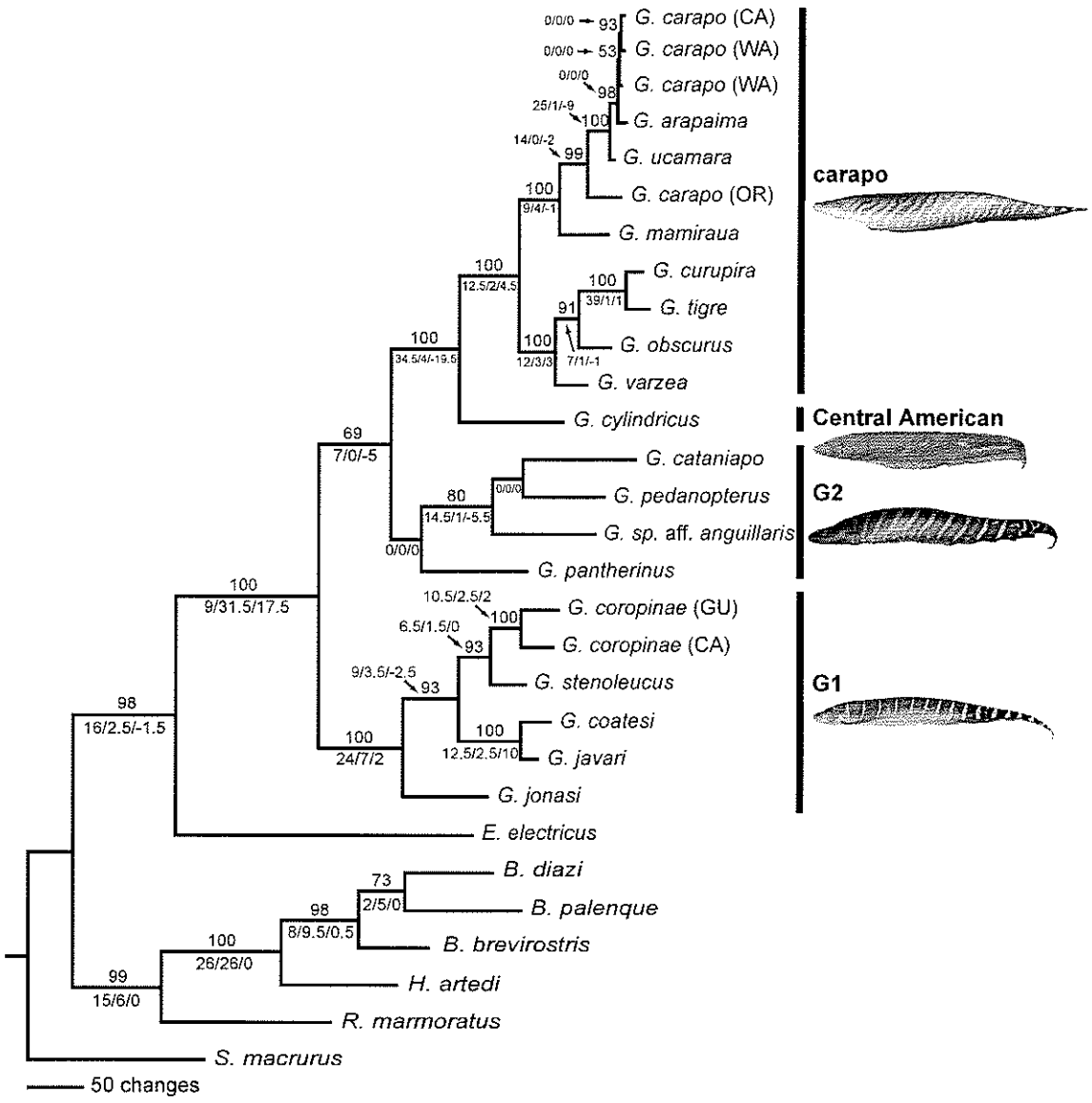

Figure 10. Single most parsimonious tree demonstrating *Gymnotus* phylogenetic relationships based on the combined analysis of nuclear (RAG2), mitochondrial (cytochrome *b* + 16S), and morphological data (2894 characters, 3256 steps, CI=48, RI=0.81). Duplicate taxa included in analysis were pruned from the tree. Numbers above nodes denote bootstrap support values based on 1000 replicates. Numbers below nodes or indicated using arrows denote Partitioned Bremer Support values (decay indices) for the mtDNA, RAG2, and morphology partitions respectively. Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon, OR = Orinoco, GU = Guyana.

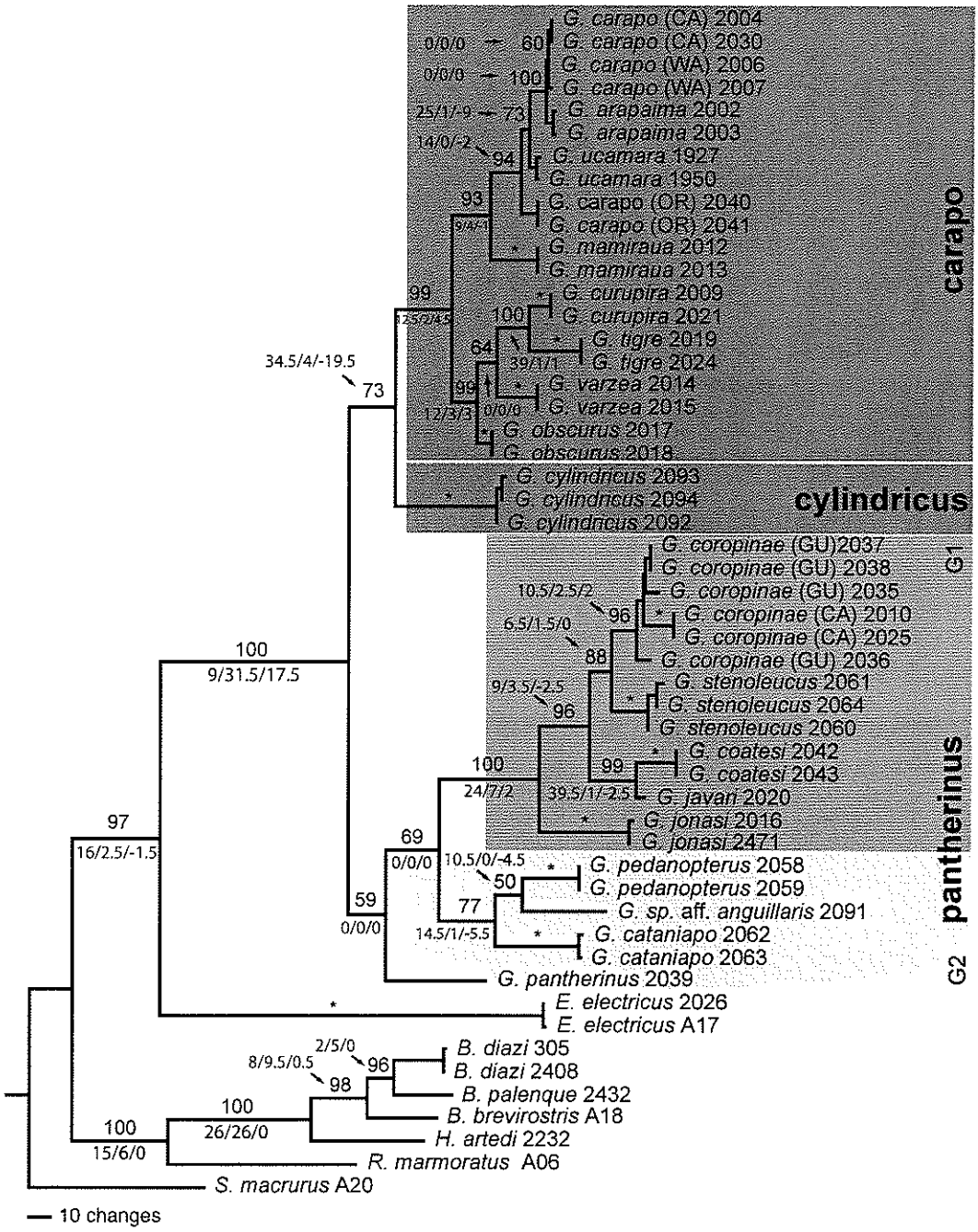

Figure 11. Single most parsimonious tree demonstrating *Gymnotus* phylogenetic relationships based on total evidence analysis excluding cytochrome *b* 3rd positions (2526 characters; 1544 steps; CI=0.59; RI=0.86). Numbers above nodes denote boostrap support values based on 1000 replicates. * indicates 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. Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon, OR = Orinoco, GU = Guyana. Species groups as per Albert et al. (2004).

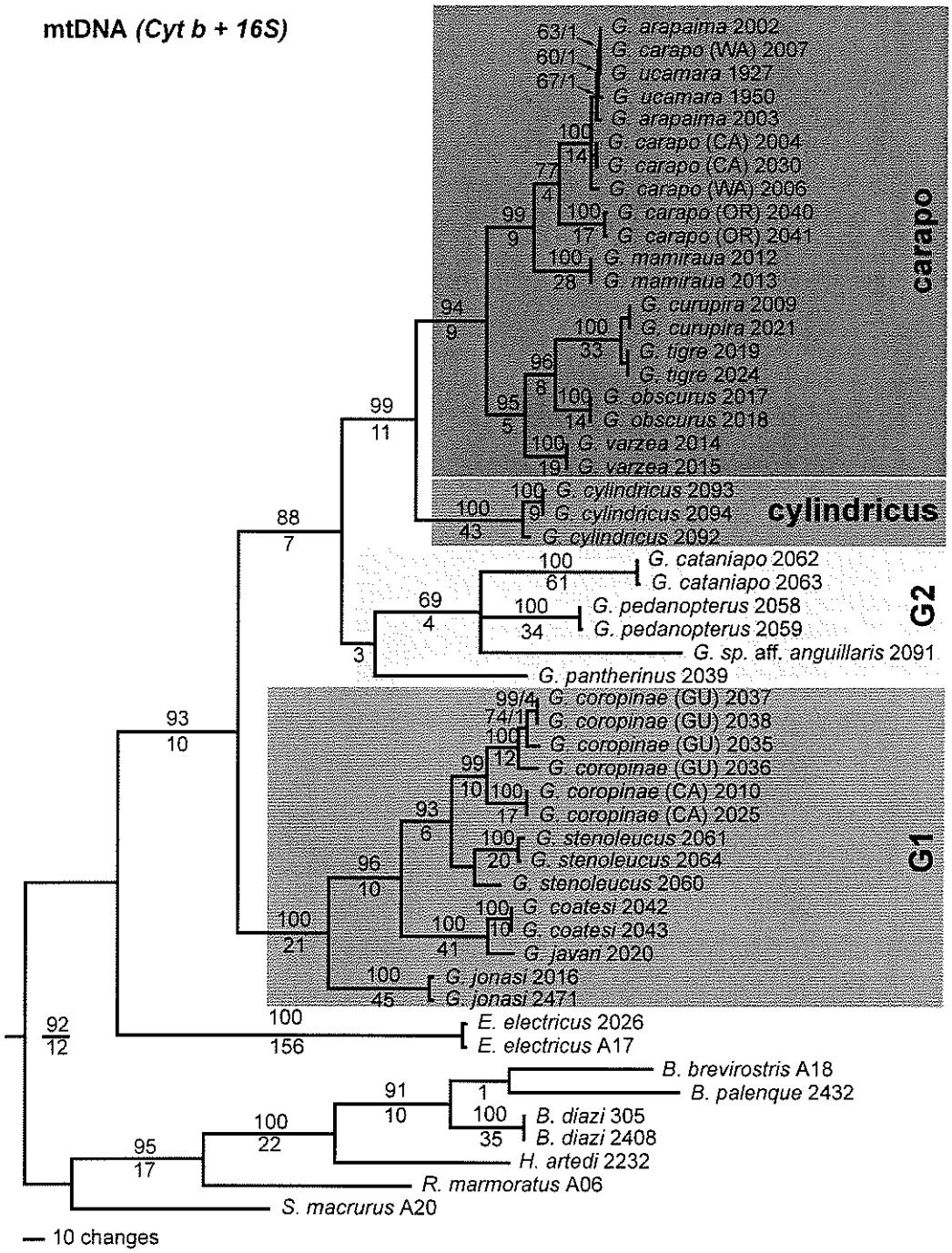

Figure 12. *Gymnotus* phylogeny based on the strict consensus of four trees produced by the analysis of the mitochondrial dataset (cytochrome *b* + 16S, excluding ambiguously aligned loop regions) including all taxa and individuals. (2411 steps; CI = 0.42; RI = 0.79). Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon, OR = Orinoco, GU = Guyana.

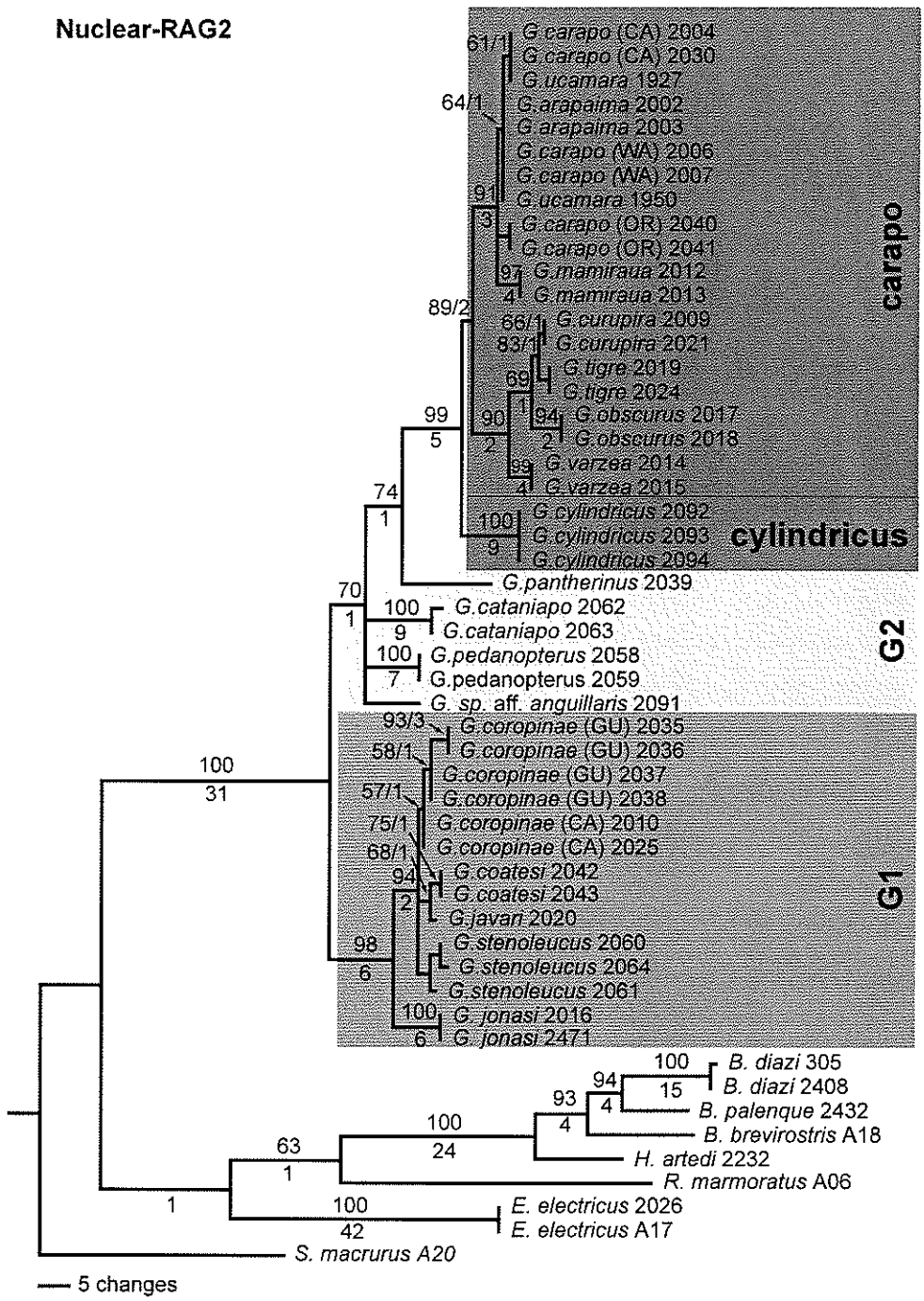

Figure 13. *Gymnotus* phylogeny based on the strict consensus of 8 most parsimonious trees produced by the analysis of the nuclear dataset (RAG2) including all taxa and individuals (489 steps; CI = 0.77; RI = 0.92). Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon; OR = Orinoco; GU = Guyana.

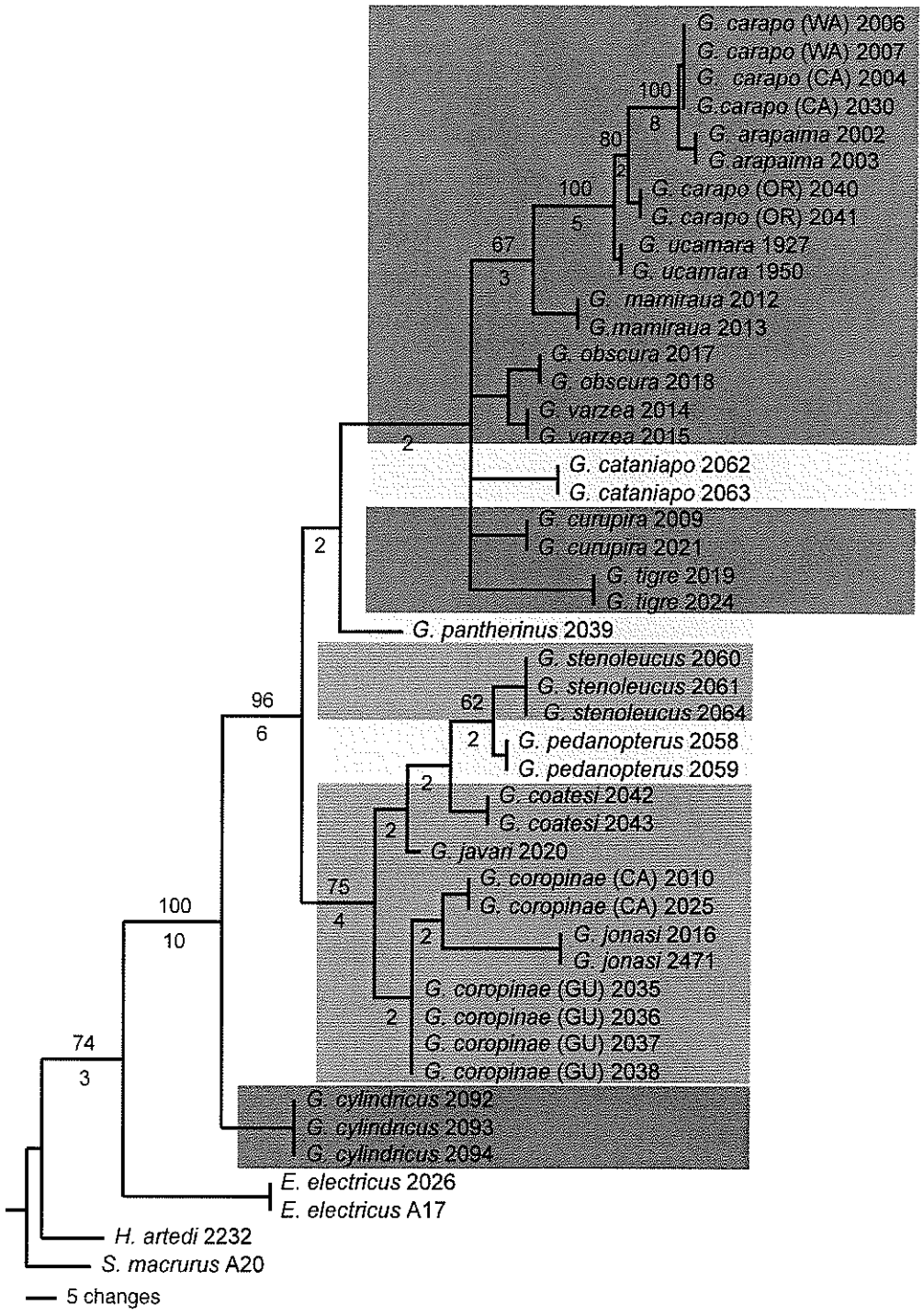

Figure 14. *Gymnotus* phylogeny based on 113 morphological characters of Albert et al. (2004) incorporating only the subset of taxa used in the present study (length=322, CI=0.463, RI=0.842).

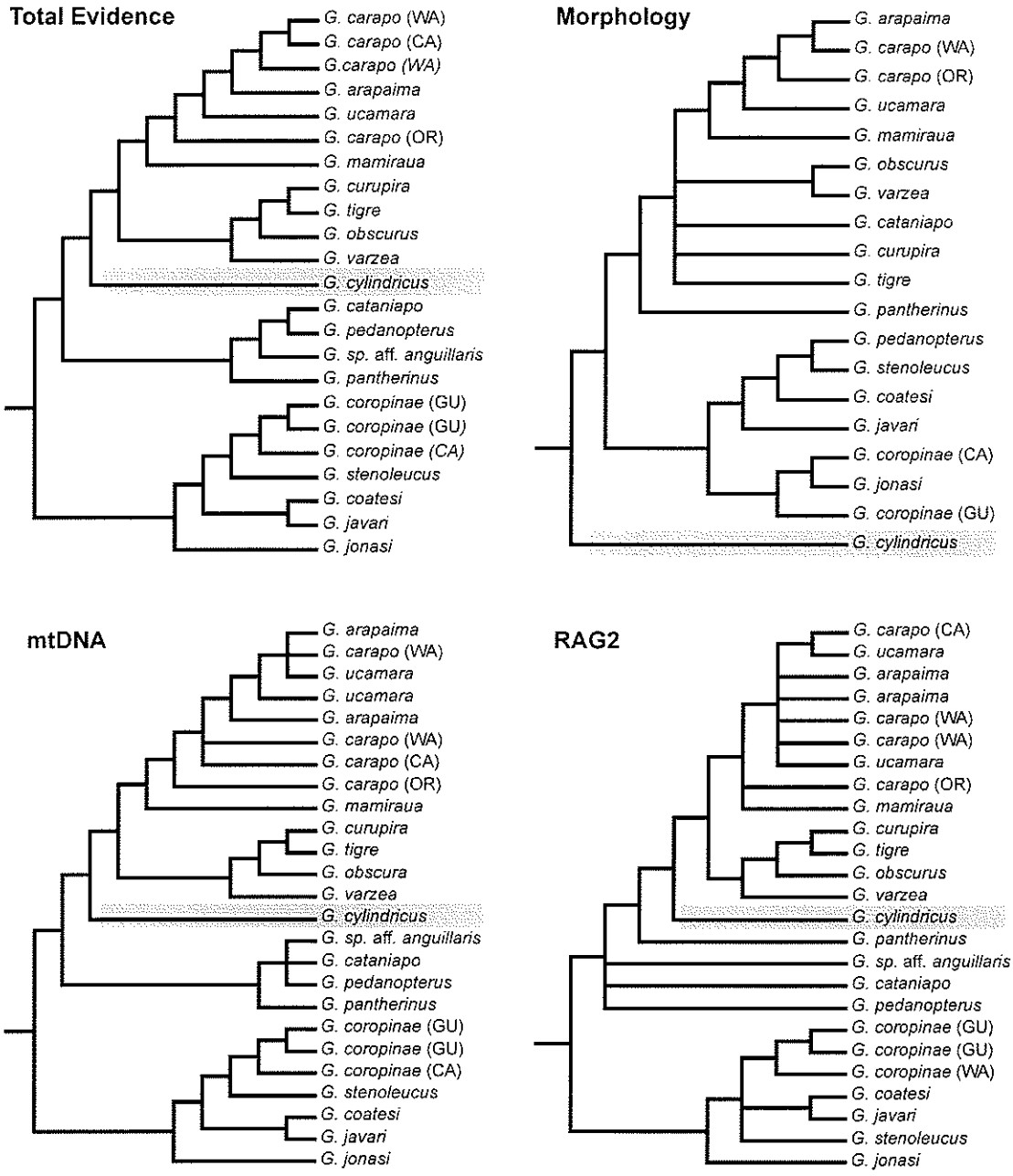

Figure 15. Pruned total evidence, mtDNA and RAG2 trees showing the derived position of the Central American clade, here represented by *G. cylindricus* (shaded) compared to its basal position in the morphology based tree. Biogeographic abbreviations: CA = Central Amazon; WA = Western Amazon, OR = Orinoco, GU = Guyana.

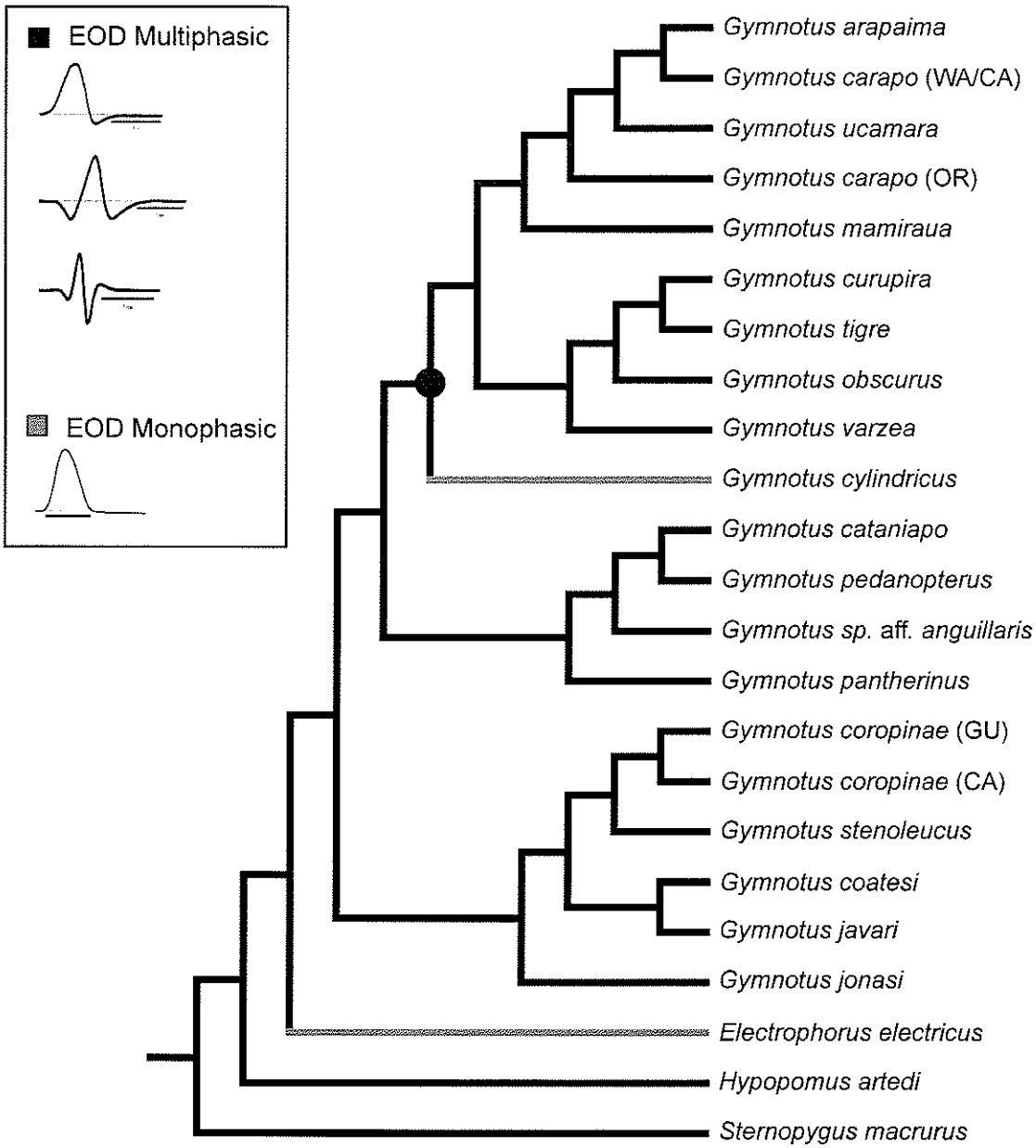

Figure 16. Monophasic and multiphasic electric organ discharges (EODs) optimized on the total evidence phylogeny showing the monophasic EOD of *G. cylindricus* to be independently derived from a multiphasic ancestor (indicated by black circle).

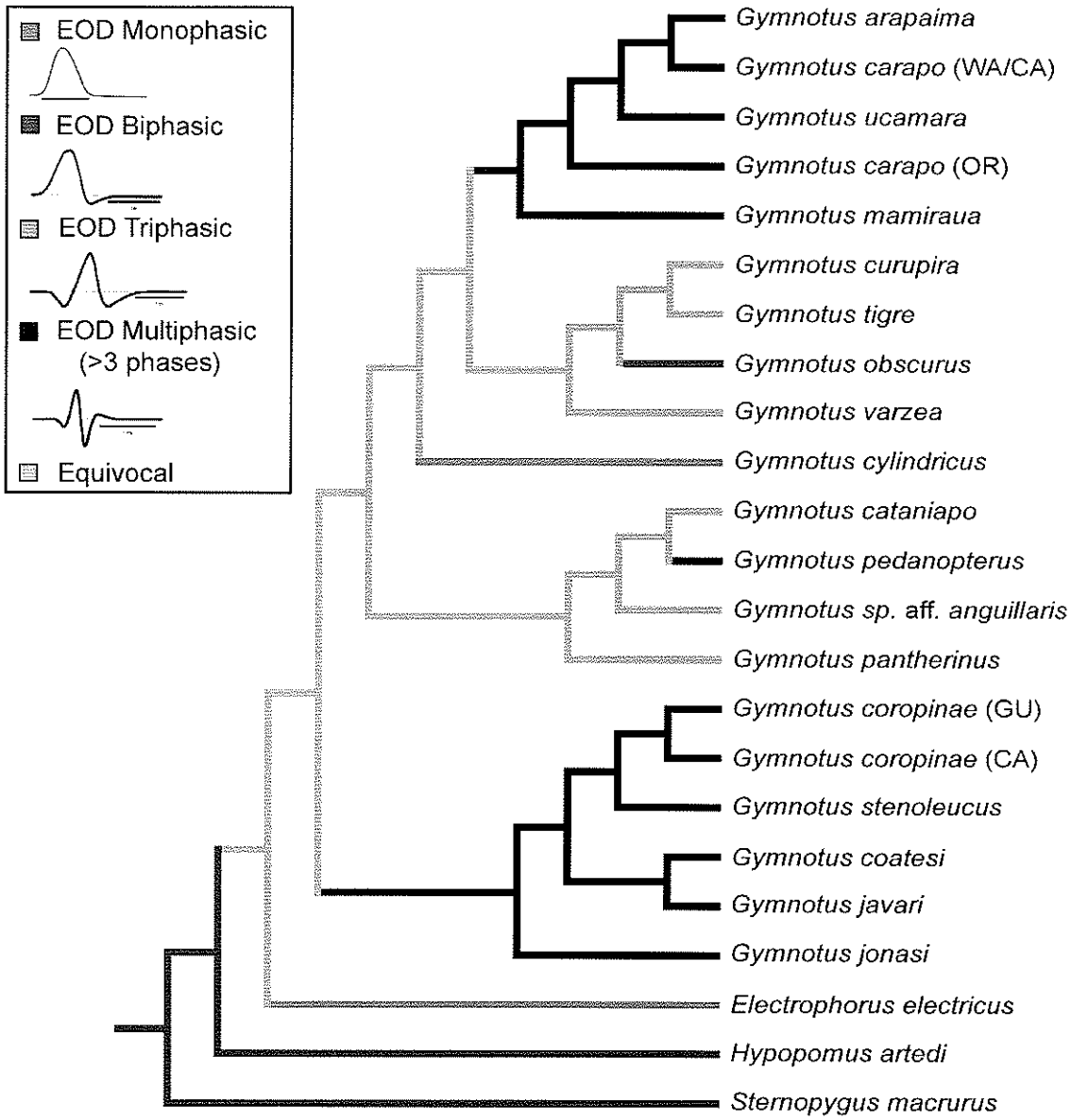

Figure 17. Number of phases above and below baseline of electric organ discharge (EOD) recordings of *Gymnotus* species optimized on the total evidence phylogeny using the ACCTRAN optimization technique.

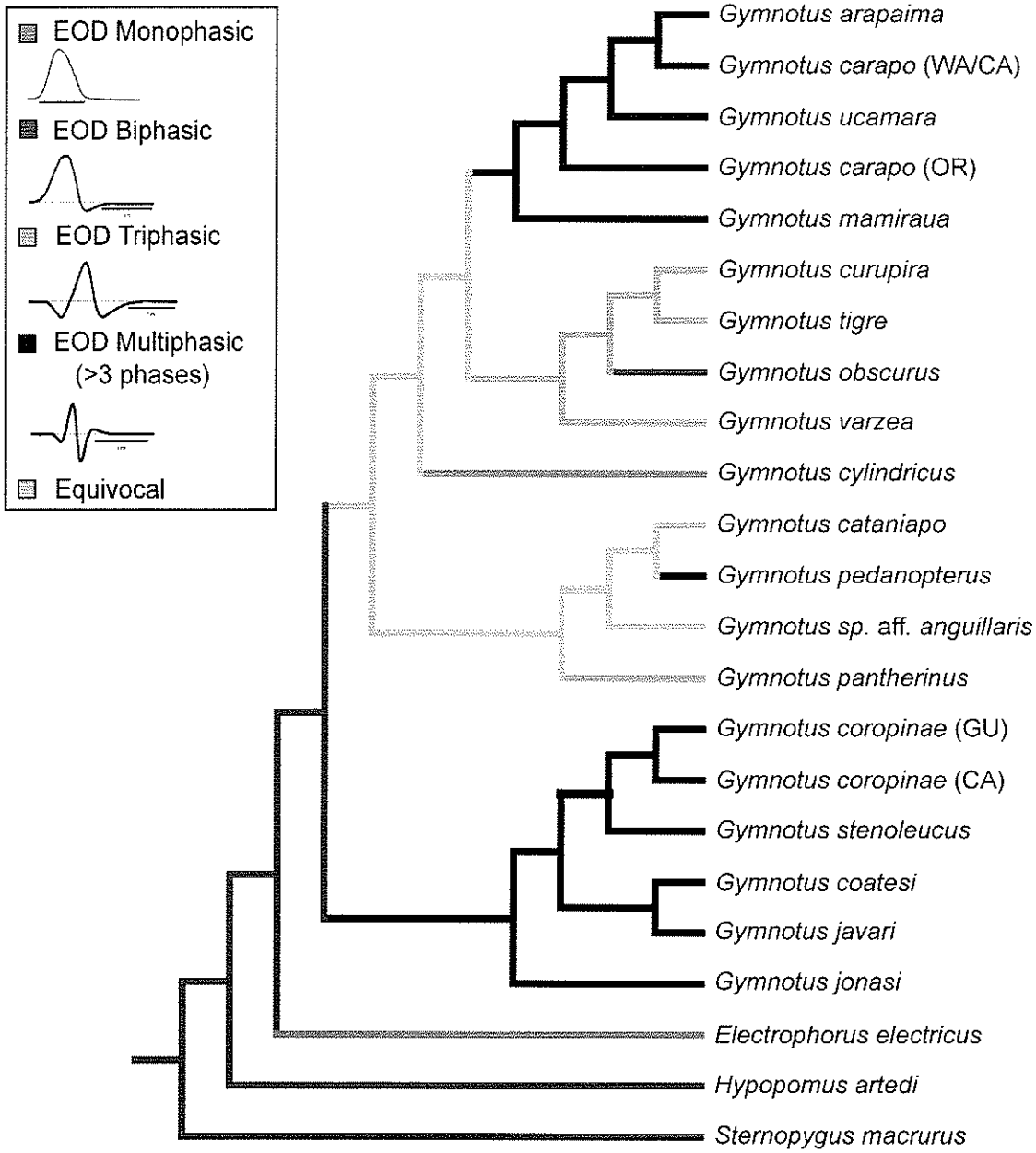

Figure 18. Number of phases above and below baseline of electric organ discharge (EOD) recordings of *Gymnotus* species optimized on the total evidence phylogeny using the DELTRAN optimization technique.

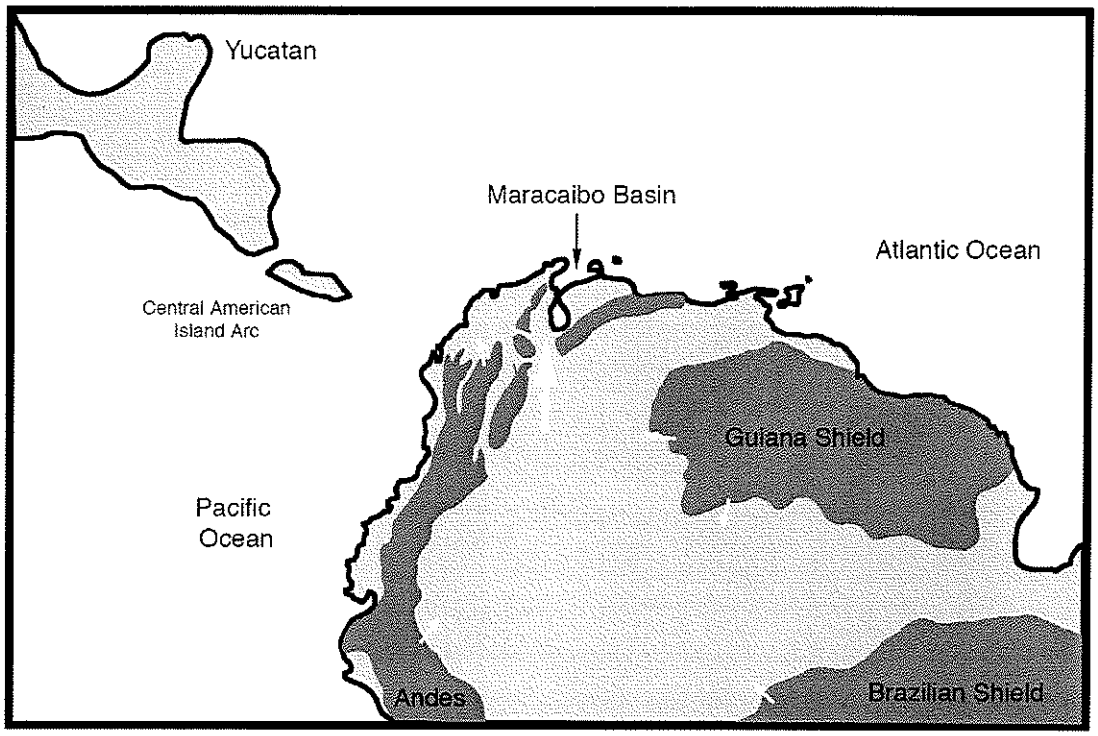

Figure 19. Miocene paleogeographic map depicting area of palaeo-Amazonas-Orinoco drainage and the postulated area of its entrance to the Caribbean (large white arrow) (after Hoorn et al. 1995).

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