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Evolutionary history of the synbranchid eels (Teleostei: Synbranchidae) in Central America and the Caribbean islands inferred from their molecular phylogeny

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Abstract

Swamp eels of the genera *Synbranchus* and *Ophisternon* are secondary freshwater fishes whose biogeography provides evidence of their long residence in Mesoamerica, while their impoverished species-level taxonomy might suggest a more recent diversification or a conservative morphology. We have inferred the phylogenetic relationships of *Synbranchus marmoratus* and *Ophisternon aenigmaticum* from 45 drainages throughout South, Central America, and Cuba based on mitochondrial genes (cytochrome *b* and ATPase 8/6). Phylogeographic analysis supported the monophyly of Mesoamerican *O. aenigmaticum* although our results suggest that *S. marmoratus* is not a monophyletic group. We found a evolutionary differentiated *Synbranchus* mtDNA lineage inhabiting Las Perlas islands (Pacific Panama) that appeared to be taxonomically distinct and separated for a long period of time from the main *Synbranchus* clade. Major synbranchid clades were also corroborated with the nuclear RAG-1 gene (1171-bp). Application of two fish-based mtDNA clocks (1.05–1.3% pairwise divergence/million year (Ma)), is in accordance with the Godwanian origin suggested for the Synbranchidae. The mtDNA lineages exhibited a remarkable geographic structure in Central America suggesting that vicariance has most likely promoted the *Synbranchus* and *Ophisternon* mtDNA diversification. Although our data indicate the importance of the Pacific area in *Synbranchus* differentiation, the mtDNA divergence between South and Central American *Synbranchus* is too small to support Cretaceous colonization via the proto-Antillean bridge suggested by Rosen [Syst. Zool. 24 (1976) 431]. Instead, our phylogeographic results suggest that *Ophisternon* and *Synbranchus* mtDNA clades most likely colonized Central America during the Miocene (12.7–23 Ma) prior the final closure of the Isthmus of Panama (3.3 Ma).
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1. Introduction

Studies of Neotropical Mesoamerican freshwater fishes have described an impoverished fauna, usually comprised of only one or a few lineages representing genera that are very diverse in South America. This generality owes to the much smaller geographic area of Central America (>500.000 km^2) versus South America (>17 million km^2), and their long separation, only recently united by the Pliocene rise of the Panamanian isthmus (Coates and Obando, 1996). Secondary freshwater fishes such as cichlids (Cichlidae), live-bearers (Poeciliidae), and killifishes (Rivulidae) provide exceptions to the extreme disparity in the species numbers of families

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found in both the northern and southern neotropical regions. By definition, all secondary freshwater fishes should have some tolerance for salty waters, and thus should be physiologically capable of occasional dispersal through marine environments (Myers, 1966). Although, the secondary freshwater fish division contains families living mainly in fresh waters, it has a number of euryhaline taxa. On the contrary, the primary freshwater fishes exclusively inhabits fresh waters and are physiologically intolerant to salinity, and thus their dispersal is restricted to continental routes (Myers, 1966). It is proposed that some families of secondary freshwater fishes colonized Mesoamerica via marine dispersal prior to the rise of the Isthmus of Panama. Therefore, they had more time for species formation in Mesoamerican rivers than did primary Neotropical freshwater fishes, which did not extend into Central America until the formation of the terrestrial bridge roughly 3.3 million years ago (Ma) (Myers, 1966).

In light of the different histories of primary and secondary freshwater fishes in Mesoamerica, swamp eels (Synbranchidae) are unusual given that their biogeography evidences a long residence in Mesoamerica consistent with their secondary physiological status. However, their current species-level taxonomy suggests a Central American diversification history more reminiscent of primary freshwater fish, as suggested by the low number of species described for each taxon. Synbranchids occur in freshwater and marshy areas in a wide variety of life habits: amphibious, fossorial, and cavernicole (Rosen and Greenwood, 1976). Although swamp eels are relatively abundant in islands that are geographically close to continents (Hubbs, 1936; Miller, 1966; Rosen and Rumney, 1972; Rosen and Greenwood, 1976; Tyler and Feller, 1996), there are few reports of their presence in more distant islands such as Cuba (Rosen and Rumney, 1972; Rosen and Greenwood, 1976). The Cuban distribution of swamp eels might attest to their potential capabilities for marine dispersal, but their absence from other main Caribbean islands implies that this potential may rarely be realized. In this regard it is worth noting that cichlids, poeciliids, and rivulins have successfully colonized other main Caribbean islands, whereas synbranchid eels have not.

A revisionary study of the synbranchid anatomy recognized 15 species included in 4 genera distributed in Asia, Australia, Africa, and Central and South America (Fig. 1) (Rosen and Greenwood, 1976). New World synbranchids are represented by two genera: *Ophisternon* shared with the Old World, and *Synbranchus* restricted to the New World. Both genera exhibit complementary ranges with a narrow area of sympatry in Central Amer-

Fig. 1. World distribution of the genera *Ophisternon* (in black and grey) and *Synbranchus* (stripes and grey) (modified from Rosen, 1976; Rosen and Greenwood, 1976). Two *Ophisternon* species are distributed in Central and South America: (1) the widespread *O. aenigmaticum* inhabiting drainages from the Isthmus of Tehuantepec (Mexico) to the vicinity of the Motagua fault, and (2) the cave species *O. infernale* known only from the Yucatan (white dot). Two *Synbranchus* species are described for Central and South America: (A) the widespread *S. marmoratus* inhabiting Central America eastward the Motagua fault to Argentina, and (B) the *S. madeirae* inhabiting the River Madeira (Amazonas) (black dots). Sympatric areas of *Synbranchus* and *Ophisternon* in Central and South America are represented in grey. The arrow indicates the Central American sympatric area in the Caribbean slope described for *Ophisternon* and *Synbranchus* (Rosen and Greenwood, 1976).

ica in the vicinity of the Motagua fault (Guatemala) (Rosen and Greenwood, 1976). This fault represents the suture between the ancient Chortis and Maya Terranes (Coates, 1997). In South and Central America, each synbranchid genera contains only two species (Fig. 1).

Swamp eels have also appeared in discussions of Central American and Caribbean biogeography. Rosen's (1976) vicariant model of Caribbean biogeography proposes that *Ophisternon* and *Synbranchus* may be members of an ancient Gondwanian biota. Over the past decades questions of how and when freshwater fishes reached Central America and the Caribbean islands have been of interest to many biogeographers (Croizat, 1962; Darlington, 1957; Rosen, 1976, 1978), however there is still no consensus regarding the origin and age of neotropical freshwater fish fauna. Questions of when and how often South and Central America were connected provide the main differences among vicariant hypotheses. All biogeographical hypotheses concede great importance to the closure of the Isthmus of Panama in mid-Pliocene (~3.3 Ma) as the main corridor for the expansion of South American freshwater fish into Central America (Bermingham and Martin, 1998; Bussing, 1976, 1985; Myers, 1966). Previous connections at the Middle Miocene (12.9–11.8 Ma) and Late Mesozoic (~70–65 Ma) are still under debate (Iturralde-Vinent and Macphee, 1999). The geological history of the Antilles and their connection with South and Central America is still subject to controversy (Hedges, 1996; Iturralde-Vinent and Macphee, 1999). The geological complexity of the Neotropics coupled with the paucity of freshwater fish fossils made the phylogenetic relationships of organisms the keystone on which the Mesoamerican biogeographical hypothesis is constructed.

However, a recent cytological study has suggested that *Synbranchus marmoratus* from South America could represent a species *complex* based on their chromosome and genome size variation (Torres, 2000). We consider that application of molecular techniques would permit enhanced detection of evolutionary structure across the widespread species *S. marmoratus* and *Ophisternon aenigmaticum*. Towards that end we sequenced the complete mitochondrial genes cytochrome *b* (cyt *b*) and ATP synthase 8 and 6 (ATPase 8/6) for synbranchid eels throughout their Central American and Cuban distribution, as well as representative *cis*-Andean populations from Venezuela, Peru, Paraguay, and Argentina to establish their phylogenetic relationships. We also analyzed a subset of the individuals using the nuclear Recombinant Activating Gene (RAG-I) to confirm major patterns of mtDNA lineages relationships, and to assess their molecular divergences. In this study, we address two major issues in Neotropical freshwater fishes. The first is the appraisal of the origin and maintenance of freshwater fish diversity in Central America using phylogenetic inferences based on mitochondrial sequences (cyt *b* and ATPase 8/6). The second is the assessment of the relative importance of marine and terrestrial dispersal in synbranchid eels for reconstructing their historical biogeography in Central America.

2. Materials and methods

Seventy eight specimens of *S. marmoratus* and *O. aenigmaticum* were sequenced from 45 drainages in Cuba and Central and South America. Fig. 2 summarizes sample locations and ichthyological regions defined in Central America. Details of sample localities are listed in Appendix A. From 1 up to 6 individuals from the same drainage were sequenced for the complete mitochondrial genes cyt *b* (1140 bp) and ATP 8/6 (842 bp). We increased our study with restriction fragment length polymorphisms (RFLPs) analysis of all sequenced individuals ($N = 78$) and 57 more synbranchid specimens. PCR amplification were digested of the cyt *b* gene with the *Dde*I endonuclease (Promega Corporation) following manufacturer's indications. *Dde*I endonuclease were used to generate specific digestion profiles for major synbranchid clades. RFLP products were visualized in 2% agarose gels and individuals with novel *Dde*I RFLPs profile were sequenced for cyt *b* and ATPase 8/6.

A subset of 12 individuals of *Synbranchus* and *Ophisternon* were selected from the main mtDNA lineages and sequenced for the nuclear RAG-1 gene (1171 bp) (Appendix A). DNA voucher specimens and their associated lots were preserved in buffered formalin and have been deposited in the permanent collection at the Smithsonian Tropical Research Institute in Panama (STRI) (Bermingham et al., 1997b) or in the Museo Nacional de Ciencias Naturales of Madrid, Spain (MNCN). One specimen of *S. marmoratus* from the GenBank (Accession No. AP004439) (Miya et al., 2003) was included in our analyses and the synbranchiforms *Monopterus albus* (NC_003192, AP002945) and *Mastacembelus favus* (NC_3193, AP003193) (Miya et al., 2001) were selected as phylogenetic outgroups. For the nuclear analysis one specimen of *Gymnotus maculosus* (STRI-1470, NC. AY35925) from Colombia was used as outgroup.

DNA extraction and mitochondrial amplification protocols were performed as previously described (Perdices et al., 2002). The entire cyt *b* gene was PCR amplified with the primers GluDG.L (5′ TGACT TGAAR A ACCA YCGTTG 3′; Palumbi (1996)) and H16460 (5′C GAYCTTCGGATTAACAAGACCG3′; (http://nmg .si .edu/bermlab). The complete ATPase 8/6 slightly overlapping mitochondrial genes were amplified with primers L8331 (5′ AAAGCRTYRGCCTTTTAAGC 3′) and CO 3.2 H9236 (5′ GTTAGTGGTCAKGGGCTTGGR TC 3′) (http://nmg.si.edu/bermlab). The nuclear RAG-1 gene was amplified with the primers RAG-1F (5′ AGCTGTAGTCAGTAYCACAARATG 3′) and

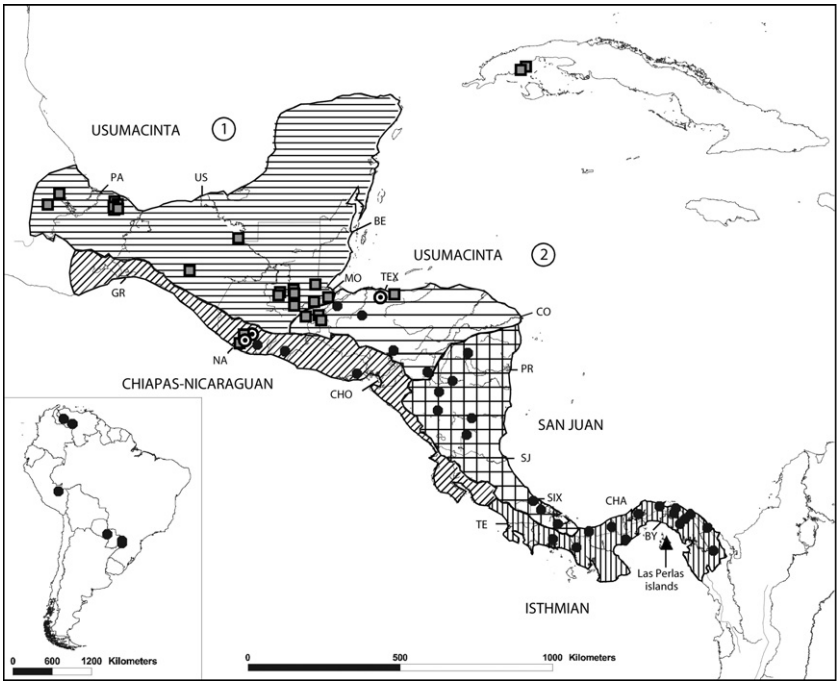

Fig. 2. Sampling localities of •, *Synbranchus*; ■, *Ophisternon*; and ▲, *Synbranchus aff. marmoratus* from Las Perlas island. ⊙ Corresponds to localities where *Synbranchus* and *Ophisternon* were collected in sympatry. Ichthyological provinces follow Bussing (1976) and are differently hatched: Usumacinta 1 and 2, San Juan, Chiapas-Nicaraguan, and Isthmian. River codes, Caribbean: BE, Belize; CHA, Chagres; CO, Coco; MO, Motagua; PA, Papaloapan; PR, Prinzapolka; SIX, Sixaola; SJ, San Juan; TEX, Texiguat; and US, Usumacinta. Pacific: BY, Bayano; CHO, Choluteca; GR, Grande; NA, Nahualate; and TE, Terraba.

RAG-9R (5′ GTGTAGAGCCAGTGRTGYTT 3′) (Quenouille and Bermingham, submitted). In all cases, the purified PCR band was used as template in a cycle sequencing reaction using the dRhodamine terminator cycle sequencing kit (PE Applied Biosystems). In some cases, the cyt *b* internal primer Cb3H (5′ GGCAA ATAGGAARTA TCATT C 3′; Palumbi (1996)) was also used for sequencing. For RAG-1 the purified PCR band was used as template, and the internal primers R AG-3F (5′ GGGTGATGTCAGYGAGAAGCA 3′), R AG-5R (5′ TRGAGTCACAGACTGCAGA 3′) and R AG-8R (5′ CGCCACACAGGYTTCATCT 3′) were always used in all sequencing reactions (Quenouille and Bermingham, submitted). After sequencing, samples were cleaned with G-50 Sephadex columns and loaded in an automated ABI 377 DNA sequencer.

2.1. Data analysis

Sequences were aligned and revised with Sequencher ver. 4.0 (Gene Codes). One less ATPase 8 codon was found in three *Synbranchus* specimens (STRI-8513, 98, and 6907) at position 124. These three specimens were not phylogenetically closely related, and this codon was excluded from further analyses. Nucleotide composition was examined for variable sites, and the χ^2 homogeneity test of base frequencies was done in PAUP* v. 4.0b10 (Swofford, 2002) for all positions. Nucleotide saturation was analyzed for mitochondrial genes by plotting uncorrected *p* distances at 1st, 2nd, and 3rd codon position against absolute distance values. Relation between genotypes were resolved by distance methods with Sequencer 6.1 (http://nmg.si.edu). Congruence among tree topologies generated with cyt *b* and ATPase 8/6 genes was tested with the partition homogeneity test in PAUP* (Farris et al., 1994; Mickevich and Farris, 1981).

We used the program Model test 3.06 (Posada and Crandall, 1998) to find the best model of evolution that fit our data. The model suggested for the mitochondrial genes was TrN + I (0.4363) + G (1.3766) selected by the Akaike criterion (empirical base frequencies A = 0.31, C = 0.31, G = 0.09, T = 0.29; substitution rates: A–C = 1.0, A–G = 14.92, A–T = 1.0, C–G = 1.0, C–T = 10.9676, G–T = 1.0). These parameters were used to estimate ML distances from minimum-evolution (ME) analyses. The mitochondrial data set was also analyzed by maximum parsimony (MP). Only minimal trees were retained and zero length branches were collapsed. Different weighting schemes were employed to adjust for transitional saturation (8:1, 5:1, 4:1, equal weights). Results found were congruent for the different weightings and only results for 5:1 are shown based on the empirical Ts:Tv ratio of 4.2. In all cases, MP analysis was performed using heuristic searches with TBR branch swapping and 10 replicates of random addition of taxa was used. Bootstrap analysis (1000 replicates) was used to assess the relative robustness of branches of ME and MP trees. All the above phylogenetic analyses were done using PAUP*.

Bayesian inference (BI) was implemented with MrBayes ver. 3.0 (Huelsenbenck and Ronquist, 2001). We ran one million generations of four simultaneous Monte Carlo Markov chains (MCMC) under the GTR model of evolution (sample frequency every 100, chain temperature 0.2). Posterior probability values were used as support for the Bayesian topology.

Nucleotide rate homogeneity of cyt *b* and ATPase 8/6 was assessed with a ML log-likelihood ratio test of clock-enforced and non-enforce trees (Page and Holmes, 1998) performed in PAUP*. Substitution rates were also evaluated with a relative rate test using RRTree v. 1.1 (Robinson-Rechavi and Huchon, 2000) on a set of taxa ($N = 18$) representing the intra-clade variation from each of the main mtDNA lineages recovered in the phylogeny (Appendix A). Under the assumption of constant rate of nucleotide substitution, we converted TrN + I + G genetic distances calculated from the ultrametric tree constructed using the non-parametric rate smoothing (NPRS) method (Sanderson, 1997) as performed in TreeEdit version 1.0 (Rambaut and Charleston, 2001) to absolute time. We used two different fish calibrations. The first is a cyt *b* calibration of 1.05% sequence divergence (TrN + G) per pairwise comparison per million years (Ma) for the freshwater fish family Cyprinidae estimated from match of fossil to molecular data (Dowling et al., 2002). The second is an ATPase 6 a calibration of 1.3% for geminate marine fishes separated by the closure of the Isthmus of Panama (Bermingham et al., 1997a).

The RAG-1 data were analyzed independently to assess the phylogenetic relationships recovered with the mitochondrial genes and their derived times of divergence across mtDNA lineages. Nucleotide composition was examined for variable sites, and the χ^2 homogeneity test of base frequencies was done in PAUP* for all positions. Nucleotide saturation was evaluated for the RAG-1 nuclear gene by plotting uncorrected *p* distances against absolute distance values considering the codon positions (data not shown). The model that best fits RAG-1 data selected under the Akaike criterion implemented in Model test was the TrN model + I (0.4454) + G (0.6234) (base frequencies (A = 0.2545; C = 0.2378; G = 0.2798; T = 0.2279; substitution rates 2.7146; A–C = 1.0, A–G = 5.7214, A–T = 1.0, C–G = 1.0, C–T = 8.9636, G–T = 1.0). We used these parameters in PAUP* to calculate ML distances for minimum-evolution (ME) trees (10,000 bootstraps).

3. Results

3.1. Mitochondrial and nuclear molecular characterization

The complete cyt *b* and ATPase 8/6 were sequenced for a total of 47 *S. marmoratus* and 31 *O. aenigmaticum*, and have been deposited in GenBank (ATPase8/6: AY354982–AY355059; cyt *b*: AY355060–AY355137). Among the synbranchids specimens used in the RFLP analysis ($N = 135$), we found seven exclusive haplotypes for *S. marmoratus*, and 9 for *O. aenigmaticum* whereas the synbranchid from Las Perlas islands (Pacific Panama) exhibited an exclusive RFLP pattern. Base frequencies were homogeneous across all variable sites and did not differ significantly within genera ($\chi^2 = 164.00$, 227.71; degrees of freedom (df) = 234, and $P = 0.99$, 0.60, for ATPase 8/6 and cyt *b*, respectively). The majority of variable and informative sites are third position with similar rates for ATPase 8/6 (29.6%) and cyt *b* (30.8%). A slight trend toward saturation was observed in the cytochrome *b* gene at 3rd codon position in excess ~45% of divergence that correspond to the pairwise distances between synbranchid genera (Fig. 3).

Independent analysis of the partitioned mitochondrial genes yielded similar topologies of the phylogenies with ME, MP or Bayesian inference. In general, the observed discrepancies across methods correspond to branches with bootstrap values <70% in some of the methods. The partition homogeneity test revealed no significant differences among the genes studied (ATPase 8 vs. ATPase 6 $P = 0.82$; ATPase 8/6 vs. cyt *b* $P = 0.24$) and then, subsequent analyses are based on the combined data. The completed data set contained 966 variable sites, 860 of which were phylogenetically informative for MP analyses (without outgroups).

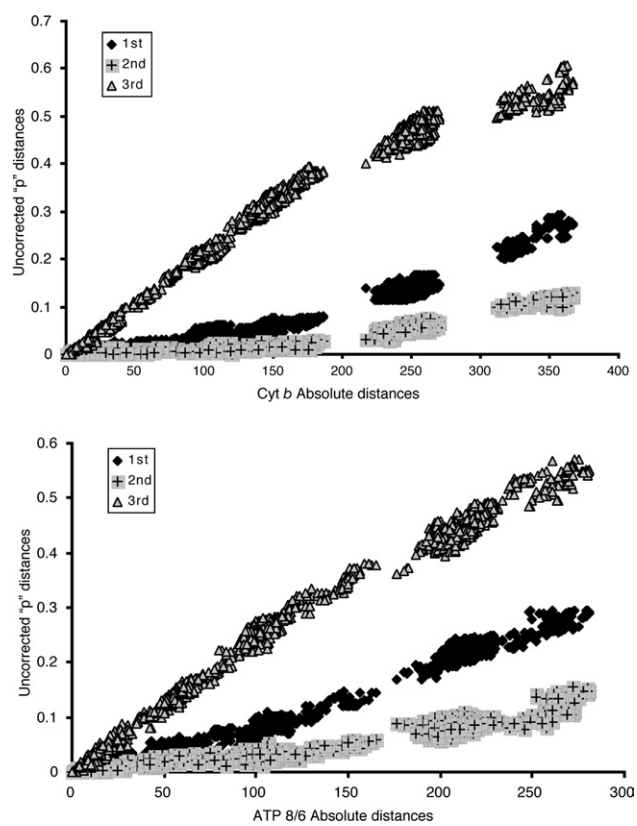

Fig. 3. Saturation plots of cyt *b*, and ATPase 8/6 mitochondrial genes. 1st, First; 2nd, second; and 3rd, third positions were plotted against absolute distances.

A total of 1171 bp were analyzed for the nuclear gene RAG-1 in 12 synbranchid eels and were deposited in the GenBank (NC. AY359213–AY359224). Similar nucleotidic percentages were found across individuals with slight G-enriched bias (29.2%) when compared to the mitochondrial genes for the same individuals. Base frequencies were homogeneous across all variable sites and did not differ significantly between genera ($\chi^2 = 0.87$, df = 33, $P = 1.0$). The RAG-1 gene contained a total of 6.7% variable and informative sites. Saturation plots revealed no trend toward saturation in any position (plots not shown).

3.2. Phylogeography of O. aenigmaticum and S. marmoratus based on mtDNA sequences

All reconstructions based on the mitochondrial genes indicate a deep split between three divergent clades: *Ophisternon*, *Synbranchus*, and one *S. marmoratus* specimen collected in Las Perlas islands (>82% bootstrap and >97% posterior probability) (Fig. 4). In spite that the specimen from Las Perlas was recovered as the sister group of the *Synbranchus* clade in the Bayesian inference, it was supported with a low value of posterior probability (68%). *S. marmoratus* is not strongly supported as a monophyletic taxon. Thus, to facilitate the results and discussion we refer to Las Perlas islands synbranchids as *S. aff. marmoratus* (Sysl Perlas). Genetic distances estimated using ML parameters (TrN + G + I) are considerably high especially for distant clades or lineages (mean 68.9, range 49.8–87.1% (Table 1).

The *Synbranchus* clade is composed of 47 *S. marmoratus* specimens representing 30 Central and South American drainages along both Central America slopes from Eastern Honduras (HO) to Argentina (ARG) (Appendix A, Fig. 2). All phylogenies identified seven mtDNA lineages with bootstrap and posterior probability values >90% (Fig. 4). Contrary to what one would predict, the *S. marmoratus* specimens from the Bayano drainage (Pacific Panamá) are the basal lineage (SyLCA Bayano) and show higher genetic distances (>28%) to the rest of the *S. marmoratus* lineages than those shown by South American individuals (>20%). South American specimens are divided in four well-supported lineages (>90% bootstrap and posterior probability), being the Northern South American (NSA) specimens from the River Orinoco (SyNCA) closely related to the Central American *S. marmoratus* (SyMCA, SyLCA). It is worth to note that the South American *S. marmoratus* from the Paraná drainage and Lower Central America individuals are not monophyletic (Fig. 4). Lower Central American specimens are included into three mtDNA lineages (SyLCA Bayano, SyLCA, and SyMCA) with high sequence divergence among them (Table 1, Fig. 5).

The *Ophisternon* clade includes 31 *O. aenigmaticum* individuals representing 14 river drainages from Upper Central America (Mexico, Guatemala, Western Honduras) and Cuba (Fig. 2, Appendix A). It is divided into four well-supported (77–100%) mtDNA lineages: OpCUBA, OpYUC, OpUCA 1, and OpUCA 2 (Figs. 4 and 5). In general, all the *Ophisternon* lineages showed allopatric ranges. Specimens from the same drainage or neighbor localities exhibited very low genetic distances. The exception are two specimens from the River Sarstung (mean genetic distance 16.1%) that belong to two different mtDNA lineages (OpYUC and OpUCA2), and the River Usumacinta (River Las Conchas) individuals (STRI-7828, 7829) with a mean divergence of 6.5% from the rest of the Usumacinta specimens.

3.3. Nuclear RAG-1 gene

The RAG-1 based phylogeny recovered the same three clades (mean TrN + G + I distances $3.7 \pm 0.7\%$; range 2.0–4.5%) as the mitochondrial groups with moderate bootstraps and posterior probability support (59–81%) (Fig. 6). The *Synbranchus aff. marmoratus* clade showed a mean nuclear genetic distance of $2.1 \pm 0.1\%$ with the *Ophisternon* clade and $3.7 \pm 0.4\%$ with the *Synbranchus* clade. The *Ophisternon* and *Synbranchus* main clade showed a mean genetic distance of $4.0 \pm 0.3\%$ (range, 3.2–4.5%). Inside each clade, there is no observable structure due to the low genetic distances found (mean $1.3 \pm 0.8\%$). The comparison between mean TrN + I + G nuclear genetic distances with the mitochondrial distances for the same 12 individuals (3.7 and 28.8%, respectively) showed that mitochondrial genes (cyt *b* and ATPase 8/6) evolved ~7–8 times faster than the nuclear RAG-1 gene.

3.4. Rate constancy and divergence time

A log-likelihood and a relative rate tests rejected the null hypothesis of evolutionary rate constancy across all *Ophisternon* and *Synbranchus* mtDNA haplotypes ($-\ln L$ enforced tree = 20.188.678, $-\ln L$ non-enforced tree = 20091.2922, df = 77, $P < 0.0001$). However, a relative rate test based on representative individuals of each of the mtDNA lineages found in the phylogeny (Fig. 4, Appendix A) accepted the null hypothesis of rate constancy, suggesting that mtDNA divergence can be used to evaluate the pattern and timing of both genera across Central America and Cuba. In the absence of a constant rate of evolution for all taxa analyzed we used the NPRS method to construct an ultrametric tree based on the phylogenetic tree obtained for the mitochondrial genes (Fig. 7). The mean level of mitochondrial sequence divergence (TrN + I + G) separating the *Synbranchus* and *Ophisternon* clades is 74.9% (range, 62.8–87.1%), and application of 1.05–1.3% sequence divergence per million year (Bermingham et al., 1997a; Dowling et al., 2002)

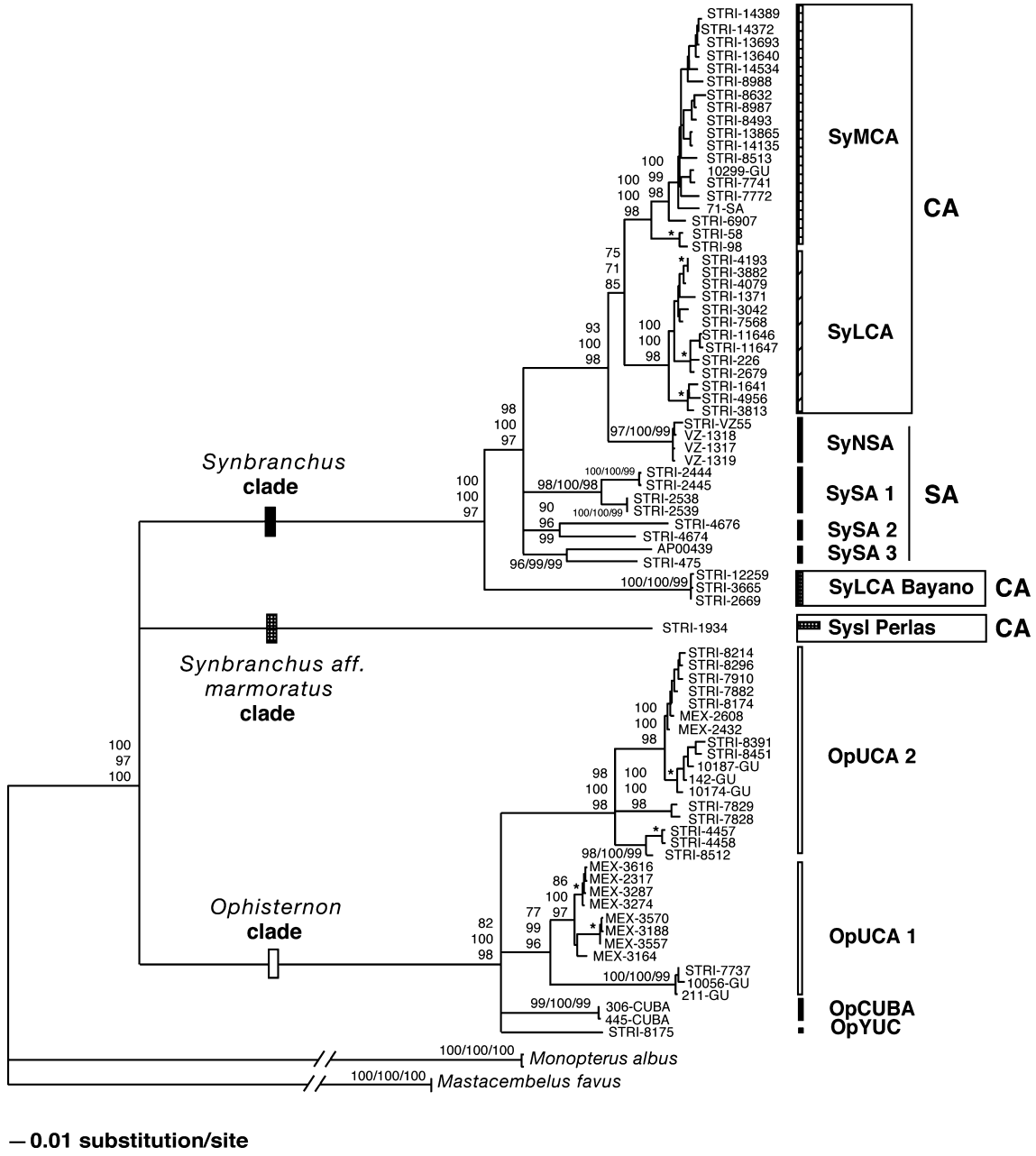

Fig. 4. Phylogenetic relationships recovered based on the combined mitochondrial genes cyt *b* and ATP8/6. The phylogeny is the single tree recovered in ME (TrN + I + G), 5:1 MP and Bayesian inference. Values on branches corresponds to ME, MP bootstraps, and Bayesian posterior probabilities, *terminal branches >80% supported in all analyses. Main branches were collapsed when branch support was inferior to 70% in any of the analyses. Named mtDNA lineages referred in the text are listed to the right: Sy, *Synbranchus*; Op, *Ophisternon*. UCA, MCA, LCA, and YUC abbreviate Upper, Middle, Lower Central America, and Yucatan, e.g., SyMCA *Synbranchus* from Middle Central America.

indicates that the age of their split varies between ~48 and 83 Ma (Table 1). For the *Ophisternon* clade, our data show high genetic distances between upper CenBA) lineages. Their mean sequence divergence is 18.5% (range, 13.0–21.2%) or ~9.9–20.2 Ma (Table 1). Inside the *Synbranchus* clade, the mean mtDNA divergence between Northern South American (SyNSA) and Central American *Synbranchus* (SyLCA + SyMCA) lineages is 11.2%, being the age of this split between ~7.7 and 12.4 Ma (Table 1).

4. Discussion

Morphological characters have traditionally been used in synbranchid systematics and biogeographical inferences (Rosen and Rumney, 1972; Rosen and Greenwood, 1976). However, the scarcity of external diagnostic characters do not offer much resolution below the genus level, and thus, synbranchids are uncommon in phylogeographical analyses (Vari and Malabarba, 1998). Mitochondrial data derived from the complete cyt *b* and

Table 1
Pairwise genetic distances between major synbranchid clades and some of the named mtDNA lineages presented in Fig. 3 (see Section 3)

	% Mean TrN + I + G distance ± SD (*range*)	Mean TrN + I + G divergence (Ma) (*range*)
Ophisternon vs *Synbranchus*	74.9 ± 4.7 (*62.8–87.1*)	57.2–71.3 (*47.9–83.0*)
Ophisternon vs *Synbranchus aff. marmoratus*	55.2 ± 2.7 (*49.8–59.0*)	42.1–52.6 (*38.0–56.2*)
Synbranchus vs *Synbranchus aff. marmoratus*	76.7 ± 2.8 (*71.6–82.4*)	58.5–73.0 (*54.7–78.5*)
All *Ophisternon* mtDNA lineages	12.0 ± 6.8 (*0.0–21.2*)	9.2–11.4
All *Synbranchus* mtDNA lineages	13.5 ± 8.6 (*0.0–32.2*)	10.3–12.9
OpCUBA vs (OpUCA 1 + OpUCA 2 + OpYUC)	18.5 ± 1.5 (*13.0–21.2*)	14.1–17.6 (*9.9–20.2*)
SyLCA Bayano vs all mtDNA *Synbranchus*	28.8 ± 1.8 (*24.8–32.2*)	22.0–27.4 (*18.9–30.7*)
SyNCA vs (SyLCA + SyMCA)	11.2 ± 0.72 (*10.1–13.0*)	8.5–10.7 (*7.7–12.4*)
(SySA1 + SySA2 + SySA3) vs (SyNSA SyMCA + SyLCA)	20.1 ± 1.50 (*16.7–24.1*)	15.3–19.1 (*12.7–23.0*)

Distances were calculated from the mitochondrial genes cyt *b* (1141 bp) and ATPase 8/6 (842 bp) and corrected using the maximum likelihood model (Tamura Nei + I + G). Divergence times (Ma) are calculated based on fish mtDNA calibrations of 1.05–1.3% (Bermingham et al., 1997a; Dowling et al., 2002).

ATPase 8/6 have permitted the reconstruction of their phylogeny and indicates the monophyly of the Mesoamerican *O. aenigmaticum* (Rosen and Greenwood, 1976). However, our phylogeny suggest that *S. marmoratus* does not represent a monophyletic taxon. Nuclear and mitochondrial data support the distinctiveness of the *S. marmoratus* specimen from Las Perlas islands (Sysl Perlas), and suggests that a possible third synbranchid group inhabit Mesoamerica. Nevertheless, more *Synbranchus* specimens from Las Perlas (Pacific Panama) need to be morphologically and genetically compared to other *Synbranchus* species to confirm their taxonomic distinctiveness.

Like other freshwater fish groups with broad Neotropical distributions (Martin and Bermingham, 2000; Montoya-Burgos, 2003; Perdices et al., 2002; Sivasundar et al., 2000), our phylogenetic data suggest that *S. marmoratus* and *O. aenigmaticum* systematics needs to be revised. In the present study, we found more than 10 mtDNA evolutionary lineages for both *S. marmoratus* and *O. aenigmaticum* (Fig. 4). For the *Synbranchus* clade, sequence divergence of specimens from the River Bayano and South America are very high, which supports their evolutionary singularity. Moreover, the genetic differentiation of *S. marmoratus* from South America is congruent with the species complex suggested for some

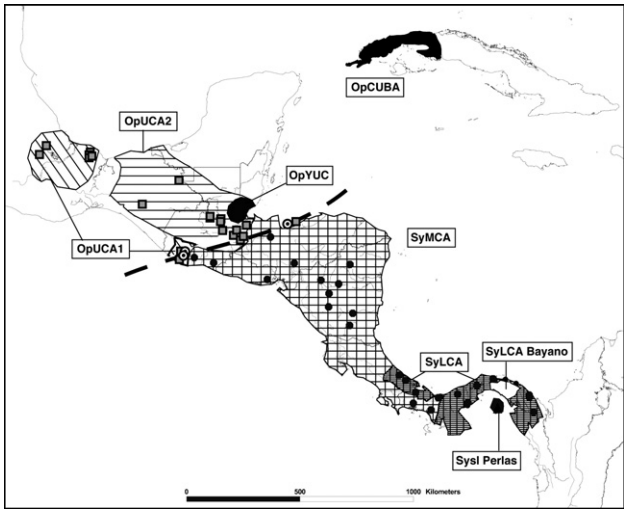

Fig. 5. Geographical distribution of the synbranchids mtDNA lineages presented in Fig. 3. UCA, MCA, LCA, and YUC abbreviate Upper, Middle, Lower Central America, and Yucatan. Dash line joint the sympatric populations of *Synbranchus* and *Ophisternon* found in the Caribbean and Pacific slopes.

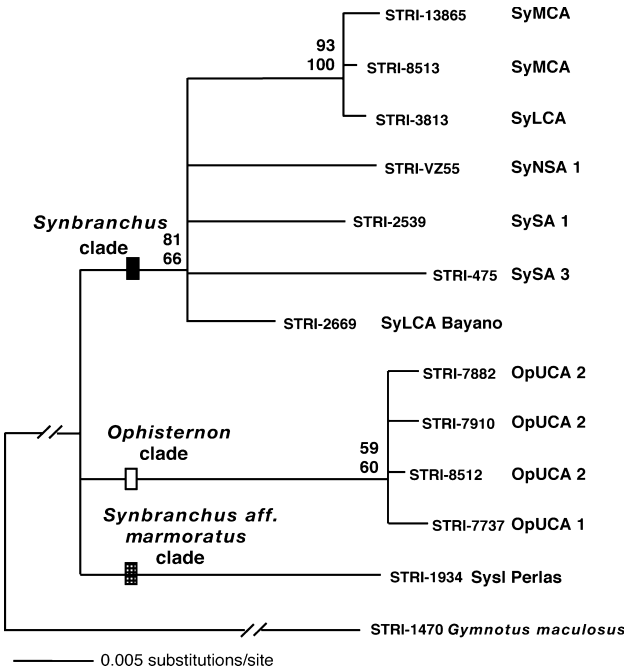

Fig. 6. Phylogenetic relationships based on RAG-1 nuclear data. The phylogeny is the ME (TrN + I + G) and ML tree. Upper values on the branches represent bootstrap values (ME above).

Brazilian populations based on their chromosome and genome size variation (Torres, 2000). For the *Ophisternon* clade, it was previously suggested that *Ophisternon* individuals from Cuba were different species (Rosen, 1976), and their evolutionary distinctiveness is supported by our data. Thus, it seems that current systematics of American synbranchids underestimates their real diversity.

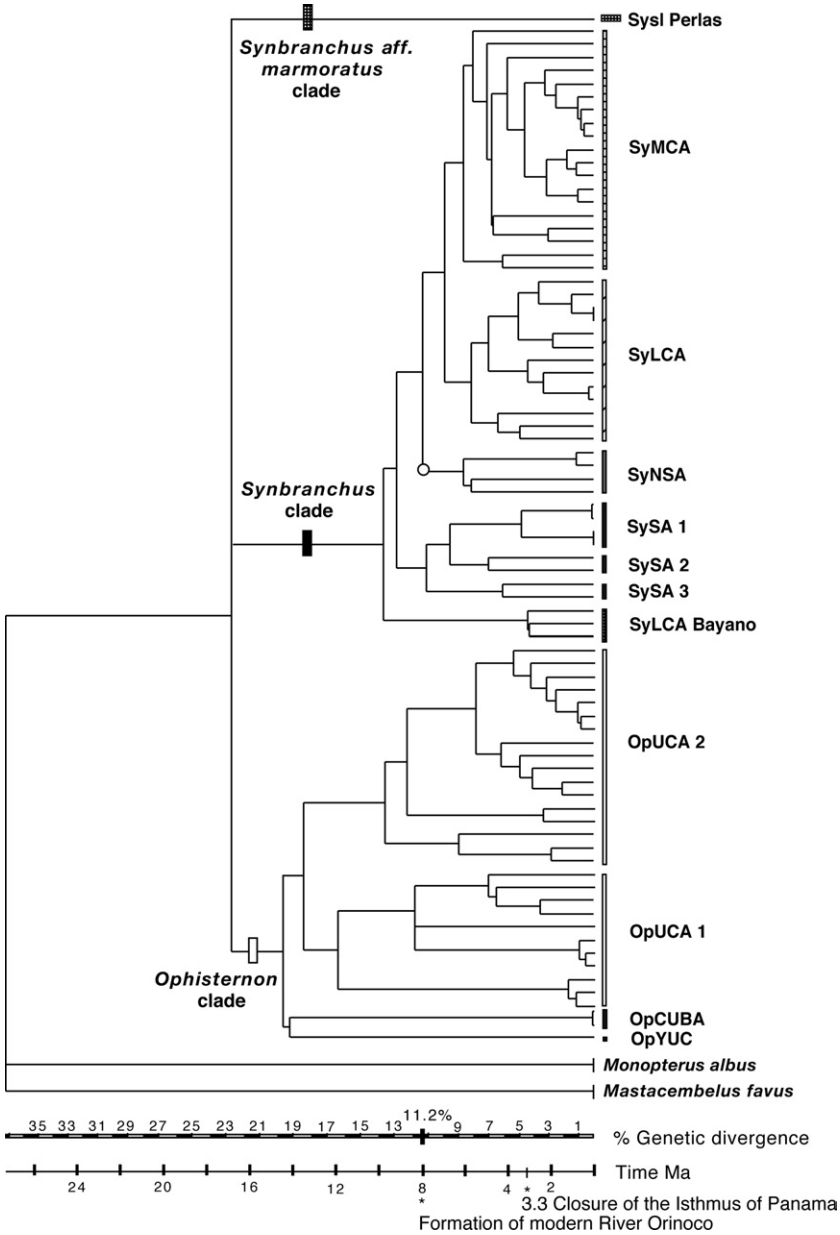

Fig. 7. Ultrametric tree based on the NPRS analysis of the mitochondrial cyt *b* and ATPase 8/6 genes. The time-scale was established assuming that the formation of the modern River Orinoco was ~8 Ma (Lundberg et al., 1998) and have promoted the isolation of the mtDNA SyNCA that inhabits the Orinoco drainage (open circle).

The phylogenetic analysis strongly supports the genetic distinctiveness of the genera *Synbranchus* and *Ophisternon* (Rosen and Greenwood, 1976). In Central America, both genera exhibit complementary distributions with a narrow sympatric area in the vicinity of the Polochic-Motagua fault (Rosen and Greenwood, 1976). Previous studies placed this sympatric area exclusively in the Caribbean however we have found sympatric populations in both slopes (Fig. 5). The Polochic-Motagua fault represents the Chortis block suture zone, and although freshwater fishes were able to survive for long periods in this active geological area, it is largely recognized as an area of change for freshwater fishes (Bussing, 1976, 1985; Myers, 1966; Perdices et al., 2002; Rosen, 1985). This narrow area of sympatry without hybrids suggests that both genera were genetically well differentiated when they came into contact with each other. Although analyzing nuclear RAG-1 sequences from few sympatric individuals (STRI-8512 and 8513, in Fig. 6), we didn't find any diagnostic position that might indicate hybridization between *Synbranchus* and *Ophisternon* specimens inhabiting the Motagua fault, thus corroborating their mitochondrial singularity.

The expansion of *Ophisternon* and *Synbranchus* across the Chortis suture was probably limited by the fact that habitats on either side of the suture were

already occupied (Hewitt, 1996, 2000; Reeves and Bermintham, submitted). Generally, most Central American drainages appear to contain a single *Ophisternom* and *Synbranchus* mtDNA lineage (Fig. 5). Most of the mtDNA lineages seem to be allopatric with few instances of sympatry, which suggests that divergence in allopatry, e.g., formation of river drainages could be one of the main evolutionary forces that promoted synbranchid speciation. However, we observed some sympatric *Ophisternon* mtDNA lineages in the Usumacinta (OpUCA 2) and Sarstung drainages (Southern Yucatan) (Sy YUC), and several *Synbranchus* sympatric lineages in Lower Central America and in the Paraná drainage (Sy SA 1 and 2). In most of these areas, other freshwater fish mtDNA lineages have also been reported in sympatry (Martin and Bermingham, 2000; Montoya-Burgos, 2003; Perdices et al., 2002), suggesting that other historical factors as river captures or confluence of drainages might have played a local role in freshwater fish diversity.

4.1. Historical biogeography

Current geographic distribution of Central American synbranchid relatives in South America, Africa, Asia, and Australia (Fig. 1) does not contradict the suggested Godwanian origin for the family Synbranchidae (Rosen, 1976). In absence of fossils, we assume a South American origin of Central American synbranchids based on present distribution of synbranchid congeners. The mtDNA phylogeny showed a basal split of all major synbranchids clades and indicates that separation of *Ophisternon* and *Synbranchus* occurred after this split (~48–83 Ma) (Table 1, Fig. 4). Although molecular clocks are rough estimates of time, the dates are in agreement with the Godwanian origin of Central American synbranchids. Current Mesoamerican distribution of *O. aenigmaticum* and its phylogeographic pattern allow some considerations about its origin in Central America. *Ophisternon aenigmaticum* inhabits Mexico, Guatemala, and Western Honduras, and its absence in suitable habitats in Lower Central America provides support that the eastern limit of *O. aenigmaticum* delineates the limits of the Chortis and Maya Terranes in the Polochic-Motagua fault. These areas were part of the North American continent during all Central America formation at least 80 million years ago (Coates, 1997), and thus were suitable habitat for freshwater fishes. The estimated age of the split between Upper Central America and Cuban *Ophisternon* suggests a Miocene (9.9–20.2 Ma) separation. The high divergences found also reject the idea that *Ophisternon* dispersal in Cuba was recent, or it was promoted by their secondary physiological status as was indicated for the genus *Gambusia* (Lydeard et al., 1995).

For the *Synbranchus* clade, the phylogeny supports a first Central American split (SyLCA Bayano) at Miocene (22.0–27.4 MYA) (Fig. 4, Table 1). Although our data showed the importance of the Pacific area on the early differentiation of Central American *Synbranchus*, these earlier lineages don't seem to be the genetic sources of current Central America synbranchid lineages. The mtDNA-based phylogeny supports a sister group relationship of the *Synbranchus* from Northern South America (SyNSA) and the *Synbranchus* from Middle and Lower Central America (SyLCA + SyMCA). The estimated dates of the SyNSA and (SyLCA + SyMCA) split (~7.7–12.4 Ma) agree with the geological dating of the formation of the modern River Orinoco (~7–8 Ma) (Lundberg et al., 1998). These results suggest that the complete formation of the River Orinoco might have favored the split of Central American and Northern South American *Synbranchus*, preventing posterior exchanges. Paleogeographical reconstructions of Central America indicate that from Middle to Late Miocene (15–6 Ma) a shallow connection might have extended from South America and Lower Central America (Coates and Obando, 1996), a link that might have favored secondary freshwater fish dispersal before the final closure of the Isthmus of Panama. The salinity tolerance of these secondary freshwater fishes supported the hypothesis that they were present earlier in Central American waters (Myers, 1966). It is likely that secondary freshwater fish such as heroine cichlids, *Rivulus* (Martin and Bermingham, 1998; Murphy and Collier, 1996) and synbranchids used this connection prior to primary freshwater fishes to colonize Central America.

Our phylogeographic analyses allow comparisons of freshwater fish faunal regions (Fig. 2). In general, the northern distribution of synbranchids in Central America coincides with the northern boundary of tropical freshwater fishes (Bussing, 1985; Miller, 1966). We observed some correspondence between the Usumacinta (1) ichthyological province (Bussing, 1976), and the geographical area defined by *Ophisternon* mtDNA lineages. However, we did not find any phylogeographical breaks in Central American synbranchids that corresponded to major ichthyological regions delimited in Middle and Lower Central America (Figs. 2 and 5). Moreover, we found some Pacific river drainages, e.g., River Bayano or Las Perlas island rivers, that harbored endemic *Synbranchus* mtDNA lineages, drainages that do not delimit any geographical boundary of freshwater fishes. This phylogeographical pattern was never reported for any other freshwater fish taxon, and more groups need to be analyzed to check how general the *Synbranchus* pattern is.

In conclusion, our phylogenetic hypothesis suggests that the *S. marmoratus* and *O. aenigmaticum* mtDNA clades were present in Central America before the final closure of the Isthmus of Panama at Pliocene. The phylogeographic structure observed is in agreement with the long residence of synbranchids in Central America (Middle to Late Miocene). Our results are similar to the

inferred dates of presence for other secondary freshwater fishes, e.g., cichlids in Central America (Martin and Bermingham, 1998). For this long residence, we suggest that the secondary physiological status of synbranchids have facilitated their expansion and maintenance in Central America.

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Appendix A

Localities and specimens analyzed for molecular systematics of Central American synbranchids

MtDNA lineages	Genus	Species	Drainage/slope A, Atlantic; C, Caribbean; P, Pacific	Country	Lat	Long	Locality	Specimen identification
OpCUBA	*Ophisternon*	*aenigmaticum*	San Francisco/C	CUBA	22.4	83.1	Pinar del Rio	306-CUBA
OpCUBA	*Ophisternon*	*aenigmaticum*	Los Palacios/C	CUBA	22.4	83.2	Rio Los Palacios	445-CUBA
OpYUC	*Ophisternon*	*aenigmaticum*	Sarstung/C	GU	16	89.3	R. San Pedro	STRI-8175
OpUCA 1	*Ophisternon*	*aenigmaticum*	Lake Catemaco/C	MEX	18.6	96.9	Lake Catemaco	MEX-2317
OpUCA 1	*Ophisternon*	*aenigmaticum*	Lake Catemaco/C	MEX	18.2	95.1	R. Chuniapan	MEX-3274
OpUCA 1	*Ophisternon*	*aenigmaticum*	Lake Catemaco/C	MEX	18.2	95.1	R. Sihuapan	MEX-3287
OpUCA 1	*Ophisternon*	*aenigmaticum*	Lake Catemaco/C	MEX	18.3	95.1	R. San Joaquin	MEX-3616
OpUCA 1	*Ophisternon*	*aenigmaticum*	Las Niñas/C	MEX	18.4	95	R. de las Niñas	MEX-3164
OpUCA 1	*Ophisternon*	*aenigmaticum*	Las Lisas/C	MEX	18.4	95.1	R. Las Lisas	MEX-3188
OpUCA 1	*Ophisternon*	*aenigmaticum*	Máquinas/C	MEX	18.3	97.1	R. Máquinas	MEX-3557
OpUCA 1	*Ophisternon*	*aenigmaticum*	La Palma/C	MEX	18.3	95.1	Afluent R. de La Palma	MEX-3570
OpUCA 1	*Ophisternon*	*aenigmaticum*	Bolas/P	GU	14.2	91.6	R. Bolas	10056-GU
OpUCA 1[a]	*Ophisternon*	*aenigmaticum*	Nahualate/P	GU	14.5	91.4	R. Pachipa	STRI-7737
OpUCA 1	*Ophisternon*	*aenigmaticum*	Nahualate/P	GU	14.5	91.3	R. Pajoca	211-GU
OpUCA 2	*Ophisternon*	*aenigmaticum*	Gri-Usumacinta/C	MEX	16.4	93	R. Mina, Afl. Grijalva	MEX-2432
OpUCA 2	*Ophisternon*	*aenigmaticum*	Gri-Usumacinta/C	MEX	17.3	91.6	R. Ashipa, Afl. Grijalva	MEX-2608
OpUCA 2	*Ophisternon*	*aenigmaticum*	Usumacinta/C	GU	15.8	90.3	R. Las Conchas	STRI-7828
OpUCA 2	*Ophisternon*	*aenigmaticum*	Usumacinta/C	GU	15.8	90.3	R. Las Conchas	STRI-7829
OpUCA 2[a]	*Ophisternon*	*aenigmaticum*	Usumacinta/C	GU	15.8	90	R. Sebol	STRI-7910
OpUCA 2[a]	*Ophisternon*	*aenigmaticum*	Usumacinta/C	GU	15.7	89.9	R. Chajmaic	STRI-7882
OpUCA 2	*Ophisternon*	*aenigmaticum*	Motagua/C	GU	15.1	89.6	R. Hato	142-GU
OpUCA 2	*Ophisternon*	*aenigmaticum*	Motagua/C	GU	15.2	89.2	R. Hondo	10174-GU
OpUCA 2	*Ophisternon*	*aenigmaticum*	Motagua/C	GU	15.1	82.2	R. Santiago	10187-GU
OpUCA 2	*Ophisternon*	*aenigmaticum*	Motagua/C	HO	14.9	89.1	R. El Sauce	STRI-8391
OpUCA 2	*Ophisternon*	*aenigmaticum*	Motagua/C	HO	14.9	89.1	R. El Sauce	STRI-8451
OpUCA 2	*Ophisternon*	*aenigmaticum*	Sarstung/C	GU	16	89.3	R. San Pedro	STRI-8174
OpUCA 2	*Ophisternon*	*aenigmaticum*	Polochic/C	GU	15.5	88.9	R. Amatillo, Lake Izabal	STRI-8214
OpUCA 2	*Ophisternon*	*aenigmaticum*	Polochic/C	GU	15.3	89.9	R. Chaguacal	STRI-8296
OpUCA 2[a]	*Ophisternon*	*aenigmaticum*	Texiguat/C	HO	15.6	87.3	Quebrada de Piedra Negra	STRI-8512

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Appendix A (*continued*)

MtDNA lineages	Genus	Species	Drainage/slope A, Atlantic; C, Caribbean; P, Pacific	Country	Lat	Long	Locality	Specimen identification
OpUCA 2	*Ophisternon*	*aenigmaticum*	Perla/C	HO	15.7	87	Utila Island/C	STRI-4457
OpUCA 2	*Ophisternon*	*aenigmaticum*	Perla/C	HO	15.7	87	Utila Island/C	STRI-4458
Sysl Perlas[a]	*Synbranchus*	*affinis marmoratus*	R. Palenque	PN	8.34	78.9	El Rey island, Las Perlas islands	STRI-1934
SyMCA	*Synbranchus*	*marmoratus*	Acome/P	GU	14.2	91	R. Chifle, Siquinala	10299-GU
SyMCA	*Synbranchus*	*marmoratus*	Nahualate/P	HO	14.5	91.4	R. Pachipa	STRI-7741
SyMCA[a]	*Synbranchus*	*marmoratus*	Texiguat/C	HO	15.6	87.3	Queb. de Piedra Negra	STRI-8513
SyMCA	*Synbranchus*	*marmoratus*	Ulua/C	HO	15.3	88.7	R. Camalote	STRI-8493 (
SyMCA	*Synbranchus*	*marmoratus*	Ulua/C	HO	15	87.9	R. Yojoa	STRI-8632
SyMCA	*Synbranchus*	*marmoratus*	Paz/P	HO	14	90.2	R. Castaño	STRI-7772
SyMCA	*Synbranchus*	*marmoratus*	Choluteca/P	HO	14	87	R. Chiquito de Guinope	STRI-8987
SyMCA	*Synbranchus*	*marmoratus*	Choluteca/P	HO	14	87	R. Chiquito de Guinope	STRI-8988
SyMCA	*Synbranchus*	*marmoratus*	Jocotal Lagoon/P	SA	13.3	88.1	Jocotal Lagoon	71-SA
SyMCA[a]	*Synbranchus*	*marmoratus*	Coco/C	NI	13.3	86	Queb. Venquilla, afl. R. Pantasma	STRI-13865
SyMCA	*Synbranchus*	*marmoratus*	Prinzapolka/C	NI	13.9	84.8	R. Ceperna	STRI-14135
SyMCA	*Synbranchus*	*marmoratus*	Grande de Matagalpa/C	NI	12.8	85.6	R. Compazagua	STRI-14389
SyMCA	*Synbranchus*	*marmoratus*	Grande de Matagalpa/C	NI	13.1	85.3	R. Blanco	STRI-14372
SyMCA	*Synbranchus*	*marmoratus*	Escondido/C	NI	12	84.7	R. Espavel, afl. R. Chimalate	STRI-13693
SyMCA	*Synbranchus*	*marmoratus*	San Juan/C	NI	12.3	85.7	R. Camoapa, afl. Lago Nicaragua	STRI-13640
SyMCA	*Synbranchus*	*marmoratus*	San Juan/C	NI	11.5	84.8	R. El Valencia, afl. L. Nicaragua	STRI-14534
SyMCA	*Synbranchus*	*marmoratus*	Tabasará/P	PN	8.2	81.6	R. Tabasará	STRI-98
SyMCA	*Synbranchus*	*marmoratus*	Está/P	PN	8.43	82.3	R. Está	STRI-58
SyMCA	*Synbranchus*	*marmoratus*	Calovebora/P	PN	8.75	81.2	R. Calovebora	STRI-6907
SyNSA	*Synbranchus*	*marmoratus*	Orinoco/A	VZ	7.01	68.2	R. Orinoco	VZ-1317
SyNSA	*Synbranchus*	*marmoratus*	Orinoco/A	VZ	7.01	68.2	R. Orinoco	VZ-1318
SyNSA	*Synbranchus*	*marmoratus*	Orinoco/A	VZ	7.01	68.2	R. Orinoco	VZ-1319
SyNSA[a]	*Synbranchus*	*marmoratus*	Orinoco/A	VZ	8.24	69.6	Caño 8 km SW Dolores	VZ-55
SyLCA	*Synbranchus*	*marmoratus*	Sixaola/C	CR	9.6	82.8	R. Sixaola	STRI-226
SyLCA	*Synbranchus*	*marmoratus*	Changuinola/C	PN	9.36	82.6	R. Bongie	STRI-2679
SyLCA	*Synbranchus*	*marmoratus*	Guarumo/C	PN	8.87	82.2	R. Cañazas	STRI-11646
SyLCA	*Synbranchus*	*marmoratus*	Guarumo/C	PN	8.87	82.2	R. Cañazas	STRI-11647
SyLCA	*Synbranchus*	*marmoratus*	Coclé del Norte/C	PN	8.82	80.6	R. Coclé del Norte	STRI-1371
SyLCA	*Synbranchus*	*marmoratus*	Mandinga/C	PN	9.47	79.1	R. Mandinga	STRI-1641
SyLCA[a]	*Synbranchus*	*marmoratus*	Azúcar/C	PN	9.42	78.6	R. Azúcar	STRI-3813
SyLCA	*Synbranchus*	*marmoratus*	Playón Chico/C	PN	9.26	78.2	R. Playón Chico	STRI-4956
SyLCA	*Synbranchus*	*marmoratus*	Acla/C	PN	8.84	77.7	R. Acla	STRI-3882
SyLCA	*Synbranchus*	*marmoratus*	Acla/C	PN	8.82	77.7	R. Acla	STRI-4193
SyLCA	*Synbranchus*	*marmoratus*	Farallón/P	PN	8.38	80.1	R. Farallón	STRI-3042
SyLCA	*Synbranchus*	*marmoratus*	Chagres/P	PN	9.2	79.8	R. Agua Salud	STRI-7568
SyLCA	*Synbranchus*	*marmoratus*	Tuira/P	PN	8.12	77.5	R. Yape	STRI-4079
SyLCA Bayano[a]	*Synbranchus*	*marmoratus*	Bayano/P	PN	9.12	78.4	Queb. upper R. Bayano	STRI-2669
SyLCA Bayano	*Synbranchus*	*marmoratus*	Bayano/P	PN	9.27	78.7	Afl. R. Aguas Claras	STRI-12259

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Appendix A (*continued*)

MtDNA lineages	Genus	Species	Drainage/slope A, Atlantic; C, Caribbean; P, Pacific	Country	Lat	Long	Locality	Specimen identification
SyLCA Bayano	*Synbranchus*	*marmoratus*	Bayano/P	PN	8.98	78.5	R. Ipeti	STRI-3665
SySA3[a]	*Synbranchus*	*marmoratus*	Amazonas/A	PE	11.8	71.4	R. Manu	STRI-475
SySA 1	*Synbranchus*	*marmoratus*	Paraná/A	ARG	25.7	54.2	Arroyo Yacuy	STRI-2444
SySA 1	*Synbranchus*	*marmoratus*	Paraná/A	ARG	25.7	54.2	Ar. Yacuy	STRI-2445
SySA 1	*Synbranchus*	*marmoratus*	Paraná/A	ARG	26	54.3	Ar. Falso, Urugua-I	STRI-2538
SySA 1[a]	*Synbranchus*	*marmoratus*	Paraná/A	ARG	26	54.3	Ar. Falso, Urugua-I	STRI-2539
SySA 2	*Synbranchus*	*marmoratus*	Paraná/A	PY	23.7	57.9	Province Chaco. Bait shop	STRI-4676
SySA 2	*Synbranchus*	*marmoratus*	Paraná/A	PY	23.7	57.9	Prov. Chaco. Bait shop	STRI-4674

Underlined specimens are those used on the relative rate test. MtDNA lineages correspond to the phylogenetic lineages recovered based on cyt *b* and ATPase 8/6 genes. Country codes: ARG, Argentina; CR, Costa Rica; GU, Guatemala; HO, Honduras; MEX, Mexico; NI, Nicaragua; PN, Panama; PE, Peru; PY, Paraguay; SA, El Salvador; and VZ, Venezuela.

[a] Specimens also studied for the nuclear RAG-1 gene.

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