Phylogenetic Analysis of the South American Electric Fishes (Order Gymnotiformes) and the Evolution of Their Electrogenic System: A Synthesis Based on Morphology, Electrophysiology, and Mitochondrial Sequence Data

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The order Gymnotiformes (South American electric fishes) is a fascinating assemblage of freshwater fishes that share the unusual ability to produce and sense electric fields used for electrolocation and social communication. In the last few decades, the electrogenic and electrosensory systems (EES) of these fish have served as an excellent model to study motor and sensory physiology in vertebrates. In an attempt to address the evolution of characters associated with the EES in the group, we applied maximum-parsimony (MP), minimum-evolution (ME), and maximum-likelihood (ML) methods to analyze 302 aligned bases of the mitochondrial 12S rRNA and 416 bases of the mitochondrial 16S rRNA of 19 gymnotiform genera representing all six recognized families. Six catfish genera (order Siluriformes) were also sequenced and used as outgroups. The phylogenetic hypothesis resultant from molecular data analysis differs in some respects from previous hypotheses based on morphological studies. Our results were most informative within the family level, as we were unable to elucidate the relationships among deeper branches in this order with sufficient confidence by using molecular data alone. The phylogenetic information of both mitochondrial DNA segments appears to be affected by functional constraints, and the resultant topologies were sensitive to different weighting schemes and the algorithm used. Nonetheless, we found unanimous support for the following phylogenetic relationships: (1) the family Sternopygidae is an unnatural group, and *Sternopygus* is the sole representative of a unique lineage within the order; (2) the family Hypopomidae is not monophyletic; and (3) the order Gymnotiformes is composed of at least six natural clades: *Sternopygus,* family Apteronotidae, a new clade consisting of the remaining sternopygids, families Hypopomidae + Rhamphichthyidae, family Electrophoridae, and family Gymnotidae. By combining molecular, morphological, and physiological information, we propose a new hypothesis for the phylogeny of this group and suggest a new family Eigenmanniidae n. (order Gymnotiformes).

Introduction

The South American electric fishes (order Gymnotiformes) occur throughout the freshwater habitats of the Neotropical region, from Guatemala to Argentina, and also on the Caribbean island of Trinidad (Mago-Leccia 1976). The clade reaches its highest abundance and diversity in the Amazonian Basin, where it inhabits a vast array of aquatic environments including the channels of main rivers, small creeks and ponds, "várzea" lakes, "igapós" (flooded forest), clefts in rocky substrates, water falls, sandy banks, and muddy pools formed during the dry season. The order currently consists of six families, 27 genera, and 94 nominal species (Mago-Leccia 1994), and the morphological diversity

within the group encompasses species like *Hypopygu lepturus,* which are sexually mature at less than 8 cm ir length and produce only weak electric fields, and the powerful electric eel (*Electrophorus electricus*) that cai reach more than 2 meters in length and discharge ove 600 V. Furthermore, with the introduction of new sam pling techniques in the South American tropics such a bottom trawling in the main-river channels and the uti lization of "electric fish detectors," new species are reg ularly being described, many of which may fall into nev genera.

Probably the most remarkable specialization of the gymnotiforms is their ability to use self-generated electri fields, not only for electrolocation of objects and othe organisms in their environment but also for social in teractions. Electric organ discharges (EODs) can be classified as either pulse or wave type (fig. 1) and ar produced by a specialized electrogenic tissue, the electri organ, which is embryologically linked to muscle cell (Keynes 1957; Kirschbaum 1983). The voltage gradien built across the skin during each EOD is monitored b
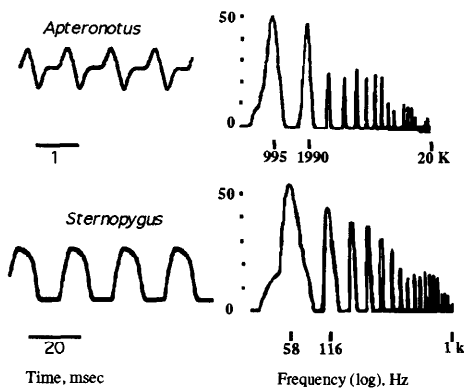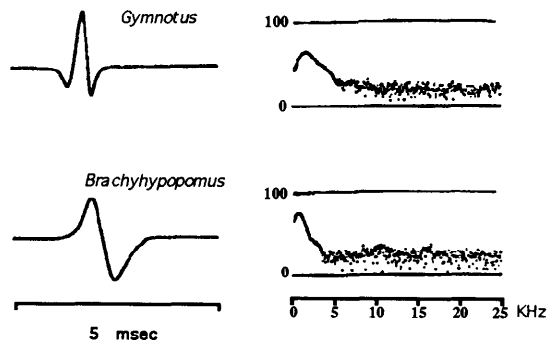
A)   Wave-type EOD

B)   Pulse-type EOD

FIG. 1.—Examples of the two basic types of EODs found in gymnotiforms. The signals were recorded differentially, with the positive electrode placed near the head of the fish and the negative electrode placed near the tail. The wave form, displaying voltage (positive up) as a function of time is shown on the left of its respective spectral frequency plots. The frequency spectra were obtained by fast Fourier analysis. *A*, Wave-type EODs of *Apteronotus* and *Sternopygus:* in the wave-type EOD, the discharge forms a distorted sinusoid, and the frequency spectrum shows narrow peaks at the fundamental frequency and higher harmonics. When played through a speaker, such signals sound like a pure tone with fundamental frequencies ranging from below 60 Hz (as in *Sternopygus*) up to almost 2,000 Hz (as in some apteronotids). *B*, Pulse-type EODs of *Gymnotus* and *Brachyhypopomus.* Pulse-type discharges are separated by relatively long intervals and have a much broader spectral profile. Repetition rates of pulse-type species vary from less than 1 Hz (in a resting *Electrophorus*) to over 100 Hz, as recorded during brief accelerations in *Brachyhypopomus.* When played through a speaker such signal sounds like a repetitive beat of a drum. (Modified from Heiligenberg 1977).

electroreceptor cells distributed over the fish's body (Lissman and Machin 1958; Machin and Lissman 1960; Bastian 1986), and modulations of the transepidermal voltage caused by any object, organism, or foreign EOD that interferes with the fish's own "electric space" are encoded by electroreceptors and transmitted to higher nervous centers where this information is processed (Heiligenberg 1987 and references therein). The electrogenic and electrosensory systems (EES), operating in tandem, allow the gymnotiforms to explore their habitat and to communicate with conspecifics by means of electric potentials. Moreover, being less dependent on visual cues, these fish are naturally active at night, when vision is ineffective and the predatory pressure reduced.

Over the past 20 yr, numerous studies have been published about various functional aspects of the EES of some gymnotiform species, in an attempt to better understand basic processes of motor and sensory physiology in vertebrates (Heiligenberg 1990, 1991). However, in order to address the differentiation in the neuronal circuitry of these fishes from an evolutionary perspective, we must also know the phylogenetic relationships among the different species being studied. Unfortunately, no consensus about gymnotiform phylogeny has been achieved. With exception of Hopkins and Heiligenberg (1978), who have suggested an evolutionary history for the group based on the physiological aspects

of their EODs, all other studies proposing taxonomic or phylogenetic relationships within the gymnotiforms are based on morphological characters (Ellis 1913; Mago-Leccia 1976, 1978, 1994; Mago-Leccia and Zaret 1978; Fink and Fink 1981; Lundberg and Mago-Leccia 1986; Gayet et al. 1992; Triques 1993). These studies did not provide a solid phylogenetic hypothesis for the order for various reasons: for instance, those that have employed a cladistic approach reached conflicting conclusions (see fig. 2). Previous studies also lacked an explicit phylogenetic perspective (Ellis 1913; Mago-Leccia 1978, 1994), dealt only with a subset of the order (Fink and Fink 1981; Lundberg and Mago-Leccia 1986), or both (Hopkins and Heiligenberg 1978; Mago-Leccia and Zaret 1978).

In the present study we present a phylogenetic hypothesis for the gymnotiforms based on 718 aligned nucleotides from two segments of the 12S and 16S mitochondrial ribosomal RNA genes. DNA sequences were obtained for 18 genera (19 species), including the majority of the genera of all described families (indicated by an asterisk in fig. 2). Six catfish genera (order Siluriformes) were also sequenced and used as the outgroups in the analyses. Catfishes were chosen as the outgroup because Fink and Fink (1981) have provided convincing evidence that catfishes and gymnotiforms are sister groups. Therefore, by using siluriform sequences
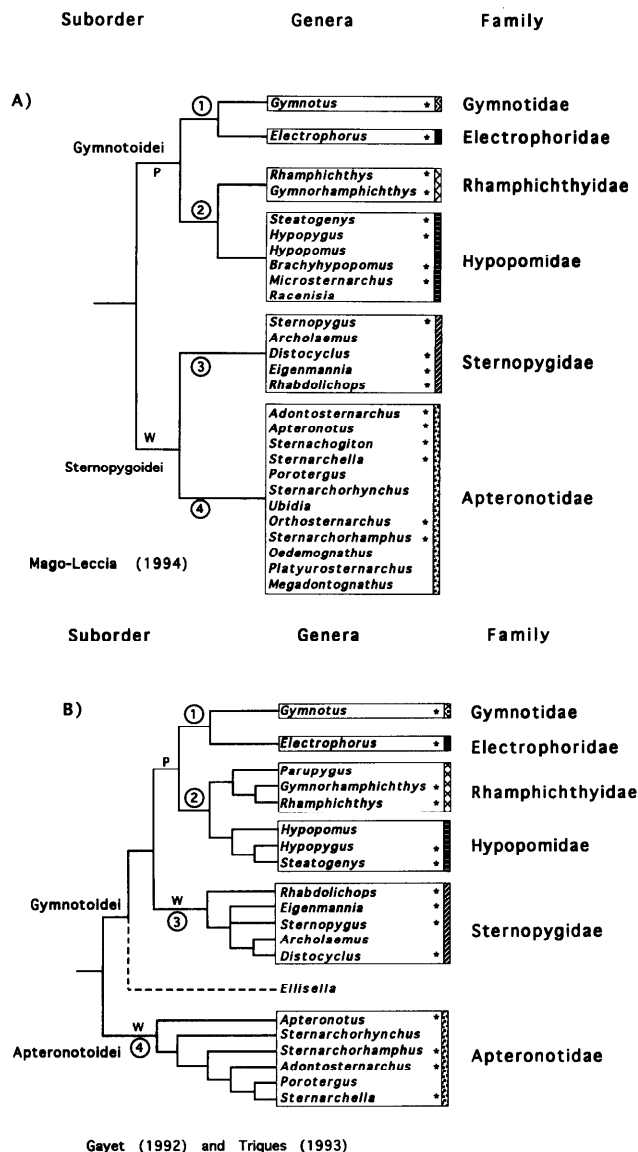
FIG. 2.—*A*, Schematic representation of the gymnotiform phylogeny based on the taxonomic classification recently proposed by Mago-Leccia (1994). Twenty-seven genera were arranged into six families. Previously, Mago-Leccia (1976) had defined four morphotypes in this order, depicted here by numbers in circles: 1, morphotype gymnotoideo; 2, morphotype rhamphichthyoideo; 3, morphotype sternopygoideo; and 4, morphotype apteronotoideo. *B*, Phylogeny of the order Gymnotiformes according to the cladistic analysis of 51 osteological characters by Triques (1993). Gayet et al. (1992) have suggested a phylogenetic hypothesis identical to that of Triques (1993) at the family level, the only difference being that the former authors included the fossil genus *Ellisella* (dotted line) in their hypothesis. No intrafamily relationships were described by Gayet et al. (1992). The four morphotypes defined by Mago-Leccia (1976) are preserved as monophyletic groups in Triques's and Gayet et al.'s hypotheses (number in circles), but noticeable discrepancies remain between these authors between and within the family level. The genus *Parupygus,* appearing in the family Rhamphichthyidae in *B* corresponds to *Hypopomus artedi*, a genus of the family Hypopomidae according to Mago-Leccia (1994). *P:* pulse-type EOD; *W*, wave-type EOD. The asterisk (∗) indicates the genera from which DNA was extracted and sequenced for this study.

we were able to homologize (align) a higher number of sites for phylogenetic analysis than if we had used more distantly related clades. Previously, authors have suggested that the characiforms and gymnotiforms shared an immediate common ancestor (Rosen and Greenwood 1970; Mago-Leccia and Zaret 1978; Mago-Leccia 1994 and references herein), but in a preliminary study (Alves-Gomes, Orti, Haygood, Meyer, and Heiligenberg 1993), we also investigated the molecular phylogeny of various gymnotiforms plus six siluriforms, five characiforms, three cypriniforms, and four osteoglossiforms genera. Our results, presented in the 1993 meeting of the American Society of Ichthyologists and Herpetologists, validated Fink and Fink's (1981) hypothesis, confirming the siluriforms as the sister group of the gymnotiforms.

The molecular phylogeny obtained in the present study was compared to current evolutionary hypotheses for the group based on morphology, and we also examined our results in conjunction with some neurophysiological and neuroanatomical data of the EES. By taking this approach we were able to generate the first phylogenetic hypothesis for the order Gymnotiformes based on cladistic analysis of molecular data. Employing this hypothesis as a framework, we discuss some basic aspects about the appearance and differentiation of the neuronal circuits involved in bioelectrogenesis in these fish.

Material and Methods
DNA

The genera utilized in the present study are listed in table 1. The majority of the specimens were obtained from field collections in the Brazilian Amazon, and some tissues were sampled from fish obtained from dealers or kept in captivity in laboratories in the United States. For preliminary identification purposes, either pictures were taken or the whole specimen was preserved. Some of the specimens were identified to species, but in most cases the identification was only to genus, because of the scarcity of reliable taxonomic keys. All preserved specimens will be deposited in the fish collection of the Instituto Nacional de Pesquisas da Amazônia, Brazil. This paper mainly addresses phylogenetic relationships at generic and family level, and therefore the only critical species names to be considered are the two species of the genus *Apteronotus* (table 1).

From each specimen, total genomic DNA was extracted by an SDS-based extraction buffer as described in Kocher et al. (1989), but using overnight incubation instead. The DNA was then purified by two extractions with equilibrated phenol, one or two with phenol/chloroform/isoamyl alcohol (25:24:1), and one with chloroform/isoamyl alcohol (24:1). DNA concentration in

the extract was inferred by comparisons with DNA size markers of known concentration in standard electrophoresis in 0.8% agarose gels with 0.5 µg/ml ethidium bromide.

Amplification and Sequencing

Polymerase chain reaction (PCR) was used to amplify one segment approximately 400 bases long from the 12S ribosomal RNA (rRNA) and a second segment about 550 bases long from the 16S rRNA gene. The 12S primers were modified from Kocher et al. (1989), and the sequences for the 16S primers were obtained from Palumbi et al. (1991). The sequences are 12S, L1091: 5'-AAACTGGGATTAGATACCCCACTAT-3' and H1478: 5'-GAGGGTGACGGGCGGTGTGT-3'; 16S, 16Sa-L: 5'-CGCCTGTTTATCAAAAACAT-3', and 16Sb-H: 5'-CCGGTCTGAACTCAGATCACGT-3'. The positions of the 3' end of each primer in the human mitochondrial genome (Anderson et al. 1981) are, respectively 1091, 1478, 2510, and 3059.

Double-stranded PCR products were obtained in a total volume of 25 µl, by following the concentrations described in Kocher et al. (1989). These amplifications were carried out in 25 cycles with the following temperature profile: denaturation for 1 min at 93°C, annealing for 1 min at temperatures varying between 50°C and 60°C depending on the primer specificity for the different genera and extension for 1 min 20 s at 72°C. The double-stranded PCR products were used either as a template for a second asymmetric PCR prior to manual sequencing (Gyllensten and Erlich 1988) or for cycle sequencing in an automated sequencer (Applied Biosystems Inc.). Asymmetric PCR products were obtained as described in Kocher et al. (1989) in a total volume of 50 µl, using the same temperature profile for the double-stranded PCR, but increasing the respective annealing temperature by 2°C and running 35 cycles. Manual sequencing was done using the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the Sequenase 2.0 kit (United States Biochemical) and following manufacturer's recommendations. Cycle sequencing (Applied Biosystems Inc.) with Taq polymerase and dye-labeled terminators was performed by following the protocol suggested by the manufacturer with a minor modification: after the double-stranded PCR product had been checked in 0.8% agarose gels with 0.5 µg/ml of ethidium bromide, the remaining 20 µl were filtered twice with 2 ml of ultrapure water in Centricon-100 (Amicon Inc.) ultrafiltration units for 30 min in a fixed-angle rotor Sorval centrifuge at 1,000 × g. From a final volume of approximately 40 µl of filtered DNA 9.5 µl were used as template during the cycle sequencing. All other steps followed the instructions accompanying the cycle sequencing kit (Applied Biosystems Inc.). The amplified product of the cycle sequencing was cleaned by one filtration with a Centri-sep column (Princeton Separations), dried under vacuum, and loaded in the automated sequencer after being rehydrated with the standard solution suggested by the manufacturer. All final sequences were obtained by reconciling sequences from both L and H strands.

Complete sequences are available from GenBank under accession numbers U15251–U15275 (12S rRNA) and U15226–U15250 (16S rRNA). Aligned sequences can be obtained on request from the first author.

Sequence Alignment

The DNA sequences were edited with the multiple-sequence editor ESEE (Cabot 1987), and preliminary alignment was achieved by using the default parameters of CLUSTAL (Higgins and Sharp 1988, 1989). The alignment of the 12S rRNA was refined by superimposing the sequences of the various genera over the proposed bovine secondary structure (Gutell et al. 1985) (fig. 3A). By visually comparing the superimposed sequences, we defined segments corresponding to loops and stems, established base-pairing, and improved the alignment. The same procedure was performed for the 16S rRNA (fig. 3B) in reference to the published secondary structure of Xenopus (Gutell and Fox 1988).

Stems, Loops, Transitions, Transversions, and Indels

We used equal weighting of stems and loops in our analysis. The primary concern was to establish a well-corroborated alignment from which homology between the nucleotide sites could be inferred unambiguously.

Before estimating the transitional bias in the data set and defining relative weights to be given to transitions (TS) and transversions (TV) in maximum parsimony, we joined the 12S and 16S sequences into a single matrix and computed the absolute distances (total number of sites with different character states between each pair of taxa) without corrections for multiple hits. Subsequently we determined the number of TS, the number of TV, and the number of indels (ID) for all possible pairs of taxa. Indels were treated as a fifth character for calculating the distances and divergences but were subsequently treated as missing character in the phylogenetic analyses. We calculated the percentage divergence between each taxon by the formula suggested by Mindell and Honeycutt (1990, p. 551). We also calculated the TS/TV ratio for all pairwise comparisons and plotted it versus the percentage divergence (fig. 4).

We used three different combinations of costs/weights for TS and TV in our maximum-parsimony analyses: TS1TV1 (meaning that both TS and TV were equally weighted); TS1TV2 (a TV has twice the weight
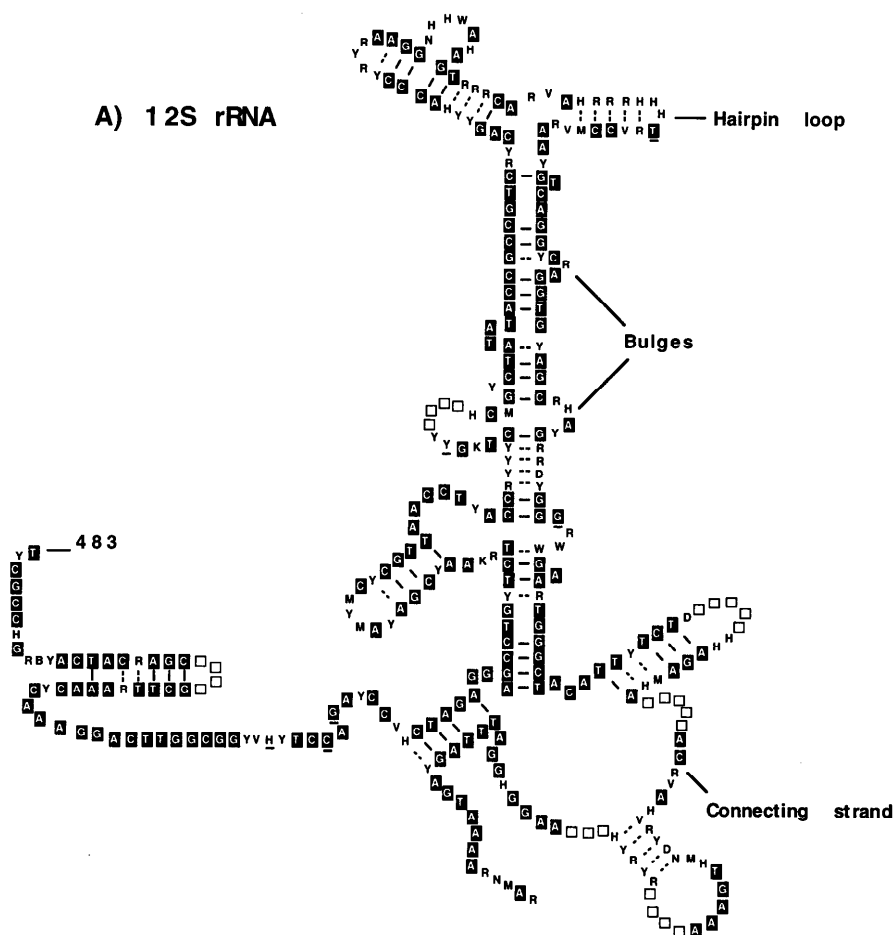
A) 12S rRNA

FIG. 3.—Proposed secondary structure of the 12S (*A*) and 16S rRNA (*B*) for the teleost fish sequenced in this study. The consensu sequences for all 25 genera were superimposed upon the published secondary structures of *Bos taurus* 12S rRNA (Guttel et al. 1985) an *Xenopus* 16S rRNA (Guttel and Fox 1988). The letters represent the standard IUPAC/IUB single-letter code for nucleotides. Invariant region are shown as filled boxes, and empty squares depict the sites that could not be aligned and were excluded from all analyses. *N* means that a four nucleotides were registered for that particular site, and underlined letters indicate that a gap was aligned at that site for at last 1 of the 2 genera. Solid lines between bases represent complementary base pair formation for all genera, and dashed lines denote that pairing was attaine in at least one genus. The number in the first base of each segment corresponds to the number of the respective site in the bovine and *Xenopu* sequences of their original publications. Bulges, connecting strands, and hairpin loops were defined after Erdmann et al. (1985).

of a TS), and TS1TV4. By adopting these three different weighting schemes, we attempted to compensate for increasing saturation of TS, as the "% divergences" increases (fig. 4).

Phylogenetic Analysis

We estimated phylogenetic relationships among the taxa sequenced by using three phylogenetic methods: maximum-parsimony (MP) analyses were performed by the PAUP program, version 3.1.1 (Swofford 1993); minimum-evolution (ME) trees were obtained with the METREE program (Rzhetsky and Nei 1993); and maximum likelihood (ML) was calculated with the FAST-DNAML program (Olsen et al. 1994) available

in the DNASYSTEM package (Smith 1988) at the Uni versity of California, San Diego. Whenever applicable either all siluriforms (for MP and ME) or the genu *Malapterurus* only (for ML) were designated as the out group.

Under MP, we first performed heuristic searche with 50 replications of random stepwise additions fo each one of the weighting schemes adopted (TS1TV1 TS1TV2, and TS1TV4). The other options in PAUI were selected as follows: uninformative characters wer ignored, only minimal trees were kept, and zero-lengt branches collapsed. Branch-swapping was performed i all starting trees, and during branch swapping, TBR (tre bisection-reconnection), MULPARS (save all minima trees), and steepest descent options were always selectec ACCTRAN (accelerated transformation) was also cho
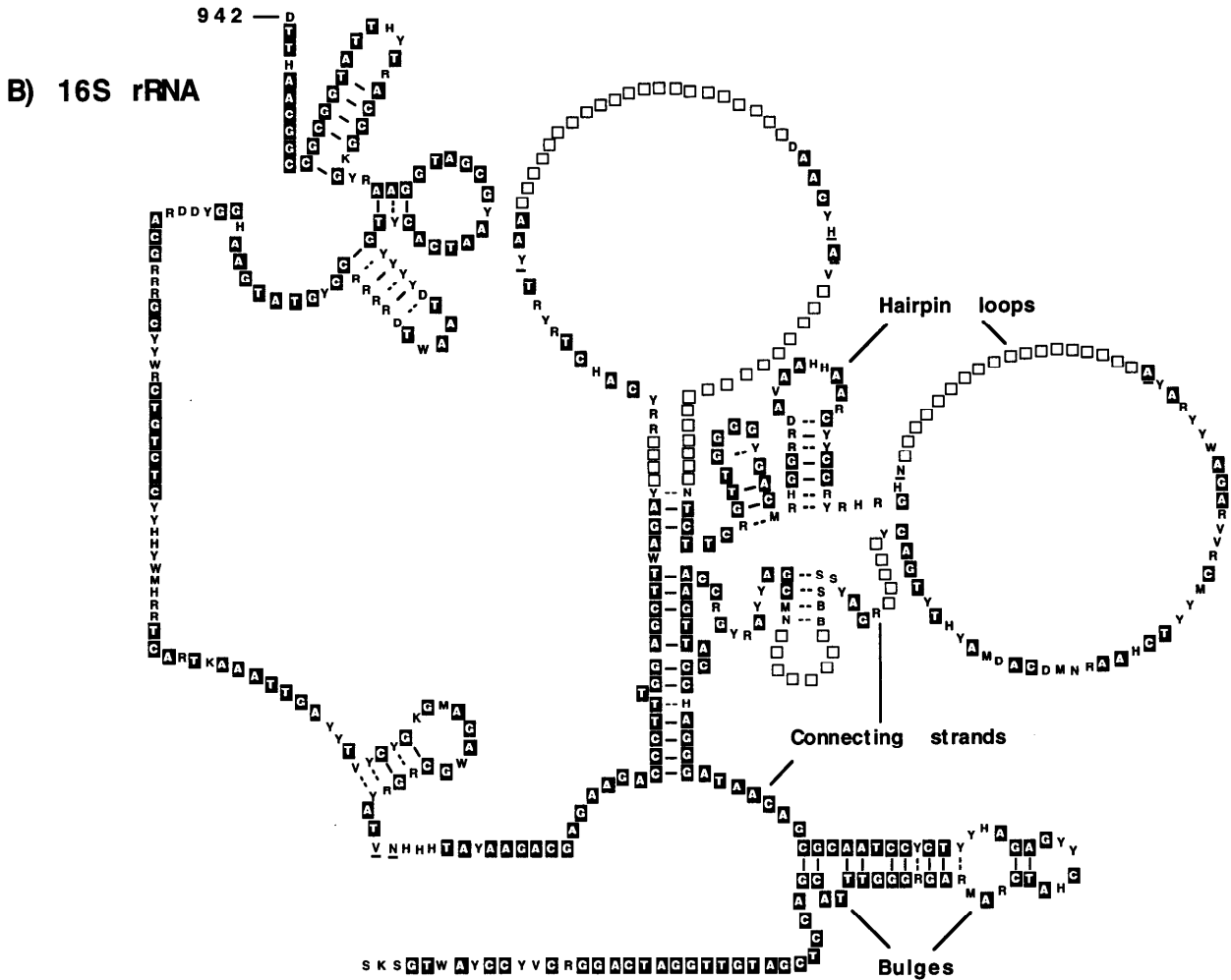
FIG. 3 (*Continued*)

sen. To have an estimate about the degree of support of our data set for the resultant tree topologies in PAUP, we executed 100 bootstraps replications (Felsenstein 1985) with five heuristic, random stepwise additions being performed at each replication, for each of the three TS/TV weights used. We also examined the content of phylogenetic information in our data set by checking the skewness of the tree distribution and the g1 values (Hillis and Huelsenbeck 1992) for 10,000 random trees for each of the above weighting sets. The g1 statistic was performed first with no constraints in the data set and subsequently by constraining the well-supported monophyletic taxa. This was done in order to investigate whether there was phylogenetic information to resolve the deeper branches of our trees (Hillis and Huelsenbeck 1992).

Minimum-evolution trees (Rzhetsky and Nei 1993) were generated using both Jukes-Cantor one-parameter and Kimura's two-parameter models to correct pairwise distances for multiple hits. Sites with ambiguous characters were excluded for all sequences. Alternative trees around the temporary minimum-evolution trees were generated by using the topological distances (d_T) method, when all trees with topological distances of two and four units from the temporary minimum-evolution trees were examined. In addition, 1,000 bootstrap topologies were examined for each correction model using the scaling factor $f = 1$.

For ML calculations, we performed 25 searches using a different seed for the "jumble" option each time. All other parameters used in FASTDNAML (Olsen et al. 1994) were the default options of the program.

Results

Before reporting our findings with the phylogenetic analysis, we will first consider some of the structural particularities of the two segments studied that are related to their utilization in phylogenetic analyses.
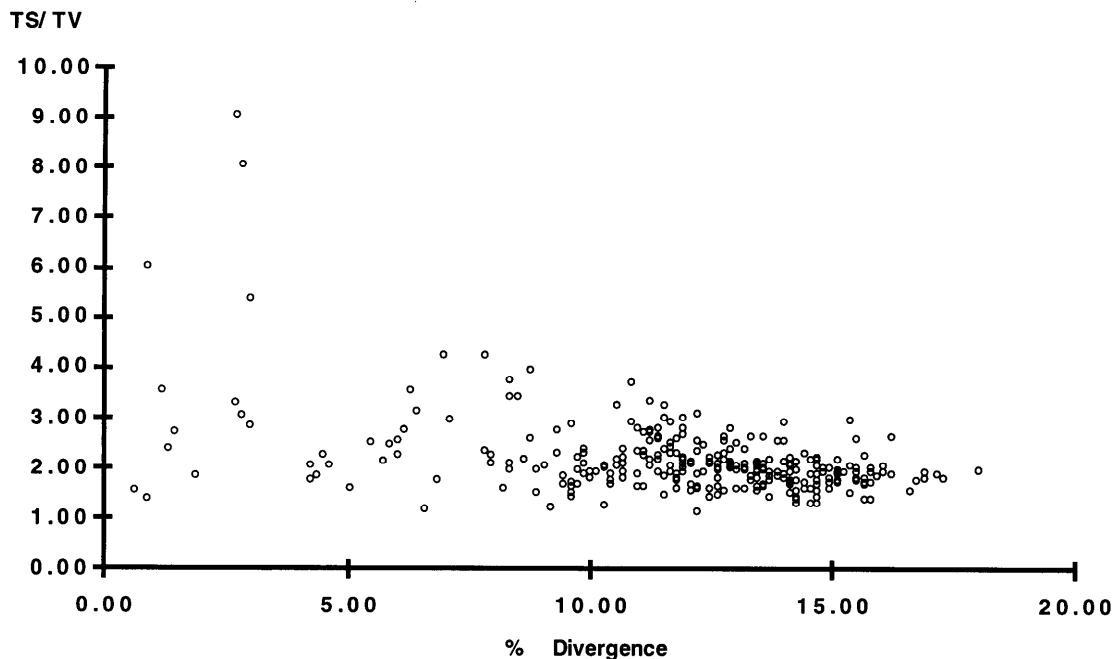
TS/ TV

FIG. 4.—Ratio of TS/TV for the combined 12S and 16S sequences as a function of percentage divergence

12S and 16S rRNA Sequences

The complete sequences used in this study are available from GenBank (IntelliGenetics Inc.). The 12S rRNA sequences are under accession numbers U15251–U15275 and the 16S rRNA sequences under numbers U15226–U15250. The aligned data set can be obtained on request from the first author.

We were able to align 302 bases of the 12S rRNA and 416 of the 16S rRNA gene unambiguously. From a total of 211 informative sites (178 in the ingroup), 94 (77 in the ingroup) were in the 12S segment and 117 (101 in the ingroup) in the 16S rRNA. Twenty-five sites in the 12S rRNA and 72 at the 16S rRNA which could not be aligned with confidence were discarded for all the analyses (see squares in fig. 3). Also, 60.9 percent and 61.5 percent of the nucleotides were invariant in all 12S and 16S sequences, respectively. In the combined data set, ID represent 4.02%, whereas TV represent 31.94% and TS 64.04% of all variable sites, averaged for all pairwise comparisons. Figure 5A and 5B show, respectively, the absolute number of each type of substitution as a function of the percentage divergence and the proportion of TS and TV in relation to the total number of bases for all pairwise comparisons.

From the aligned sequences we computed a single consensus sequence for all 25 genera and compared it with the proposed secondary structure for the cow's 12S rRNA (Gutell et al. 1985) and *Xenopus's* 16S rRNA (Gutell and Fox 1988) (fig. 3A and B). The geometric/spatial relationships between loops and stems as pro-

posed for land vertebrates is well preserved in fish, and the primary discrepancies among our sequences are length variations in the bigger hairpin loops in the 16S rRNA.

The base compositions of both 12S and 16S rRNA are similarly biased. No consistent differences in base composition were detected between the different genera (table 1). There is a significant excess of adenine, and thymine is underrepresented in both segments ($F < 0.001$ for 12 fish; $0.001 < P < 0.01$ for 12 fish; and $0.01 < P < 0.02$ for 1 fish) χ^2, one-sample test, df = 3: (Siegel 1956, pp. 35–59).

In the teleost fishes used in this study, as has been documented for the mt rRNA of other vertebrates (Brown et al. 1979; Hixson and Brown 1986; Mindell and Honeycutt 1990), there is a clear tendency for the accumulation of TS in recently diverged taxa. Between closely related species (up to 5% divergence), TS can account for up to 90% of the substitutions (fig. 4). However, as the divergence among taxa increases the TS/TV ratio declines and stabilizes around two for divergences between 10% and 15%. For taxa with divergences above 15%, the average ratio goes to values between one and two.

Phylogenetic Relationships within Gymnotiforms

Depending on the tree reconstruction method as well as the TS/TV weight utilized, slightly different resultant topologies were obtained. Rather than showing a large number of clado/phylograms of limited signifi-
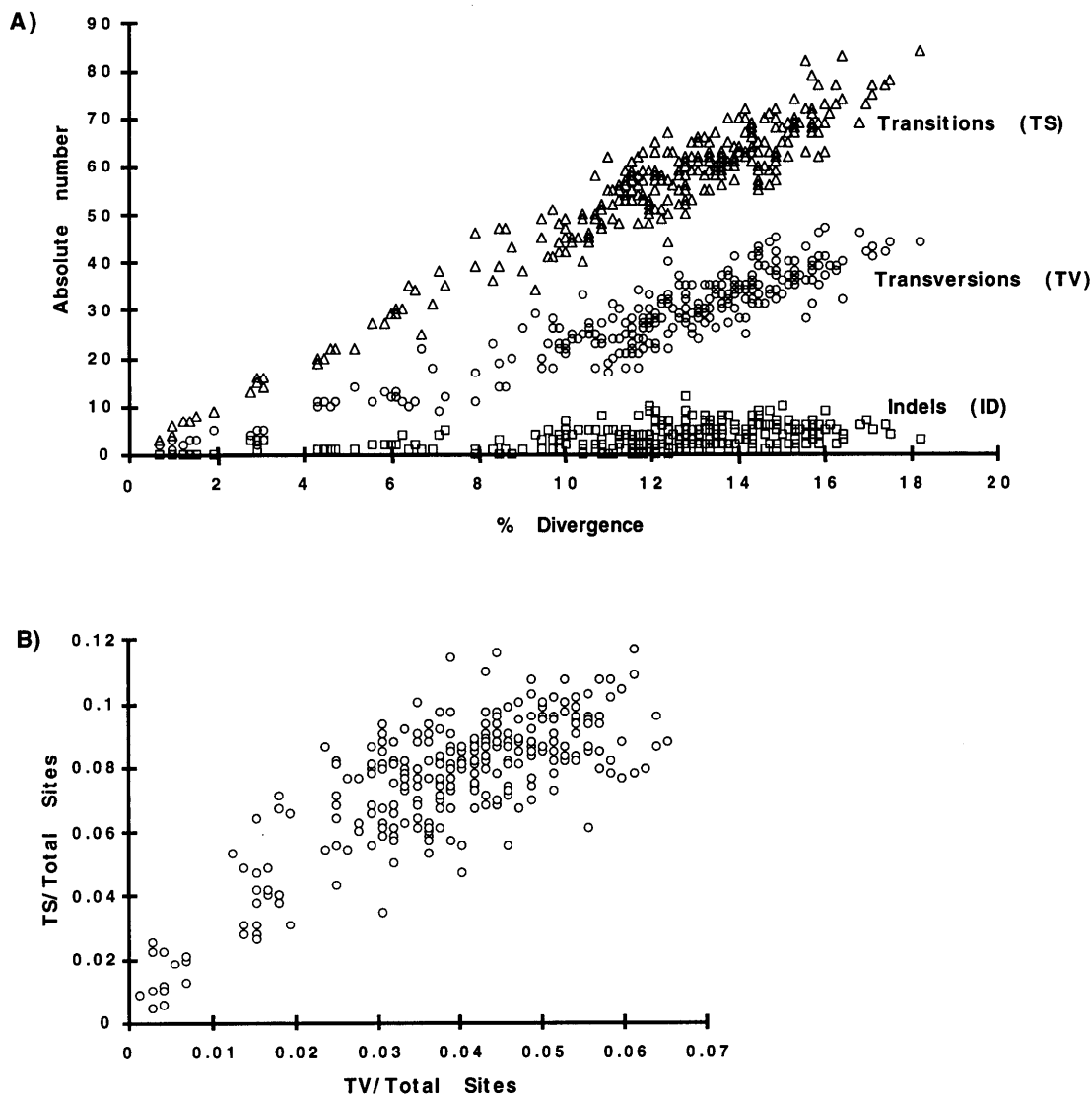
FIG. 5.—*A*, Absolute number of transitions (TS), transversions (TV), and indels (ID) for all pairwise comparisons as a function of the percentage divergence of the combined 12S and 16S data set. *B*, Proportion of TS and TV in relation to the total number of bases plotted for every pairwise comparison.

cance, we will present only the strict consensus topology for all MP trees, the consensus between Jukes-Cantor's (1969) and Kimura's (1980) correction for ME, and the phylogram with best Ln likelihood for ML. Variations from the topologies shown will be discussed in the text.

Using maximum parsimony, we obtained nine equally most parsimonious trees for TS1TV1 with 670 steps, 20 trees for TS1TV2 with 882 steps, and three trees for TS1TV4 with 1,269 steps. The strict consensus for all 32 most parsimonious trees has the same topology as the strict consensus for TS1TV2 alone (fig. 6). The main discrepancy in the phylogenetic resolution between the three weighting schemes is the position of the clade formed by *Ele* + *Gym* (please see abbreviations in table 1). Other less conspicuous changes occur within the

family Apteronotidae and within the clade formed by the families Hypopomidae and Rhamphichthyidae, as we change from TS1TV1 to TS1TV4. Under TS1TV1, the strict consensus of nine trees shows *Ele* + *Gym* as the sister group of all remaining genera, and the remaining genera form a polytomy with four lineages: Spy, family Apteronotidae, *Rha* + *Eig* + *Dis*, and families Hypopomidae + Rhamphichthyidae. The strict consensus of TS1TV2 is the same shown in fig. 6. For TS1TV4, *Sternopygus* is the sister group of all gymnotiforms. The remaining genera are organized as follows: ((families Rhamphichthyidae + Hypopomidae) ((*Rha* + *Eig* + *Dis*) ((*Ele* + *Gym*) (family Apteronotidae)))).

Using METREE (Rzhetsky and Nei 1993), for the Jukes-Cantor model, the sum of branches (S) of the

Table 1
Genera Sequenced and Base Composition (%) of the Aligned 12S and 16S rRNA Segments

			12 S rRNA				16S rRNA			
GENERA	ABBREVIATION	FAMILY[a]	A	C	G	T	A	C	G	T
Gymnotiformes:										
Sternopygus	*Spy*	S	32.55	25.84	22.82	18.79	30.77	24.28	24.04	20.9
Eigenmannia	*Eig*	S (E)	30.20	28.52	23.49	17.79	30.53	24.28	23.08	22.1;
Distocyclus	*Dis*	S (E)	30.54	27.18	23.15	19.13	30.77	23.56	23.56	22.1;
Rhabdolichops	*Rha*	S (E)	30.10	28.43	23.08	18.39	30.53	24.04	23.80	21.6;
Apteronotus albifrons	*Aal*	A	31.65	25.93	23.23	19.19	31.16	24.64	23.43	20.7'
Apteronotus leptorhynchus	*Ale*	A	31.65	25.59	23.23	19.53	30.58	24.03	24.03	21.3(
Adontosternarchus	*Ado*	A	31.31	25.93	23.23	19.53	30.92	24.64	23.91	20.5;
Sternarchorhamphus	*Sam*	A	31.99	24.92	21.89	21.21	31.16	24.88	23.43	20.5;
Orthosternarchus	*Ort*	A	31.42	25.68	22.64	20.27	31.64	24.64	22.95	20.7'
Sternarchella	*Sla*	A	31.99	26.26	23.23	18.52	30.92	24.88	23.67	20.5;
Sternarchogiton	*Sgi*	A	31.65	26.26	23.23	18.86	31.48	23.97	23.49	21.0'
Rhamphichthys	*Rhp*	R	29.77	26.76	23.08	20.40	29.64	25.54	24.58	20.2«
Gymnorhamphichthys	*Grh*	R	29.10	27.42	24.08	19.40	29.81	25.24	24.04	20.9
Hypopygus	*Hgu*	H (R)	29.43	28.09	22.74	19.73	28.92	25.78	25.06	20.2«
Steatogenys	*Ste*	H (R)	27.33	27.33	24.67	20.67	29.81	25.24	25.24	19.7
Microsternarchus	*Mic*	H	29.77	28.09	23.75	18.39	31.49	24.52	21.63	22.3(
Brachyhypopomus	*Bra*	H	30.67	26.67	23.67	19.00	30.53	24.52	22.84	22.1;
Electrophorus	*Ele*	E	32.11	25.42	21.74	20.74	30.60	24.34	22.65	22.4
Gymnotus	*Gym*	G	32.55	22.82	22.15	22.48	30.99	24.94	23.73	20.3·
Siluriformes:[b]										
Trichomycterus	*Tri*	T	30.18	26.67	22.81	20.35	31.25	24.04	23.08	21.6;
Cetopsis	*Cet*	C	31.10	25.08	22.41	21.40	30.51	23.49	23.97	22.0;
Hemicetopsis	*Hem*	C	31.44	25.75	22.41	20.40	31.73	24.28	23.32	20.6'
Corydoras	*Cor*	Ca	33.44	25.42	22.07	19.06	31.73	22.12	22.84	23.3;
Hypostomus	*Hus*	L	30.43	25.75	23.75	20.07	31.25	24.04	23.80	20.9
Malapterurus	*Mal*	M	31.44	25.75	22.74	20.07	31.73	23.56	23.32	21.3¹
Average			30.95	26.30	23.01	19.73	30.82	24.38	23.58	21.2;

NOTE.—A total of 302 and 416 sites were aligned for the 12S rRNA and 16S rRNA, respectively.

[a] The gymnotiform families are according to Mago-Leccia (1994): S, Sternopygidae; E, Eigenmannidae; A, Apteronotidae; H, Hypopomidae; R, Rhamphicthyidae; E, Electrophoridae; G, Gymnotidae. The letters in parentheses indicate the family suggested by this study.

[b] The siluriform families are from Nelson (1994): T, Trichomycteridae; C, Cetopsidae; Ca, Callichthyidae; L, Loricariidae; M, Malapteruridae.

neighbor-joining tree (NJ) was $S = 0.945954$. Five trees were retained after the trees with topological distances (d_T) of two and four units around the NJ tree were examined. The best (lowest) sum of branch lengths among the ME trees with Jukes-Cantor correction was $S = 0.940671$. For the two-parameter (Kimura's) model, the NJ tree had $S = 0.956797$, and the best ME tree was $S = 0.951624$. Once more, five shorter trees were found under Kimura's model after trees with $d_T = 2$ and $d_T = 4$ around the NJ tree were examined. The topologies of the best ME trees were identical for both type of correction; therefore, in figure 7 we show the resultant topology by keeping the clades that are preserved in more than 70% of the bootstrap replications in both models. As we have observed before under MP, between all 12 ME trees, the position of *Gymnotus* and *Electrophorus* varies most. Among the nonoptimal ME trees, the two genera shift between three positions: being placed as the

sister group of all other gymnotiforms, as the first clade to diverge after *Sternopygus,* or as the sister group of the apteronotids.

In 25 FASTDNAML searches with random addition (jumbling) of taxa, the tree with the best likelihood was recovered three times, and the second best topology another three times. This indicates that the algorithm converged on the same two optimal topologies about 25% of the trials, regardless of the order of taxa addition. The best value obtained for the likelihood was Ln likelihood = $-4,804.4061$, and the tree is depicted in figure 8. Between the two best topologies, the only difference is the position of *Electrophorus* and *Gymnotus,* which changes from being the sister group of Hypopomidae + Rhamphichthyidae to the sister group of the Apteronotidae in the second best topology.

The g1 statistics for 10,000 randomly generated trees was -0.637 for TS1TV1, -0.737 for TS1TV2, and
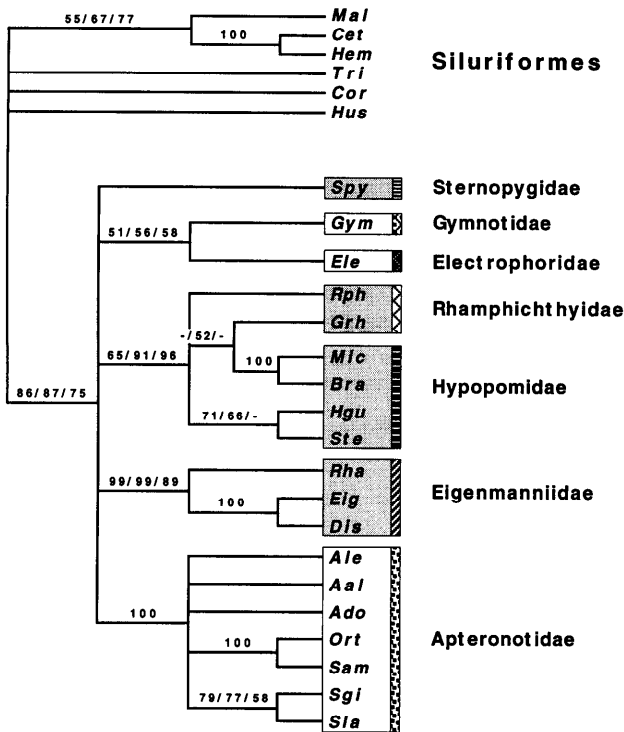
FIG. 6.—Strict consensus cladogram of all 32 most parsimonious trees found by PAUP (Swofford 1993) for the three weighting schemes used in this study. The Mickevich's consensus information (Swofford, 1993) for TS1TV1, TS1TV2, and TS1TV4 were, respectively 0.333, 0.22, and 0.538. Numbers above the branches correspond to bootstrap values (100 replications with five random stepwise additions being performed at each replication) for the three respective TS/TV costs used. Single values over the branches indicate that the same bootstrap value was obtained for all treatments. Dashes were used when the clade was not recovered in more than 50% of the bootstrap topologies for the respective weighting applied. Clades defined on the basis of morphological characters (Mago-Leccia 1994) that are not recovered as monophyletic by parsimony are depicted with shaded backgrounds. Abbreviations for genera are listed in table 1.

−0.708 for TS1TV4. When we used a constrained tree where only the well corroborated monophyletic clades were specified in the form: (Siluriformes, (*Spy,* Apteronotidae (*Ele* + *Gym*), (Rhamphichthyidae + Hypopomidae), (*Rha* + *Eig* + *Dis*))), the g1 values changed to: TS1TV1, g1 = −0.686; TS1TV2, g1 = −0.693; TS1TV4, g1 = −0.695. All g1 values are significant at $P = 0.1$ level, according to significance intervals calculated by Hillis and Huelsenbeck (1992), and suggest that the data set also contains phylogenetic information at more basal branches in the tree.

If we compute a consensus topology for all the methods and weights used in this study, there are at least six stable, constant, and well-supported monophyletic groups within the order: (1) *Sternopygus;* (2) *Rhabdolichops* + (*Eigenmannia* + *Distocyclus*); (3) Apteronotidae; (4) Rhamphichthyidae + Hypopomidae; (5)

Electrophorus; and (6) *Gymnotus.* The latter two tend to group as a monophyletic clade in the great majority of the cases. However, in none of our topologies is *Sternopygus* grouped with the other sternopygids; neither does *Hypopygus* and *Steatogenys* form a monophyletic clade with *Brachyhypopomus* and *Microsternarchus.* These results render both families Sternopygidae and Hypopomidae, as defined by morphological characters, as paraphyletic taxa.

Discussion

In our discussion, we will first address some structural constraints that may influence the dynamics of base substitution in the two DNA segments used and consequently the phylogeny inferred, and subsequently we will consider the evolutionary implications of the phylogeny of the gymnotiforms based on mitochondrial DNA sequences.

Functional Constraints versus Phylogenetic Information in the 12S and 16S rRNAs

The secondary structure of both segments sequenced is fairly constant among the entire range of genera studied (fig. 3). Conservation of primary and secondary structures from fish to land vertebrates including humans is evident (compare fig. 3*A* and *B* with fig. 11 of Gutell et al. 1985 and fig. 21*b* of Gutell and Fox 1988, respectively), suggesting that functional constraints indeed exert a selective pressure at the molecular level (Fitch and Markowitz 1970; Shoemaker and Fitch 1989). The existence of certain extremely conserved regions implies that fixed mutational events are accumulating in discrete portions of the molecules which are more free to change. The rate of nucleotide substitutions, therefore, is not constant across all nucleotide sites, and the presence of "hot spots" (sites with elevated mutation rate) is likely. Considering further that for every initial nucleotide only one transition and two types of transversions are possible, the less constrained (faster-evolving) regions will reach saturation level earlier, and homoplasic substitutions or multiple hits will start to accumulate in those regions, as the time of divergence increases.

Our results offer evidence that both 12S and 16S rRNA are not entirely saturated with homoplasic substitutions. This is corroborated by results of the g1 test, as well as by the plots depicted in figure 5. The g1 statistics imply a strong skewness in the distribution of randomly generated trees, according to the confidence limits presented by Hillis and Huelsenbeck (1992). Since the skewness is maintained after we impose topological constraints to our searches, it appears that we should have sufficient phylogenetic information in the data to
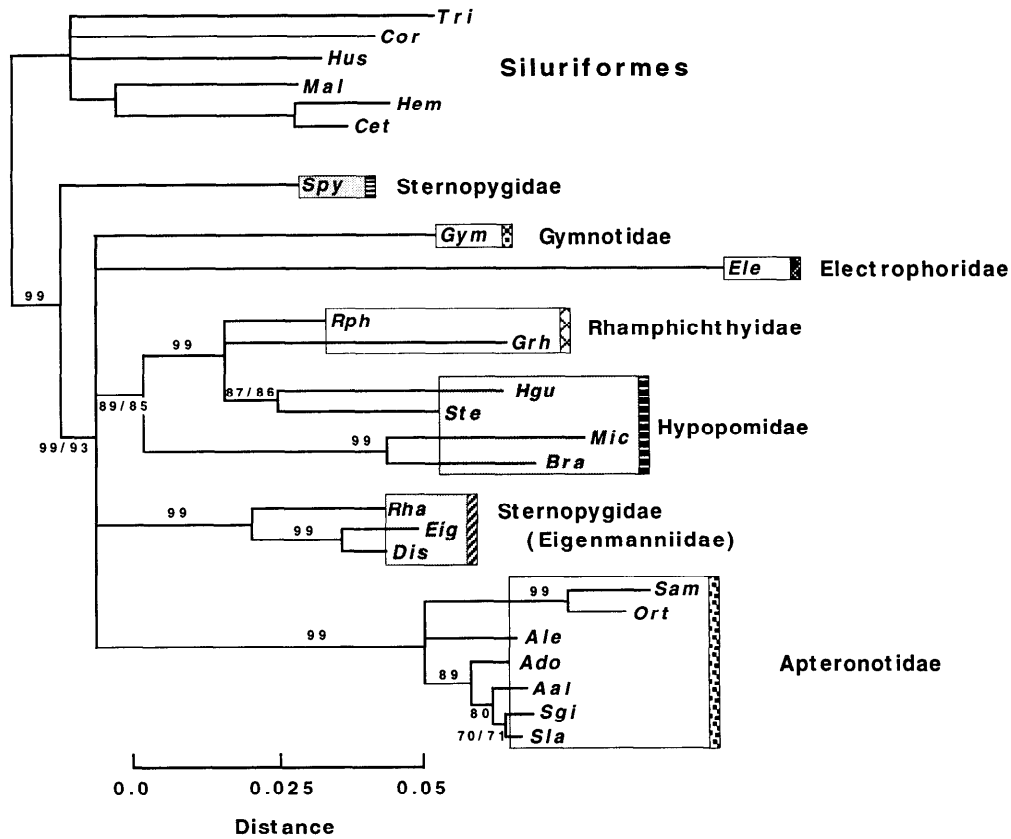
FIG. 7.—Using the minimum-evolution approach (METREE program; Rzhetsky and Nei 1993), we corrected the pairwise distances using both Jukes-Cantor (1969) and Kimura's (1980) models. The best trees for both models have the same topology. We show here a consensus topology, using Kimura's distance correction, obtained by preserving only the clades that are supported in more than 70% of the bootstrap topologies for both models. The first and second values above the branches correspond to bootstrap values after 1,000 replications for Jukes-Cantor and Kimura's models, respectively. Single values above the branches imply that the bootstrap values were the same in both trees. Clades defined on the basis of morphological characters (Mago-Leccia 1994) that are not recovered as monophyletic by the minimum evolution (distance) model are depicted with shaded backgrounds. Abbreviations for genera are listed in table 1.

resolve the relationships of the deeper branches (inter-family level) of our cladograms (Hillis and Huelsenbeck 1992). In the plot shown in figure 5A, when more distantly related fish were included in the analysis, such as representatives of the distantly related order Osteoglossiformes, the "% divergence" reaches values around 23% (Alves-Gomes et al., 1993), as opposed to 18.25% when we compare gymnotiforms and siluriforms only. Because percentage divergences can still increase at least one-fifth of its current maximum value when osteoglossiforms are included, we believe that we have not yet reached complete saturation in our comparisons between gymnotiforms and siluriforms, although we do not know how far from it we may be. In figure 5B, saturation would be evident if the proportion of TS and TV for all pairwise comparisons had reached an asymptote as the number of TS or TV increased. This is clearly not the case over most of the range observed, although at the highest levels a leveling off may be beginning to appear.

In addition, comparing a variety of organisms, Mindell and Honeycutt (1990, p. 552, fig. 2A and B) have shown that transitions in both small and large mitochondrial ribosomal subunits do not saturate at divergences up to 30%. The maximum divergence we found between electric fishes and the siluriforms sequenced is less than 19%.

One possible cause for our lack of success to resolve the deeper branches of the gymnotiform phylogeny with confidence may be due to a partial saturation of phylogenetically informative characters in our sequences, as a result of the different substitution rates for different sites along the molecule. The fast-evolving sites/regions, distributed along the molecule, are accumulating substitutions faster because they are probably less involved in the functional performance of the molecules. If the phylogenetically informative sites are located mainly at those most variable regions, although the overall dissimilarity between two sequences may still increase, the majority of the informative sites could be saturated with

Siluriformes

Mal
Hem
Cet
Hus
Cor
Trl

Spy — Sternopygidae

Gym — Gymnotidae

Ele — Electrophoridae

Rph — Rhamphichthyidae
Grnh

Hgu
Ste — Hypopomidae
Mlc
Bra

Elg
Dia — Sternopygidae
Rha — (Eigenmannlidae)

Aal
Sla
Sgl
Sam — Apteronotidae
Ort
Ado
Ale

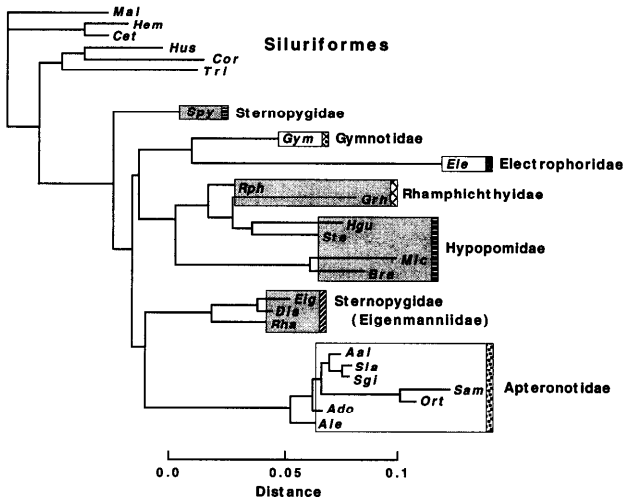0.0   0.05   0.1
Distance

FIG. 8.—Best maximum-likelihood tree obtained by FAST-DNAML (Olsen et al. 1994) after 25 searches with jumbling of taxa. Clades defined on the basis of morphological features (Mago-Leccia 1994) that are not recovered as monophyletic by the maximum likelihood model are depicted with shaded backgrounds. As in MP and ME analyses, ML fails to support Mago-Leccia's (1994) families Sternopygidae, Hypopomidae, and Rhamphichthyidae as monophyletic clades (shaded background). Abbreviations for genera are listed in table 1.

divergences that occurred over 250–300 million years ago, provided one has sequences of appropriate lengths. We apparently do not have enough of these sites in our sequences to resolve the relationships between different gymnotiform families with confidence.

Graybeal (1994) recently addressed the suitability of various genes with different rates of evolution for recovering vertebrate phylogenies for divergences at particular time windows. Our results suggest that the same argument could be applied for distinct regions within one gene. The faster-evolving sites of both 12S and 16S rRNAs mutate too rapidly and are saturated with homoplasies to provide resolution for branching events that have occurred within the same time window in which different gymnotiform families have evolved. The more slowly evolving sites, on the other hand, are too few in our sequences to solve the phylogeny within the same time window. This rate heterogeneity is probably present in the great majority, if not in all, genes currently used in phylogenetic analyses (Fitch and Markowitz 1970; Shoemaker and Fitch 1989), but more detailed analyses of the variable and informative sites in our sequences are necessary before we can identify these fast-evolving sites and address this hypothesis properly.

Another hypothesis that could account at least for part of the weak resolution in the deeper branches of our cladograms concerns the possibility of a narrow time window in which differentiation may have occurred in the main gymnotiform lineages. If there had been a sudden availability of ecological niches after the evolution of an ancestral gymnotiform, divergences could have been accelerated during speciational events. Fast radiations are not easily testable at the molecular level (Avise and Ayala 1975, 1976; Avise 1977; Mayden 1986; Mindell et al. 1989), but we cannot rule out this hypothesis (see also Meyer et al. 1990; Sturmbauer and Meyer 1992).

Stems versus Loops

The relative weight of stems in relation to single-stranded regions in the phylogenetic analysis of ribosomal genes has been addressed by Wheeler and Honeycutt (1988), Smith (1989), and Dixon and Hillis (1993), among others. A consensus about this matter was apparently achieved when Dixon and Hillis (1993) demonstrated that some percentage of the presumably "compensatory mutations" in the ribosomal RNAs fails to occur, and therefore stem bases should be weighted no less than 0.8 in relation to loops.

We decided to disregard any differential weighting between loops and stems regions in our analysis primarily because Dixon and Hillis (1993) still recovered the correct topology when stems and loops were weighted equally but also because the secondary structure alone

homoplasic substitutions relatively early in time. If this is the case, an obvious consequence is that the phylogenetic information in the two segments can be suitable for studying evolutionary relationships at various hierarchical/taxonomic levels. For instance, the unaligned regions in our sequences are probably regions that do not play a major role in the molecule's function, and therefore they are too variable and saturated with homoplasy in order to provide phylogenetic information for events that have occurred far in the past. These regions, however, can be very useful for studying closely related taxa, such as different species within a genus. The remaining (aligned) sites of our sequences that are variable can probably be associated with at least two types of regions. The first one evolves relatively fast and saturates with homoplasies for taxa between 7% and 12% divergent. Sites located at these regions could have provided the necessary phylogenetic information to resolve the phylogeny within each family, but the signal/noise ratio drops quickly for divergences above 10% (fig. 4). Divergence between genera of two gymnotiform families are normally above 8%, and the interfamily relationships are least well resolved in our trees. The second type of region is less free to change, probably due to functional constraints, and accumulates mutations at a much slower pace. The phylogenetically informative sites located in these regions are probably few, and they are useful for studying more distantly related taxa, such as

does not reveal any pairing interactions that may occur in the tertiary or quaternary structures of the molecules, or even during the ribosomal assembly. Since we do not know the complete three-dimensional structures of these molecules, at this point we cannot differentiate the unpaired regions in the secondary structure that accumulate mutations truly independently from those unpaired bases for which the assumption of independence no longer holds when more complex folding in the molecule is considered. Furthermore, the evolutionary constraints of different single-stranded regions as seen in the secondary structure are not equal (fig. 3) so that, ideally, different single-stranded regions should also be weighted differently. As a more defensible approach, we decided to weight all alignable regions of both molecules equally, regardless of their status as a stem or a loop base.

Gymnotiform Phylogeny Inferred from DNA Sequences

Considering only the phylogenetic relationships that are preserved in all resultant trees, regardless of the method or TS/TV weights used, molecular data suggest that there are at least six main evolutionary lines within the gymnotiforms. These lineages are represented by the following taxa: (1) *Sternopygus*, (2) family Apteronotidae, (3) *Rhabdolichops* + *Eigenmannia* + *Distocyclus*, (4) families Hypopomidae + Rhamphichthyidae, (5) family Gymnotidae, and (6) family Electrophoridae. By using molecular information alone, we could not resolve the relationships between these main lineages with a high degree of confidence, but in our discussion we will include morphological, physiological, and anatomical data related to the EES of gymnotiforms and argue in favor of one or another topology obtained in our analyses. Ultimately, we present a hypothesis for the gymnotiform phylogeny that combines molecular, physiological, and morphological evidence.

Mago-Leccia (1976) defined four distinct morphotypes in the order Gymnotiformes: "sternopygoideo," "rhamphichthyoideo," "apteronotoideo," and "gymnotoideo" (numbers 1–4 in figs. 2*A*, *B*, and 10). The monophyly of these clades are preserved in his new hypothesis (Mago-Leccia 1994) as well as in the two other phylogenetic studies dealing with representatives of all gymnotiform families (Gayet et al. 1992; Triques 1993). However, there are conflicts between these authors regarding the phylogenetic relationships between and within these four major clades (compare fig. 2*A* and *B*).

Molecular data also support Mago-Leccia's (1976) original hypothesis of four morphotypes almost entirely. From the four clades defined by each morphotype, only the sternopygoideo appears as an unnatural (paraphyletic) group according to 12S and 16S mtDNA phylogenies, since *Sternopygus* is never depicted as the sister group of the other genera of the family Sternopygidae in our results. We find other minor disagreements between DNA sequences and morphological data when we address the intergeneric relationships within the four main morphotypes. We will discuss them separately.

Morphotype Sternopygoideo—Family Sternopygidae

When Mago-Leccia (1976) defined the family Sternopygidae, *Sternopygus* was placed in the same clade with *Archolaemus* (not sequenced in this study) + (*Rha* + *Eig* + *Dis*). The monophyly of the family has not been questioned by subsequent studies (Mago-Leccia 1978; Mago-Leccia and Zaret 1978; Fink and Fink 1981; Lundberg and Mago-Leccia 1986; Gayet et al. 1992; Triques 1993). Until the last couple of years, the monophyly of Sternopygidae had been supported by only two morphological characters: villiform teeth in the mandible and the complete and well-developed bones of the infraorbital series (Mago-Leccia 1976; Mago-Leccia and Zaret 1978; Lundberg and Mago-Leccia 1986). Fink and Fink (1981) had pointed only to the enlarged infraorbitals as the unique synapomorphy (shared derived characters) between *Sternopygus* and the other genera in the family. The authors (Fink and Fink 1981, p. 309) also assert (text in brackets is our addition), "All of the [other] characters used by both Mago-Leccia (1978) and Mago-Leccia & Zaret (1978) to define the family [Sternopygidae] are either primitive teleostean features, primitive for gymnotoids, or absent in *Sternopygus*." Very recently, Gayet et al. (1992) and Triques (1993) added, jointly, nine new characters uniting *Sternopygus* with the other genera in the family. Because the matrix containing the character states for the different genera was not available from Gayet et al.'s (1992) study, we will only discuss Triques's (1993) hypothesis further. Triques (1993) listed six new characters corroborating the monophyly of Sternopygidae, namely, (1) frontal bone with an ante-orbital process; (2) enlargement of the supraorbital channel and their respective pores; (3) enlargement of the nasal bone; (4) extreme enlargement of the mandibular channel and respective pores; (5) presence of a spiny process in the anterolateral surface of the fifth ceratobranchial, and (6) fusion of the two proximal radials in the pectoral fin. The ante-orbital process in the frontal is character number 3 in Triques's (1993) matrix and appears to correspond to the lateral ethmoid of Fink and Fink's (1981) figure 2*E*. The character is not explicitly depicted in Triques's figure 2 (a lateral view of the ethmoidean region of *Eigenmannia trilineata*), and it is also not obvious in figure 5 of Lundberg and Mago-Leccia (1986) (lateral view of the neurocranium of *Rhabdolichops caviceps*), in figure 2 of Fink and Fink (1981) (lateral view of the ethmoid region of five ostariophysan genera, including *Sternopygus*), or

in figures 8, 13, and 26 of Mago-Leccia (1976) (respectively, the lateral view of *Rhabdolichops troscheli, Eigenmannia virescens,* and *Sternopygus macrurus's* neurocranium). Consequently, it is not clear which particular morphological feature Triques (1993) refers to and to what extent this character may or may not differ between *Sternopygus* and the other genera. The character listed here as number 6 (three radials in the pectoral fin) is number 47 in Triques's matrix, and corresponds to Lundberg and Mago-Leccia's (1986) character number 15. Opposing Triques's interpretation, Lundberg and Mago-Leccia's (1986, p. 60) state, "*Sternopygus, Archolaemus,* and nearly all other gymnotiforms have four separate pectoral radials." Mago-Leccia (1976) also reports four pectoral radials for *Sternopygus.* From the remaining four synapomorphies listed by Triques (1993), character numbers 2, 3, and 4 listed above are enlargements of bony processes in the head. One of the premises when phylogenetic hypotheses are inferred by parsimony is that the characters utilized are evolving independently from each other. Without discussing the subjectivity of defining enlargements (see fig. 2 of Lundberg and Mago-Leccia [1986], and note that the "enlargement" of the infraorbitals is much more conspicuous in *Rhabdolichops* than in *Eigenmannia* or *Distocyclus*), we cannot be sure that they are evolving independently. These characters could represent a more general trend happening independently in *Sternopygus* and in the other genera as, for instance, the enlargement of particular portions of the neurocranium. In summary, we would argue that the features listed by Triques (1993) do not constitute strong evidence supporting the monophyly of Sternopygidae.

Almost every author has referred to *Sternopygus* as one of the less derived genera among gymnotiforms (Mago-Leccia 1976, 1978; Mago-Leccia and Zaret 1978; Fink and Fink 1981). Molecular phylogeny corroborates this notion, since *Sternopygus* is depicted as the sister group of all gymnotiforms in the ML, ME, and almost all MP trees (figs. 6–8). In none of our resultant topologies, considering all three different algorithms as well as the weights used, does the genus *Sternopygus* form a monophyletic clade with the other genera of the family Sternopygidae. Instead, *Sternopygus* appears as a single representative of a unique evolutionary line within the order. The percentage divergence between *Sternopygus* and *Eigenmannia* is 8.3%, a value comparable to the distance between *Sternopygus* and any other pulse-type genus: 9.6% to *Hypopygus,* 6.51% for *Rhamphichthys,* and 9.4% for *Steatogenys,* for example.

Even at the morphological level there are many distinctions between *Sternopygus* and the rest of the genera in the family, supporting the molecular hypoth-

esis that *Sternopygus* represents a distinct line from the remaining sternopygids. According to Mago-Leccia (1976), *Sternopygus* differs from the other genera by having an incipient mesocoracoid in the scapular girdle whereas the remaining genera do not; by not having the posttemporal fused with the supracleithrum; by having 24–26 precaudal vertebrae, whereas the other genera have no more than 16; by the absence of scapular foramen, which is present in the other genera; and also in contrast to the other genera, by having no skin covering the border of the eye. In addition, Lundberg and Mago-Leccia (1986) and Fink and Fink (1981) report about 15 morphological character states that are shared by *Archolaemus + (Rha + Eig + Dis)* but which are different in Sternopygus. As a recognition of the evident morphological distinctions and specializations of the members of the Sternopygidae, Mago-Leccia (1976) suggested that the three subfamilies he had defined within the Sternopygidae, (Sternopyginae, Archaeogymnotinae, and Eigenmanninae) could eventually be elevated to the rank of families.

Additional distinctions between *Sternopygus* and the other genera in the family are found at the level of physiological features associated with the EES. The genus has the lowest EOD repetition rate among all the wave species, being as low as 60 Hz in some individuals, a value well in the range of some pulse-type EODs, whereas the other sternopygids have fundamental frequencies normally above 150 Hz. Another trait shared between *Sternopygus* and the pulse-type fish is the anatomical organization of the pacemaker nucleus, the endogenous oscillator which determines the rhythm of the electric organ firing rate. Unlike *Rha + Eig + Dis* or all apteronotids, in which pacemaker and relay neurons intermingle in the pacemaker, these two types of cells are segregated in the pacemaker nucleus of *Sternopygus,* as they are in every pulse-type fish (Elekes and Szabo 1980; Ellis and Szabo 1980; Kawasaki and Heiligenberg 1988; Keller et al. 1991). Moreover, in contrast to what is known for *Eigenmannia, Rhabdolichops,* and the apteronotids, the neuronal mechanisms required to perform the jamming avoidance response (JAR) or to produce "chirps" (rapid upward modulations of the EOD repetition rate) were never identified in *Sternopygus* (Dye 1987; Dye and Heiligenberg 1987; Kawasaki and Heiligenberg 1989; Keller et al. 1991).

Considering these varied sources of information, it is likely that the genus *Sternopygus* diverged very early in the gymnotiform history and has retained a good number of plesiomorphic characters, including morphological, molecular, and physiological ones. We also believe that the family Sternopygidae can no longer be considered monophyletic and that the presumed syna-

pomorphies listed by previous authors represent homoplasies between *Sternopygus* and the other genera. Sufficient evidence exists, in our opinion, to propose that the genus *Sternopygus* represents a distinct and unique lineage within the order, and the family Sternopygidae should become a monogeneric family. As a natural consequence, the new clade consisting of the remaining genera (*Archolaemus* + (*Rha* + *Eig* + *Dis*)) should be named. We propose Eigenmanniidae n. (order Gymnotiformes) as the name for the new family, following a previous suggestion by Mago-Leccia (1976), because *Eigenmannia* is a well-known gymnotiform genus due to its broad utilization in physiological and behavioral experiments in various laboratories. In brief, according to molecular, anatomical, and physiological data, the new family Eigenmanniidae would be composed of four genera, in which *Archolaemus* was probably the first to diverge and is the sister group of *Rha* + *Eig* + *Dis*. However, until we obtain molecular as well as physiological data from *Archolaemus* this will remain a conjecture. *Rhabdolichops* is the next genus to diverge and is the sister taxon of *Eigenmannia* and *Distocyclus*. The relationship among *Rha* + *Eig* + *Dis* found at the molecular level is the same as proposed by Fink and Fink (1981) with morphological data.

Morphotype Apteronotoideo—Family Apteronotidae

The monophyly of the Apteronotidae is well supported. Morphologically all genera of the family can readily be distinguished on the basis of three synapomorphies that are unique to this family among gymnotiforms: first, the presence of a caudal fin; second, a dorsal filament which is embedded within a groove; and third, a "neurogenic" electric organ. In the apteronotids, a larval myogenic electric organ degenerates early during ontogeny and is replaced by the neurogenic organ of the adult form (Kirschbaum 1983), consisting of the modified axons of the spinal electromotor neurons (review in Bass 1986). Moreover, since all neurons involved in the generation of the EODs in apteronotids are coupled electrotonically (Dye and Meyer 1986), the fish of this family generate EODs of the highest frequencies among gymnotiforms, being as high as 1,800 Hz in some genera. Four subfamilies (Apteronotinae, Sternarchorhynchinae, Adontosternarchinae, and Oedemognathinae) and 12 genera are recognized by Mago-Leccia (1976, 1994).

The monophyly of the Apteronotidae is also very well characterized at the molecular level, but the internal topology of the family changes accordingly to the method and the weighting scheme adopted (figs. 6–8). For most genera, therefore, it was not possible to determine their phylogenetic relationships conclusively. This could be a direct consequence of the small number of phylogenetically informative characters supporting alternative topologies. The average divergence among apteronotids is only 3.67%, a value that may explain why so few stable sister taxa in the clade could be established, as we go from one tree-reconstructing method or weighting scheme to another. However, in all topologies, independently of the method utilized, two monophyletic clades are preserved: *Sternarchella* + *Sternachogiton* and *Sternarchorhamphus* + *Orthosternarchus*. *Apteronotus albifrons* is the sister group of the former two genera in the best ME and ML trees as well as in all 50% majority-rule MP trees, which validates the subfamily Apteronotinae of Mago-Leccia's (1976) work. The clade *Sternarchorhamphus* + *Orthosternarchus*, always depicted as sister taxa by molecular data, validates the subfamily Sternarchorhynchidae, and *Adontosternarchus* represents a unique line according to our data, corroborating Mago-Leccia's (1976) monogeneric subfamily Adontosternarchinae.

A rather unexpected result within the Apteronotidae is that the two species of *Apteronotus* (*A. albifrons* and *A. leptorhynchus*) have never emerged as a monophyletic group. Thus, the genus *Apteronotus* is likely to be paraphyletic.

In view of the diversity of the clade, the scarcity of taxonomic and phylogenetic studies of the group, and the relatively small number of genera used in this study, we think it is premature to propose any additional phylogenetic hypothesis for the family beyond the obvious relationships depicted in our cladograms, but we disagree with Triques (1993) and Gayet et al. (1992) about the position of the family Apteronotidae within the order (fig. 2B). Although the relationship is not obvious from molecular data alone, by combining molecular and physiological data, the family Apteronotidae appears to be the sister group of the Eigenmanniidae, as suggested recently by Mago-Leccia (1994). These two clades are depicted as sister groups in the 50% majority-rule consensus tree in TS1TV1 and TS1TV2 in MP analyses, as well as in the best ML tree. From a physiological perspective, the genera of these two families have a wave-type EOD, intermingled pacemaker and relay cells in the pacemaker nucleus; they also produce chirps and have the neuronal circuitry involved in the JAR (Heiligenberg 1977). We predict that future morphological studies will find additional synapomorphies grouping the eigenmannids and the apteronotids, with the exclusion of *Sternopygus*.

Morphotype Rhamphichthyoideo—Families Hypopomidae + Rhamphichthyidae

According to 12S and 16S rRNA sequences, the genera of the families Hypopomidae + Rhamphichthyidae form a monophyletic assemblage in which *Brachyhypopomus* and *Microsternarchus* are sister taxa, and

Steatogenys and *Hypopygus,* with the exception of MP under TS1TV4, are also always depicted as sister taxa. Our results, however, do not support the traditional view of morphological studies (Mago-Leccia 1976, 1978, 1994; Triques 1993), in which the families Hypopomidae and Rhamphichthyidae are considered as two well-defined monophyletic groups.

According to Mago-Leccia (1994) the family Hypopomidae contains six genera: *Hypopomus, Brachyhypopomus, Microsternarchus, Hypopygus, Steatogenys,* and *Racenisia;* and the family Rhamphichthyidae, the genera *Rhamphichthys* and *Gymnorhamphichthys.* In all the ME analyses, and in the best ML tree generated from molecular data, *Steatogenys* + *Hypopygus* form a monophyletic clade with *Rhamphichthys* and *Gymnorhamphichthys* rather than join the family Hypopomidae with *Brachyhypopomus* and *Microsternarchus.* For MP, the topology within the Hypopomidae + Rhamphichthyidae changes according to the relative weights of TS and TV, but *Steatogenys* and/or *Hypopygus* are never found in a monophyletic clade with the other hypopomids. For TS1TV1 and TS1TV2 the topology of the strict consensus of all most parsimonious trees shows the same polytomy depicted in figure 6. For TS1TV4, the strict consensus of the three most parsimonious trees is (*Rph* (*Hgu* (*Ste* (*Ghr* (*Bra* + *Mic*))))).

From the entire range of morphological characters analyzed by Mago-Leccia (1976) to resolve the relationship between the genera of the morphotype rhamphichthyoideo, there are only three claimed synapomorphies, which are probably not evolving independently, that support the monophyly of his family Hypopomidae: short snout, posterior nasal aperture near the eye, and fixed position of the anus during ontogeny. All other characters are either plesiomorphic for the "rhamphichthyoid" morphotype, are not registered for all genera, or support the grouping of either *Steatogenys* or *Hypopygus* with the family Rhamphichthyidae. Among those characters, the fusion of the posttemporal and supracleithrum, a ventral process in the coracoid, and the lack of a mesocoracoid place *Steatogenys* in the Rhamphichthyidae, and the absence of lateral ethmoid place both *Steatogenys* and *Hypopygus* in the family Rhamphichthyidae.

There are two characters in Triques's data matrix supporting the monophyly of *Rhamphichthys* and *Gymnorhamphichthys* and a couple of synapomorphies supporting the monophyly of *Steatogenys* + *Hypopygus,* but there are none supporting the monophyly of *Steatogenys* + *Hypopygus* + *Hypopomus.* In fact, in Triques's (1993) matrix, the absence of lateral ethmoid places *Steatogenys* + *Hypopygus* with *Gymnorhamphichthys,* the separation of the laminar

and globular parts of the vomer unites *Steatogenys* + *Gymnorhamphichthys,* and the square format of the opercle groups *Rhamphichthys* with *Steatogenys* + *Hypopygus* and *Hypopomus.*

From the evidence above, it is obvious that the phylogenetic relationships between the genera of these two families are not completely settled by morphology. There are as many morphological characters supporting the monophyly of Hypopomidae including *Steatogenys* + *Hypopygus* as there are characters grouping these two genera with the rhamphichthyids, as mitochondrial sequence data suggest. However, the topology derived from sequence data is also corroborated by physiological data associated with the EOD wave form of these genera. The EOD wave form of *Steatogenys* and *Hypopygus* shares more similarities with the EOD of *Rhamphichthys* and *Gymnorhamphichthys* than with the other hypopomids (fig. 9). In summary, although we have reasonably strong evidence pointing to the fact that *Hypopygus* and *Steatogenys* are probably being incorrectly placed in the family Hypopomidae, further studies are necessary to elucidate the phylogenetic relationships among these genera conclusively.
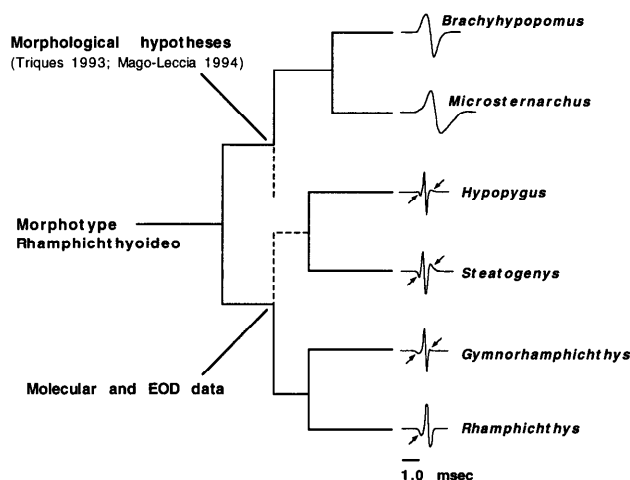

FIG. 9.—Two alternative hypotheses for the phylogenetic relationship between the genera of the families Rhamphichthyidae and Hypopomidae. Molecular data place *Steatogenys* and *Hypopygus* with *Rhamphichthys* and *Gymnorhamphichthys,* in contrast to previous studies (Triques 1993; Mago-Leccia 1994). The EOD wave form, which is the result of a combination of factors including the innervation pattern, physiology, and distribution of electrocytes along the fish's body, supports the molecular hypothesis. Note that the EOD of *Hypopygus, Steatogenys, Gymnorhamphichthys,* and *Rhamphichthys* are very similar, being much shorter than the EODs of *Brachyhypopomus* and *Microsternarchus* and having an initial negative phase (*up arrows*) not found in the latter two genera. *Hypopygus, Steatogenys,* and *Gymnorhamphichthys* also have an unique positive fourth phase (*down arrow*). The EODs shown here where obtained as described in the legend of fig. 1.

Morphotype Gymnotoideo—Families Gymnotidae + Electrophoridae

The phylogenetic position of the families Electrophoridae and Gymnotidae is the most variable in the order. In MP, for the strict consensus tree under TS1TV1, *Ele + Gym* is the sister group of all other gymnotiforms; for TS1TV2 the position of the clade is shown in figure 6, and with TS1TV4 the clade is placed as the sister group of the apteronotids. A similarly variable position of *Ele + Gym* is found among the six best ME trees, whereas in the best ML tree, *Ele + Gym* are placed as the sister group of Hypopomidae + Rhamphichthyidae, forming a clade in which all representatives have a pulse-type EOD. The ML tree agrees with the suggestion by Triques (1993) and Mago-Leccia (1994) (fig. 2) for the phylogenetic position of the Gymnotoidea; that is, they are the sister group of the remaining pulse-type genera.

Electrophorus is the sole genus in the family Electrophoridae and the only gymnotiform having three electric organs that may discharge over 600 V when activated synchronously. *Electrophorus* is also unique in the order by having a scaleless body and a unique respiratory physiology, being an obligatory air breather, and being able to release CO_2 through the skin (Farber and Rahn 1970). The family Gymnotidae is also monotypic, and the close relationship between *Gymnotus* and *Electrophorus* is corroborated not only by DNA sequences but also by many morphological (Ellis 1913; Mago-Leccia 1976; Gayet et al. 1992; Triques 1993) and physiological synapomorphies. *Gymnotus* can also breathe air, and with *Electrophorus* the genus shares the status of the only gymnotiforms without cranial fontanels, having a cylindrical body, a premaxilla longer than the maxilla, a prognate mandible, a rounded lateral processes in the mesethmoid that covers the anteromedial spine, an elongated mouth, a reduction of the cleithrum, and an absence of riblike bones (Ellis 1913; Mago-Leccia 1976; Gayet et al. 1992; Triques 1993).

Despite many synapomorphies, the divergence found between *Electrophorus* and *Gymnotus* at the molecular level is considerable (13.93%), what suggests an old origin for these taxa in relation to the other gymnotiforms. Part of this divergence can probably be linked to the long branch (high number of autapomorphies) associated with *Electrophorus*, as can be seen in both ML and ME trees (figs. 7 and 8). Long branches are problematic in phylogenetic analyses because they may attract each other due to chance of homoplasic substitutions and mask the correct topology (Felsenstein 1978). The long branches associated with *Electrophorus* and the family Apteronotidae (figs. 7 and 8) may be the reason that these two clades tend to be grouped together

in some of our topologies. The reason that *Electrophoru* has such a long branch in relation to the other gymno tiforms is unknown. One possibility is that *Electrophoru* is indeed a very old genus and had enough time to ac cumulate mutations, and the second is that its mito chondrial genome is evolving faster than that of the othe genera. We cannot distinguish between these two alter natives without fossil records.

A Synthetic Hypothesis for the Gymnotiform Phylogeny and the Evolution of Their Electrogenic System

By analyzing the different sources of evidence in cluding molecular, morphological, and physiologica data, we suggest the phylogenetic hypothesis for the orde Gymnotiformes depicted in figure 10. A detailed com parative study of the neurophysiological circuitry in volved in the EOD modulations in the various gym notiform genera will be published elsewhere; nevertheles in table 2 we show some of the features associated t the EES considered in our current phylogenetic hypoth esis.
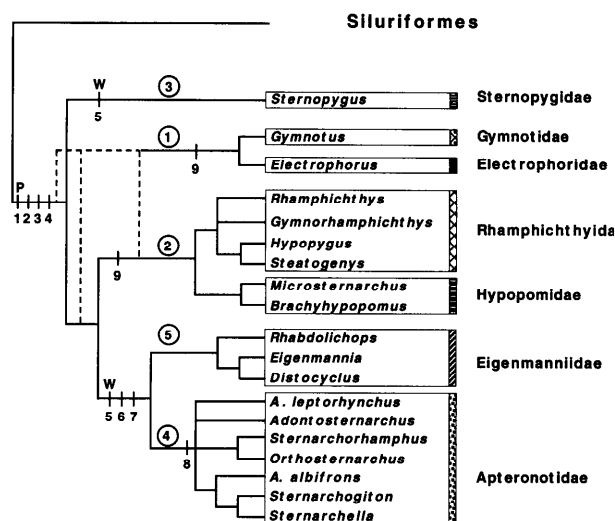

FIG. 10.—Hypothesis for gymnotiform phylogeny based on m tochondrial DNA sequence data, morphology, and electrophysiolog The position of *Electrophorus + Gymnotus* is uncertain. In contra to previous hypotheses shown in fig. 1, molecular and physiologic data do not support the monophyly of the families Sternopygidae an Hypopomidae. A new family Eigenmanniidae including *Archolaem i Rhabdolichops + Eigenmannia + Distocyclus* is suggested. Additior studies are still necessary in order to elucidate the relationship amor the genera of Rhamphichthyidae and Hypopomidae conclusively, b we believe that *Hypopygus* and *Steatogenys* are more likely to belor to the family Rhamphichthyidae. Numbered bars in the branches co respond to characters related to the electrogenic system of the gyr notiforms listed in table 2. Numbers 1–4 in circles correspond to th morphotypes of fig. 2. Number 5 represents the morphotype define after the new family Eigenmanniidae.

Table 2
List of Physiological and Morphological Characters
Associated with the EES of Gymnotiform Fishes
Used in Figure 10

Character Number	Description
1	Appearance of EOD (pulse-type)
2	S-PPn: synaptic contact on relay cells of the pacemaker nucleus
3	Pacemaker nucleus with segregated pacemaker and relay cells
4	PPn-G: gradual rises in EOD-excitatory synapses on pacemaker cells of the pacemaker nucleus
5	Transformation from pulse-type to wave-type EOD
6	Jamming avoidance response (JAR)
7	Pacemaker nucleus with intermingled pacemaker and relay cells
8	Myogenic electric organ is replaced by neurogenic electric organ.
9	PPn-I: long interruptions of the EOD-inhibitory synapses on the pacemaker cells of the pacemaker nucleus

In our hypothesis, the position of the clade *Electrophorus + Gymnotus* is not definitive, but we believe that it represents an old gymnotiform lineage that could be the sister group of all remaining genera, have diverged immediately after *Sternopygus,* or branched very early from the line culminating in Hypopomidae and Rhamphichthyidae. We favor the last scenario, also suggested by Gayet et al. (1992), Triques (1993), and Mago-Leccia (1994), because it is corroborated by the following additional evidence: all these fish have a pulse-type EOD, spatially segregated pacemaker and relay cells in their pacemaker nucleus (Pn), and they are also capable of long interruptions (more than 10 s) in their EODs. Such capability is normally associated with the PPn-I, a specialized region of the diencephalic prepacemaker nucleus that makes inhibitory synapses onto pacemaker cells of the Pn (Kawasaki and Heiligenberg 1989) and has not been found in any wave-type species studied. Also, similarly to *Gymnotus,* some hypopomids are not obligatory air breathers but do gulp air to be used as an alternative source of oxygen in their respiration.

In consonance with molecular, morphological, and physiological data, in our hypothesis *Sternopygus* represents an old and unique lineage within the order probably representing a descendent of the most plesiomorphic (least derived) gymnotiform lineage. However, according to some of our resultant topologies, and since we cannot explain the long branches associated with *Electrophorus* and *Gymnotus,* we do not discard the possibility of these genera representing the oldest gymnotiform lineage (dashed lines in fig. 10).

The remaining genera are assigned to two monophyletic groups. One is represented by the Hypopomidae and Rhamphichthyidae, in which all genera have a pulse-type EOD, and the second by Eigenmanniidae and Apteronotidae, in which all genera have a wave-type EOD.

Hopkins and Heiligenberg (1978) suggested that the first gymnotiform had a pulse-type EOD derived from plesiomorphic muscle action potentials, and the wave-type EOD is a derived condition. In our hypothesis, either Sternopygus (a wave-type) or *Ele + Gym* (two pulse-type species) could be the sister group of all other gymnotiforms. Two genera of the order Siluriformes, the immediate sister group of the gymnotiforms (Fink and Fink 1981), are known for possessing electric organs, and both produce pulse-type EODs. In the catfish *Malapterurus,* the electric organ is believed to be derived from an anterior trunk muscle (Johnels 1956; Bennet 1971), and in *Synodontis* the EODs are generated by the sonic muscle associated with the swim bladder (Hagedorn et al. 1990). Embryologically the gymnotiform electric organs (review in Bass 1986) are derived from a different set of muscles; therefore, since they are not homologous to the catfish electric organs, we cannot infer the type of EOD in the ancestral gymnotiform using cladistic argumentation. Nonetheless, considering that muscles are activated by discrete and relatively short nerve pulses, we propose that the first EODs of gymnotiforms, similarly to what is observed presently in *Synodontis* and *Malapterurus,* were of a pulse-type nature.

Sternopygus has our preference as the plesiomorphic gymnotiform type for the following reasons: First, it has retained more morphological features found in older ostariophysan fishes than any other gymnotiform (Mago-Leccia 1976, 1978; Mago-Leccia and Zaret 1978; Fink and Fink 1981); Second, it has the simplest circuitry involved in the EOD control in the Gymnotiformes—that is, the only EOD modulations observed or elicited in *Sternopygus* are gradual rises and sudden stops (Keller et al. 1991). Third, unlike *Electrophorus,* the other candidate for the plesiomorphic type of gymnotiform, *Sternopygus* is unable to generate brief and rapid rises of its EOD frequency, which are known as "chirps" and are found in almost all other gymnotiforms. Finally, the genus is repeatedly depicted as the sister group of all gymnotiforms in our molecular results, independently of the tree constructing model or weighting scheme used.

Since we believe that a pulse-type EOD is more likely to be the character state at the base of gymnotiform phylogeny, *Sternopygus* must have acquired a wave-type signal independently from the families Ei-

genmanniidae and Apteronotidae. Physiologically, the basic requirement to go from pulse-type to wave-type EOD is the broadening of the duration of the EOD pulse, paired with an increase in the frequency stability of the pacemaker. This evolutionary transformation is very similar to what can be observed in the hypopomid genus *Microsternarchus,* a pulse-type species with a relatively broad EOD pulse (fig. 9) and a rather stable frequency.

Similarly to what is presently found in *Sternopygus,* the common ancestor of the order might also have had a pacemaker nucleus with segregated pacemaker and relay cells, a relatively low EOD repetition rate, a limited ability to produce modulations in the EODs rhythm, and no neuronal circuitry involved in the JAR, a neuroethological synapomorphy which probably arose later in the common ancestor of the eigenmannids and apteronotids. Later, jointly with the transformation from pulse- to wave-type signal, the eigenmannids and apteronotids also increased their EOD repetition rate, a condition that required a faster synaptic coupling between pacemaker and relay cells in the pacemaker nucleus. This was achieved by coupling pacemaker cells and relay cells by electrotonic junctions. Whereas we still find mixed synapses in *Eigenmannia,* only gap junctions are seen in these synapses in apteronotids (Dye and Meyer 1986). The apteronotids raised their EOD rate further by replacing the plesiomorphic myogenic organ by a much faster neurogenic organ which consists of the modified efferent axons of the spinal electromotor neurons (Bennet 1971; Bass 1986).

In summary, we have taken an initial step toward a unifying hypothesis regarding gymnotiform phylogeny, by combining analyses of mitochondrial sequence data, morphology and electrophysiology. The results presented here are a conservative estimate of the phylogenetic relationships, and only the clades strongly corroborated by different sources of evidence are endorsed. Most notably, we found strong evidence and propose that the transformation from pulse-type to wave-type EOD occurred twice and independently in the order, first in the genus *Sternopygus* and later in the common ancestor of eigenmannids and apteronotids. However, further work remains to be done. Electrophysiological experiments associated with the EES of species never studied before are currently being performed. Mitochondrial sequences from more rapidly evolving regions promise to be suitable for analyzing relationships among genera and species of gymnotiforms, but complete resolution of interfamilial relationships as well as testing the phylogenetic position of the Gymnotiformes within the superorder Ostariophysi may require additional data from more slowly evolving genes. A better scrutiny of the morphological evidence is also necessary.

Acknowledgments

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