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Molecular Phylogeny of the Live-Bearing Fish Genus *Poecilia* (Cyprinodontiformes: Poeciliidae)

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Members of the genus *Poecilia* exhibit extensive morphological, behavioral, and life history variation within and between species. This natural variation, coupled with short generation times and the ease with which members of this genus can be cultured in the lab, have made several species model systems for studying the effects of sexual and natural selection on the evolution of natural populations. Given that there is no clear understanding of the phylogenetic relationships within the genus, these studies have not been put into a historical context, and between-species comparisons have been limited. We sequenced the complete NADH Dehydrogenase Subunit 2 (ND2) mitochondrial gene (1047 bp) in representatives of the major divisions of the genus in order to examine these relationships. The subgeneric groups of Rosen and Bailey (1963) are, for the most part, supported, with some adjustment within the subgenera *Poecilia* and *Pamphorichthys*. The morphological distinctness of the groups within *Poecilia* suggest that the original generic designations be reinstated, but this awaits a more thorough analysis. Two implications from the phylogeny are particularly relevant to sexual selection studies: within the North and Central American mollies, the three species of sailfin mollies form a monophyletic group, and within the subgenus *Lebistes*, the sister taxon to the guppy, *P. reticulata*, is most likely the group of species previously designated as *Micropoecilia*.

INTRODUCTION

As part of their revision of the family Poeciliidae, Rosen and Bailey (1963) placed several well-established genera, including *Mollienesia*, *Allopoecilia*, *Limia*, *Pamphorichthys*, *Lebistes*, and *Micropoecilia*, into one genus, *Poecilia*, retaining four subgenera. The resulting genus, *Poecilia*, is a complicated and widely distributed group, ranging from the southeastern United States to Bolivia and southern Brazil. Species of *Poecilia* are found in a wide range of habitats, exhibit morphological and behavioral differentiation within and between species, and have been studied extensively for the effects of natural and sexual selection. We present a phylogeny of this genus based on NADH Dehydrogenase Subunit 2 (ND2) sequence variation, in order to resolve some of the relationships within *Poecilia* and to provide a phylogenetic context for studies of sexual selection in this group.

Taxonomy

The following description of four subgenera follows Rosen and Bailey (1963), with notes from Rauchenberger's (1989) annotated list of species in the subfamily Poeciliinae. Table 1 outlines these major divisions within the genus and the specimens examined in this study. We also review proposed taxonomies that have reinstated or adopted the genus rank for some of the assemblages within *Poecilia* (e.g., Costa, 1991; Meyer, 1993; Rodriquez, 1997).

Subgenus *Poecilia* includes at least 20 named species from North and Central America and northeastern South America that are commonly referred to as mollies. This subgenus includes the previously named genera, *Mollienesia* LeSueur, 1821 and *Allopoecilia* Hubbs, 1924. The North and Central American mollies (referred to as group *Mollienesia* within the subgenus *Poecilia* by Rauchenberger (1989)) have been divided into two species complexes: *P. latipinna* and *P. sphenops* (Hubbs, 1933; Miller, 1983). The *P. latipinna* complex, or "sailfin" mollies, includes three species, *P. latipinna*, *P. petenensis*, and *P. velifera*, and males of all three species are sexually dimorphic, having an enlarged dorsal fin used in a courtship display directed toward females (Ptacek and Travis, 1997). These three species are confined to the Atlantic slope and range from the southern United States southeastward into northern Guatemala and northern Belize. Possession of the

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"sailfin" distinguishes these species from the remaining "shortfin" molly species in the *P. sphenops* complex. The *P. sphenops* complex was originally thought to include a single, variable, and widespread species, but more recently Quesada (1971) suggested dividing it into two species complexes: *P. sphenops* and *P. mexicana*. The *P. mexicana* complex contains eight described species found on the Atlantic slope of Mexico and into Central America to Nicaragua. The *P. sphenops* complex contains four described species from the Pacific slope of Mexico. The gynogenetic unisexual Amazon molly, *P. formosa*, is included in the *P. mexicana* species complex, but is a known hybrid between one member of the sailfin complex, *P. latipinna*, and one shortfin species, *P. mexicana* (Avise *et al.*, 1991; Schartl *et al.*, 1995). We examined six species of the *Mollienesia* group, including three shortfin species and all three sailfin species, and one species outside of *Mollienesia*, *P. vivipara*, to test for monophyly of the sailfin mollies and to examine the relationship of *P. vivipara* to the *Mollienesia* group (Table 1).

Subgenus *Lebistes* consists of the guppy, *P. reticulata*, three species previously placed in the genus *Micropoecilia* (*P. parae, P. picta,* and *P. branneri*), and a fifth species, *P. amazonica*, closely allied to *P. parae*. Recently, Meyer (1993) redescribed *Micropoecilia bifurca* and reinstated the genus *Micropoecilia*. The guppy has been studied extensively for variation in life history, behavior, and morphology as a response to variation in habitat and sexual selection. In our study we examined two *P. reticulata* specimens, one from the Oropuche drainage (Oro) and one from the Caroni drainage (Car) in Trinidad. These are two genetically distinct groups (Carvalho *et al.*, 1991; Fajen and Breden, 1992). We also included one specimen of *P. picta* and one of *P. parae* (Table 1).

Subgenus *Pamphorichthys*, as described by Rosen and Bailey (1963), consists of *P. minor* from the Amazon drainage, *P. hollandi* from Rio Sao Francisco drainage and south, *P. hasemani*, poorly described and listed from the Rio Paraguay drainage in Bolivia, and *P. heterandria* from a restricted coastal area of Venezuela. Costa (1991) confirmed *P. hasemani* from several localities in Brazil, included *Cnesterodon scalpridens* from the Amazon basin in the genus (thought to be in the subgenus *Lebistes* by Rosen and Bailey (1963)), and described a sixth species, *Pamphorichthys araguaiensis*, from the Rio Araguaia drainage in Brazil, retaining the original generic name. We have examined three species in subgenus *Pamphorichthys: P. minor* and *P. araguaiensis* from Brazil and *P. heterandria* from coastal Venezuela (Table 1).

The fourth subgenus, *Limia*, consists of at least 11 species confined to the Greater Antilles. Rivas (1980) refers to this group as the genus *Limia*, divided into two subgenera, *Limia* and *Ondontolimia*. We examined 2 species from the subgenus *Limia* (sensu Rosen and Bailey, 1963), but these do not include species placed in *Odontolimia* (Rivas, 1980) (Table 1).

Rodriquez (1997) provides the most recent revision of the genus *Poecilia* (sensu Rosen and Bailey, 1963) based on a cladistic analysis of 27 morphological characters. His analysis follows Costa (1991) by recognizing the genus *Pamphorichthys* and Rivas (1980) by recognizing the genus *Limia*, while retaining species from the subgenera *Lebistes* and *Poecilia* in a genus that he recognized as *Poecilia*.

Sexual Selection Studies

A number of species in the genus *Poecilia* exhibit highly colorful males, elaborate courtship, and sexual dimorphism associated with sexual selection (reviewed in Farr, 1989). Two groups in particular, sailfin mollies and the guppy, have become model systems for the study of sexual selection. Evaluating the role of sexual selection in the differentiation and speciation of these groups will be possible only in the context of a resolved phylogeny.

In the subgenus *Poecilia*, female preference for morphological and behavioral features associated with the sailfin phenotype has been demonstrated for the sailfin species *P. latipinna* (Ptacek and Travis, 1997). Since the same morphological and behavioral traits that differ among conspecific populations also distinguish sailfin species from shortfin species (Ptacek, 1998), it is likely that sexual selection played an important role in speciation of sailfin mollies as well. One of our goals is to resolve whether or not the sailfin molly species are the result of a single speciation event and subsequent diversification, leading to the three extant species, or are the result of multiple independent origins of the sailfin phenotype.

The subgenus *Lebistes* is considered to exhibit the greatest degree of sexual dichromatism and male polymorphism in the family Poeciliidae (Rosen and Bailey, 1963; Farr, 1989). The amount of male coloration and courtship activity varies dramatically among populations of the guppy, *P. reticulata*, depending in part on level of predation (Houde, 1997). There is also a wide range of male coloration patterns exhibited among the species previously described as *Micropoecilia*. *P. picta* is strongly dichromatic, but males are essentially monomorphic throughout the geographic range (Breden *et al.*, unpublished data). There are three distinct morphs of *P. parae* males, all occurring in the same population (Rosen and Bailey, 1963; Liley, 1966). Males of *P. bifurca* have little coloration, perhaps due to secondary loss. Understanding the sister taxon relationships within the subgenus is critical to evaluating the various models for the evolution of female preference and male secondary sexual characters due to sexual selection (Basolo, 1996; Ryan and Rand, 1993). One of our goals is to determine the relationships of the species presently in the subgenus *Lebistes* to examine the

possible sister taxon relationship between *P. reticulata* and the *Micropoecilia* group.

In summary, this complex group includes several species that have been used as model systems for the study of natural and sexual selection. Such studies would benefit from knowledge of sister taxon relationships, particularly to polarize changes observed within species. The genus also includes several species complexes whose utility in studies of speciation would greatly benefit from a more resolved phylogeny. Finally, there are many unresolved taxonomic issues within the genus. For these reasons, we sequenced ND2 from representatives of the four subgenera in an attempt to examine the cohesiveness of the subgeneric classifications of Rosen and Bailey (1963) and to elucidate relationships within and among these groups.

MATERIALS AND METHODS

DNA Sources

Table 1 lists the collection sites for the 18 taxa studied. We compared sequences of 15 species in the genus *Poecilia* (16 specimens, including 1 from each of two major divisions within *P. reticulata*) and 2 other species: *Xiphophorus nigrensis*, in the same tribe as the genus *Poecilia*, tribe Poeciliini, and *Heterandria formosa*, in the tribe Heterandriini, all within the subfamily Poeciliinae (Parenti, 1981).

TABLE 1

Species Examined and Collection Sites

Subfamily Heterandriini		
Heterandria formosa		Savanah River, South Carolina
Subfamily Poeciliini		
Xiphophorus nigrensis		Rio Choy, Mexico
Subgenus *Poecilia* ("mollies")		
P. vivipara	Georgetown, Guyana	South American Molly
P. gilli	Lake Nicaragua, Nicaragua	Short fin, Atlantic Slope
P. orri	Costa Rica	Short fin, Atlantic Slope
P. sphenops	Rio Cautla, Mexico	Short fin, Pacific Slope
P. latipinna	Tampico, Mexico	Sailfin
P. petenensis	Laguna Caobas, Quintana Roo, Mexico	Sailfin
P. velifera	Cenote Azul, Quintana Roo, Mexico	Sailfin
Subgenus *Lebistes*		
P. reticulata	Oropuche River, Trinidad	Oropuche River (Oro)
P. reticulata	Lower Aripo River, Trinidad	Caroni Drainage (Car)
P. picta	Georgetown, Guyana	
P. parae	Georgetown, Guyana	
Subgenus *Pamphorichthys*		
P. minor	Parintins, Amazonas, Brazil	
P. araguaiensis	Rio Araguaia, Goias, Brazil	
P. heterandria	Estado Falcon, Venezuela	
Subgenus *Limia*		
P. perugiae	Dominican Republic, Hispaniola	
P. nigrofasciata	Haiti, Hispaniola	

ND2 Sequences

The sequencing strategy is displayed in Fig. 1 and primer sequences are given in Table 2. For most species, the ND2 gene was first amplified using primers directed at the methionine and tryptophan tRNAs. For *H. formosa*, amplification primers were directed at glutamine and asparagine tRNAs (Kocher *et al.*, 1995). Typical reaction conditions were approximately 100 ng of genomic DNA used as template for a 50-µl reaction solution containing each dNTP at 1 mM, each primer at 0.5 µM, 3 mM $MgCl_2$, 5 µl of 10× PCR buffer (200 mM Tris, pH 8.4, 500 mM KCl), and 2.5 units of Taq DNA Polymerase (Gibco-BRL, Life Technologies Inc.). Reactions were amplified for 35 cycles at 94°C for 70 s, 50°C for 90 s, and 72°C for 150 s. This set of cycles was preceded by heating to 94°C for 100 s and followed by extension at 72°C for 240 s. An approximately 1150-bp PCR product was acrylamide gel-purified and cloned using the pT7Blue T-Vector system (Novagen) for all but three of the species listed in Table 1. Cloned DNA was sequenced by the University Core DNA Services of the University of Calgary, Alberta using cycle sequencing and an ABI automated sequencer Model 377. The three species that were not cloned (*P. orri*, *P. velifera*, and *P. petenensis*) were sequenced directly using the forward and reverse amplification primers and internal primers. Complete ND2 sequence and flanking regions (1105 bp) were obtained for all taxa. Sequences were confirmed in two ways. First, the forward primers were close enough such that all regions, but the first, were sequenced two times. Second, sequences produced from two reverse primers, vector primer U19 and internal primer Int-1R, confirmed 40% of the gene, including the first region, for which there was no overlapping forward sequence. As a check on the frequency of cloning artifacts, partial sequences from cloning and from direct sequencing of acrylamide-purified PCR product were compared for four species; there was exact agreement between the two techniques for over 2000 bp.

Phylogenetic Analysis

The data set for phylogenetic analysis consisted of the complete ND2 gene and flanking regions in the methionine and tryptophan tRNA. Sequences were aligned with ClustalV, using multiple alignment gap penalties of 25. Nucleotide variation and substitution patterns were examined using MEGA (Kumar *et al.*, 1993; version 1.01). Aligned sequence data were ana-

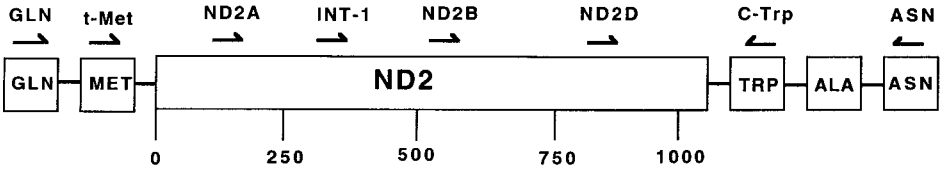

FIG. 1. Amplification and sequencing strategy for the ND2 gene. Bars depict the ND2 and flanking tRNA genes. Arrows indicate the directions of the sets of primers used. Primer sequences are given in Table 2.

lyzed by maximum parsimony and neighbor joining algorithms as implemented in test version 4.0d64 of PAUP*, written by David L. Swofford, and maximum likelihood by DNAML in the PHYLIP 3.572 (Felsenstein, 1993).

The first step in the analysis was to search for the most parsimonious tree by the branch and bound method and optimal trees under neighbor joining and maximum likelihood algorithms. Support for the nodes of these trees was examined by 500 bootstrap iterations. Because the various optimality criteria assume different models of evolution, we assume that agreement among these models indicates a robust result. Based on the results of simulation studies (Hillis and Bull, 1993), we consider bootstrap values greater than 80% as indicating moderate support for a node and greater than 90% as strong support. The neighbor joining searches assumed the HKY85 substitution model for calculating distances (Swofford *et al.*, 1996). The bootstrap iterations of the maximum parsimony algorithm utilized a heuristic search, with the following options: keep minimal trees only, collapse zero-length branches, random step-wise addition of taxa with 10 replications, and tree bisection and reconnection. The model of evolution employed for the likelihood analysis was determined by the following steps. First, an optimal tree was obtained under the neighbor joining algorithm, assuming uncorrected distances, equal frequencies of bases, and no among-site rate variation. The likelihood of this tree was compared, assuming equal base frequencies versus the HKY85 model of substitution. Then, this likelihood was compared to that calculated for the same tree, assuming a 2:1 transition to transversion ratio versus estimating this ratio from the data during the likelihood estimation. Finally, the likelihood assuming equal rates of substitution among sites was compared to that obtained assuming variable rates approximating a gamma distribution with three rate categories. The likelihood of this tree was significantly improved by the addition of these parameters to the model at each step (Huelsenbeck and Crandall, 1997). Therefore, we used the following model of evolution to obtain the optimal tree and for the bootstrap iterations of the maximum likelihood algorithm: maximum likelihood estimates of the base pair frequencies under the HKY85 model (frequencies of A, C, G, and T set to 0.286, 0.330, 0.110, 0.274, respectively), transition to transversion ratio of 2.79, and gamma distribution shape factor of 0.323, with three categories of substitution rate.

We included two outgroups in the data set, *X. nigrensis* and *H. formosa*. However, we analyzed the data with *H. formosa* as the sole outgroup to examine the relationship of *X. nigrensis* relative to species in the genus *Poecilia*.

RESULTS

ND2 Sequence Variation

Eighteen taxa in the genera *Poecilia*, *Xiphophorus*, and *Heterandria* were sequenced for ND2 and flanking regions for a total of 1105 bp. Sequences and translated proteins have been deposited in GenBank under Accession Nos. AF031386–AF031402 and AF084973. The ND2 gene was 1047 bp and showed no indels within these three genera or compared to 31 cichlid species (Kocher *et al.*, 1995). All predicted proteins were 348 amino acids long.

We observed a high degree of sequence divergence for the ND2 gene within the genus *Poecilia* (Table 3). Percentage sequence divergence ranged to 19% within the genus *Poecilia* and from 20 to 23% between *Poecilia* and *X. nigrensis*. The range of divergence was 20 to 25% between *H. formosa* and *X. nigrensis* or between *H. formosa* and species of *Poecilia*. Thus, variation between species within *Poecilia* was almost as great as that observed between Poeciliini and Heterandriini.

The base composition at the three codon positions for all taxa showed a bias similar to that found in two studies of cichlid mitochondrial genes: cytochrome *b* (Roe *et al.*, 1997) and ND2 (Kocher *et al.*, 1995). Most notably, there was a strong anti-G bias at the second and third positions (11.6 and 3.5%, averaged across all taxa). To examine possible saturation at the various positions, we plotted divergences for first, second, and third position transitions and first and second position transversions against third position transversion divergence (Fig. 2). Third position transversions were used to examine saturation, because these transversions occur relatively rarely in a nearly Poisson fashion and therefore saturate most slowly (Kocher *et al.*, 1995), and to provide a comparison to the extensive data set on ND2 sequence variation among East African cichlids

TABLE 2

Primers Used for Amplification and Sequencing of NADH Dehydrogenase Subunit 2 (ND2)

Primary amplification and sequencing of PCR product

Primer pair	Primer sequence (5′ to 3′)	Species amplified
t-Met[a]	AAG CTA TCG GGC CCA TAC CC	All except *P. reticulata* Car, *P. minor,* and *H. formosa*
C-Trp[a]	CTG AGG GCT TTG AAG GCC C	
t-MetCT[b]	ACC CTG AAC ATG ACS GYT AAA A	*P. reticulata* Car and *P. minor*
C-Trp-Pam[c]	GTC TAA GGA ATT ATC CTA AG	
GLN[d]	CTA CCT GAA GAG ATC AAA AC	*H. formosa*
ASN[d]	CGC GTT TAG CTG TTA ACT AA	

Internal sequencing primers

Primer	Primer sequence (5′ to 3′)	Species sequenced
ND2A	TGA AGC YAC CAC TAA AT	All except below
ND2AM	TGA AGC CGC CAC TAA AT	*P. parae, P. minor,* and *P. araguaiensis*
ND2AG	TGA GGC CAC CAC TAA AT	*P. reticulata* Car
INT-1	TGA ATR CCA GAA GTA AT	All except below
INT-1X	TGA ATG CCA GAA GTA CT	*X. nigrensis*
INT-1M	TGA ATR CCA GAA GTT AT	*P. parae, P. minor,* and *P. araguaiensis*
INT-1R	ATT ACT TCT GGY ATT CA	All species (reverse direction of INT-1)
ND2B	CAA CTC CGA AAA ATC CTA GC	All except below
ND2BX	CAA CTC CGA AAA ATT CTT GC	*X. nigrensis*
ND2BM	CAA CTW CGA AAA ATC CTA GC	*P. latipinna* and *P. sphenops*
ND2DX	GCT GCC CTT TCA TCC CT	*X. nigrensis*
ND2D1	GTA GCA CTT TCA TCT TT	*P. picta*
ND2D2	GCT GCA CTC TCA TCC CT	*P. gilli, P. latipinna, P. minor, P. araguaiensis,* and *P. heterandria*
ND2D3	GCC GCA CTA TCA TCC CT	*P. nigrofasciata* and *P. perugiae*
ND2D4	GCT GCA CTA TCA TCC CT	*P. sphenops*
ND2D5	GCA GCA CTT TCA TCC CT	*P. vivipara*
ND2D6	GCC GCA CTT TCA TCC CT	*P. reticulata* and *P. parae*

Note. R, A/G; S, C/G; W, A/T; Y, C/T.

[a] Park *et al.* (1993).

[b] Designed from methionine tRNA sequence, downstream from t-Met.

[c] Designed from tryptophan tRNA sequence, upstream from C-Trp.

[d] Kocher *et al.* (1995).

(Kocher *et al.*, 1995; Kocher and Carleton, 1997). There appears to be saturation of third position transitions starting at approximately 5% third position transversion divergence, while transitions and transversion divergences at other positions appear to increase linearly with third position transversion divergence (Fig. 2). In fact, only the relationship of third position transitions to third position transversion divergence showed a significant quadratic regression term ($P < 0.001$).

Phylogenetic Analysis

We employed three approaches in our phylogenetic analysis: neighbor joining, maximum likelihood, and maximum parsimony. The maximum parsimony criterion gave two most parsimonious trees. The strict consensus of these two most parsimonious trees and the optimal trees obtained from neighbor joining and maximum parsimony is presented in Fig. 3, along with the proportion of the 500 bootstrap iterations supporting each node of this consensus tree. Using *H. formosa* as the outgroup, species in the genus *Poecilia* form a clade relative to *X. nigrensis* in 100% of the bootstrap iterations for all three algorithms.

Several groups within the genus *Poecilia* are supported at the 99–100% level in all three analyses: the two species of the subgenus *Limia,* the two species of the subgenus *Pamphorichthys* from Brazil, and the six species of Central and North American mollies (group *Mollienesia;* Rauchenberger, 1989). Within this *Mollienesia* group, the sailfin mollies, *P. latipinna, P. petenensis,* and *P. velifera,* form a strongly supported group (94–100%), and the species *P. orri* and *P. gilli* cluster in 100% of the bootstrap iterations. The three "shortfin" species form a moderately supported clade in the neighbor joining and maximum likelihood analyses, but not with the maximum parsimony analysis.

The subgenus *Lebistes* forms a clade with a high degree of support (90–93%). Within the subgenus *Lebistes,* the two individuals of the Trinidad guppy, *P. reticulata,* are clearly associated (100%). However, there is only weak support for a clade composed of the two species previously classified as *Micropoecilia* (64–68%).

The relationships among these strongly supported groups are much less clear. The optimal tree obtained under neighbor joining criterion showed a split between the subgenus *Lebistes* and the other three subgenera, *Poecilia, Limia,* and *Pamphorichthys,* with *P. vivipara* basal to these two divisions. The maximum likelihood optimal tree showed a similar split, but in this case included *P. vivipara* within the clade formed by subgenera *Poecilia, Limia,* and *Pamphorichthys.* However, one of the two most parsimonious trees suggested a very different topology, linking subgenus *Pamphorichthys* and *P. vivipara* with the subgenus *Lebistes.* None of these three tree topologies represent a significantly more likely tree than the others (Likelihood Ratio Tests $P > 0.7$; Huelsenbeck and Crandall, 1997). Furthermore, bootstrap support for any of the possible relationships among the monophyletic groups in Fig. 3 was less than 80%.

The inferred position of *P. heterandria,* placed by Rosen and Bailey (1963) in the subgenus *Pamphorich-*

TABLE 3

Uncorrected Pairwise Differences in ND2 Sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *H. formosa*																	
2. *X. nigrensis*	0.208																
3. *P. vivipara*	0.203	0.199															
4. *P. gilli*	0.240	0.216	0.140														
5. *P. latipinna*	0.233	0.199	0.127	0.098													
6. *P. sphenops*	0.232	0.213	0.134	0.104	0.099												
7. *P. orri*	0.234	0.208	0.135	0.136	0.089	0.093											
8. *P. petenensis*	0.226	0.202	0.120	0.106	0.053	0.098	0.097										
9. *P. velifera*	0.229	0.197	0.116	0.099	0.038	0.093	0.092	0.048									
10. *P. nigrofasciata*	0.221	0.207	0.113	0.136	0.133	0.131	0.129	0.124	0.126								
11. *P. perugiae*	0.220	0.215	0.120	0.140	0.130	0.130	0.133	0.127	0.122	0.020							
12. *P. reticulata* Oro	0.236	0.211	0.135	0.163	0.158	0.168	0.159	0.162	0.158	0.150	0.154						
13. *P. reticulata* Car	0.235	0.220	0.146	0.170	0.172	0.172	0.163	0.166	0.167	0.150	0.156	0.053					
14. *P. picta*	0.241	0.216	0.148	0.174	0.169	0.182	0.168	0.173	0.164	0.171	0.173	0.143	0.158				
15. *P. parae*	0.237	0.216	0.164	0.189	0.178	0.187	0.179	0.188	0.172	0.168	0.168	0.154	0.160	0.157			
16. *P. minor*	0.253	0.227	0.157	0.167	0.166	0.172	0.159	0.158	0.162	0.155	0.161	0.177	0.176	0.182	0.189		
17. *P. araguaiensis*	0.242	0.217	0.143	0.155	0.161	0.159	0.147	0.151	0.153	0.143	0.150	0.165	0.171	0.177	0.181	0.034	
18. *P. heterandria*	0.225	0.212	0.126	0.150	0.132	0.133	0.141	0.131	0.129	0.126	0.130	0.162	0.166	0.167	0.175	0.162	0.150

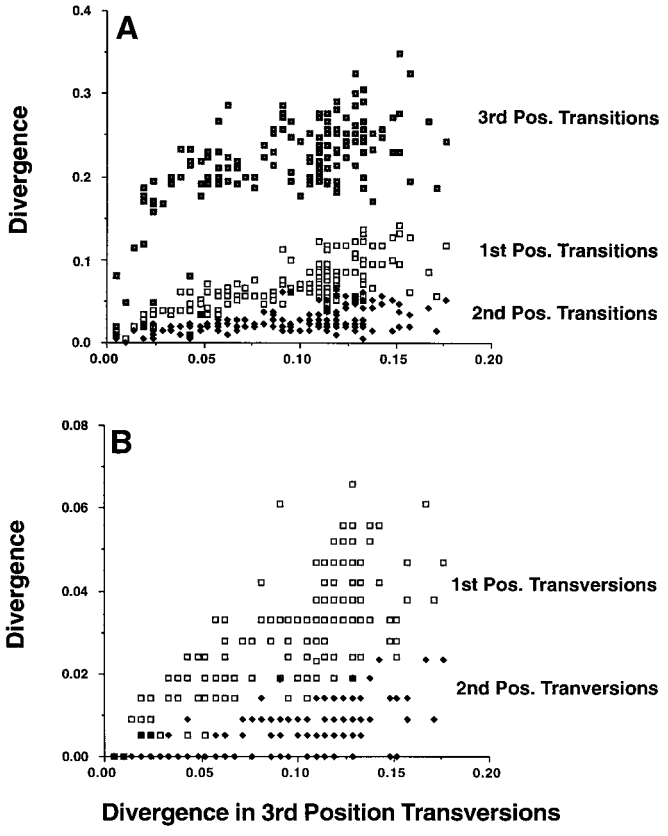

FIG. 2. Accumulation of sequence divergence between taxa at first, second, and third position transitions (A) and first and second position transversions (B) of the ND2 gene, plotted against third position transversion divergence.

thys, also changes depending on the approach taken. Only the maximum likelihood criterion places this species in a group with the other *Pamphorichthys* species, and this relationship is upheld by very low bootstrap support (47%). The most parsimonious tree obtained when constraining the three species of subgenus *Pamphorichthys* to one clade is the same as the optimal tree obtained under maximum likelihood, and as stated above, none of these trees is significantly more likely than the others.

Because of saturation in third position transitions, we conducted three further types of analyses on the ND2 region: maximum parsimony and neighbor joining employing third position transversions only or excluding the third position entirely and protein parsimony as implemented in PHYLIP 3.572 (Felsenstein, 1993). None of these approaches produced results that differed from the analysis of the full data set; i.e., all of the monophyletic clades in Fig. 3 were supported by these analyses, but the relationships among these clades were not further resolved.

DISCUSSION

Pattern of Substitution in ND2

The pattern of substitution at the various codon positions is similar to that reported for East African cichlids (Kocher *et al.*, 1995; Kocher and Carleton, 1997). The rate of first and second position transitions is much lower than that for the third position, indicating selective constraints at the first and second position (Kocher and Carleton, 1997). Divergence between taxa in third position transitions saturates at a level of approximately 25% divergence (Fig. 2), while diver-

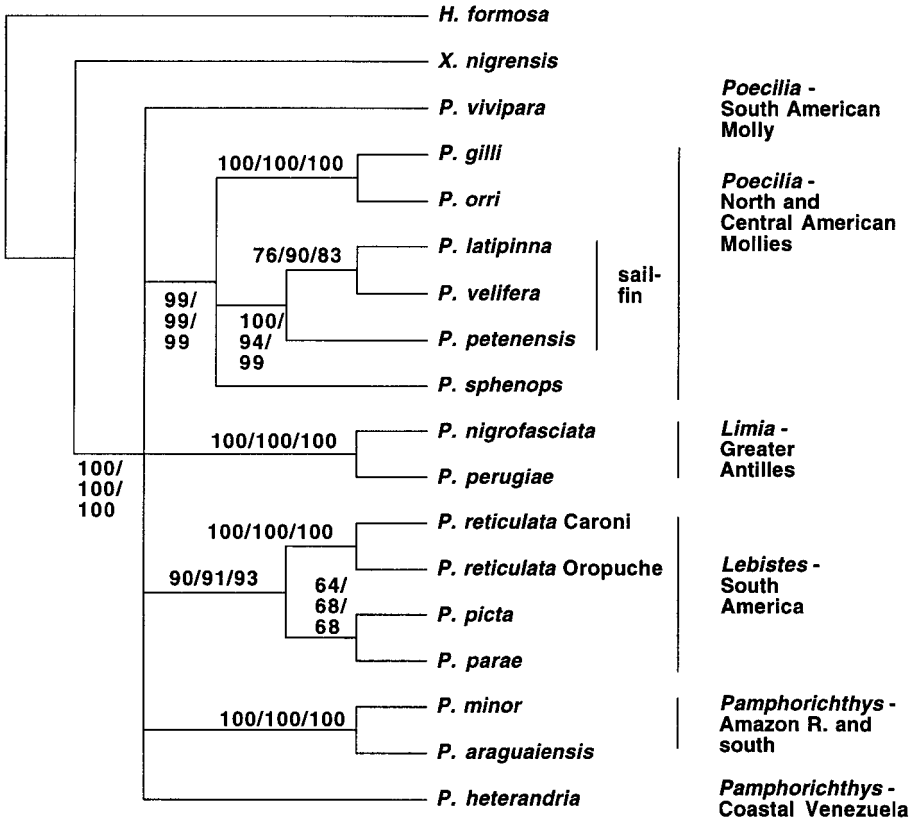

FIG. 3. Strict consensus of the optimal trees obtained from neighbor joining, maximum likelihood, and maximum parsimony analyses of ND2 sequence variation in 18 taxa in the subfamily Poeciliinae (Parenti, 1981). Bootstrap support from 500 iterations is given at each node for neighbor joining/maximum likelihood/maximum parsimony algorithms. The length of the two most parsimonious trees was 1347 steps; Consistency Index = 0.530; Rescaled Consistency Index = 0.290; Homoplasy Index = 0.470.

gence in the other substitution classes increases linearly with divergence in third position transversions over the range observed in our study. Over a greater range of divergence in third position transversions, Kocher and Carleton (1997) observed that differences at the first position reached a plateau at approximately 8%, while second position differences leveled at approximately 3%. The pattern of substitution within *Poecilia* may be approaching similar saturation levels (Fig. 2). These levels of saturation are much lower than the theoretical levels determined by nucleotide composition, and this would again imply that selective constraints are dominating the substitution process at these positions.

Phylogenetic Relationships

Our results from neighbor joining, maximum likelihood, and maximum parsimony analyses for 15 species of *Poecilia* support the subgeneric categories of Rosen and Bailey (1963), with adjustments to the subgenera *Poecilia* and *Pamphorichthys*. The subgenus *Limia* appears monophyletic, although it will be necessary to analyze more species, especially in the group *Odontolimia*. The subgenus *Lebistes* also appears monophyletic. Sequences from the mitochondrial D-loop, including individuals from more populations of *P. picta* and two populations of *Micropoecilia bifurca* (sensu Meyer, 1993) support this conclusion as well (Breden *et al.*, unpublished data). The six species of the *Mollienesia* group within the subgenus *Poecilia*, previously placed in the genus *Mollienesia*, form a highly supported clade. However, it is unlikely that the subgenus *Poecilia* is monophyletic, since none of the analyses closely ally the South American molly, *P. vivipara*, with the *Mollienesia* group. In fact, the most parsimonious tree obtained under the constraint of monophyly for the subgenus *Poecilia* was significantly less likely than any of the optimal trees (Likelihood Ratio Test, $P < 0.01$. The two Brazilian species of the South American subgenus *Pamphorichthys* form a well-supported clade, but the species of *Pamphorichthys* from Venezuela, *P. heterandria*, may not be closely related to the other two species of *Pamphorichthys*. In summary, except for the uncertain placement of *P. heterandria* and *P. vivipara*, the subgeneric classifications of Rosen and Bailey

(1963) form well-defined clades according to the analysis of ND2 sequence variation.

The position of *P. heterandria* may be problematic, even with further study. Much of the taxonomy of this group is based on characteristics of the gonopodium (the modified anal fin used in sperm transfer) and supporting structures. *P. heterandria* is unique in the genus in not possessing a gonopodial palp, the fleshy covering of the gonopodium, and the gonopodium does not possess the barbs and hooks characteristic of the other species in the genus. Still, a cladistic analysis of several morphological characters shows three synapomorphies supporting a clade formed by *P. heterandria* and species in the subgenus *Limia* (Figueiredo and Costa, unpublished data). Thus, *P. heterandria* may not be aligned with any of the subgenera, based on the lack of a gonopodial palp and specialized barbs and hooks, or it may be linked with subgenus *Limia*.

The relationships among the subgenera remain unresolved. The optimal tree obtained from maximum likelihood supports two clades within *Poecilia*, one comprising three subgenera, *Poecilia*, *Limia*, and *Pamphorichthys*, and the second comprising subgenus *Lebistes*. Neighbor joining analysis supports a similar division, except with *P. vivipara* as a sister taxon to all other species in genus *Poecilia*. However, maximum parsimony does not support this division within *Poecilia*, but rather links *P. vivipara* and subgenus *Pamphorichthys* with subgenus *Lebistes*. We are obtaining more mitochondrial and nuclear sequences to address this question.

Our results agree with some of the relationships suggested in Rodriquez's (1997) recent revision of the genus *Poecilia*, but differ from it in several important ways. His revision, based on 27 morphological characters, supports the monophyly of subgenera *Limia* and *Pamphorichthys* (although *P. heterandria* was not examined). However, he recognized a genus *Poecilia*, composed of members of the subgenera *Lebistes* and *Poecilia* (sensu Rosen and Bailey, 1963), including *P. vivipara*. None of the three analyses of the ND2 data support this relationship; *P. vivipara* does not cluster with other members of the subgenus *Poecilia*, and there is no suggestion of a relationship between group *Mollienesia* and subgenus *Lebistes*. In fact, the two most parsimonious trees obtained under the constraint of a clade comprising *Lebistes*, group *Mollienesia*, and *P. vivipara*, were significantly less likely than any of the three optimal trees (Likelihood Ratio Test, $P < 0.005$). One of the sources of the variance between our results and those of Rodriquez may be that he examined only a single species in the subgenus *Lebistes*, *P. reticulata*, even though this subgenus also includes several species previously included in the genus *Micropoecilia* (e.g., *P. picta* and *P. parae*).

Taxonomy

Several authors (Rivas, 1980; Costa, 1991; Meyer, 1993; Rodriquez, 1997) have reinstated or adopted the genus rank for assemblages within the genus *Poecilia*, sensu Rosen and Bailey (1963), rather than follow the system of Rosen and Bailey (1963), in which several genera were placed in one genus with four subgenera. Nevertheless, none of these authors, including Rosen and Bailey (1963), provided a complete classification based on an inclusive phylogenetic analysis. Rodriquez (1997) revised the genus *Poecilia* based on derived features, recognizing three genera: *Pamphorichthys*, *Limia*, and *Poecilia*. However, for reasons stated above, our phylogenetic analysis of ND2 sequence variation strongly rejects Rodriquez's genus *Poecilia*, consisting of the previous subgenus *Lebistes*, the *Mollienesia group*, and *P. vivipara*. Also, his analysis excluded at least one entire group of species within *Poecilia*. Therefore, we consider the taxonomy of this genus unresolved.

Poecilia's subgenera as defined by Rosen and Bailey (1963) are morphologically quite different from each other, and several lines of evidence suggest that there are monophyletic assemblages within *Poecilia* (e.g., *Limia*, *Pamphorichthys* with the exception of *P. heterandria*, *Mollienesia*, and *Lebistes*). They justified their decision to establish broad genera by stating that such groupings would best demonstrate patterns of relatedness among species, especially to nonsystematists (Rosen and Bailey, 1963:6). However, given the number of morphologically distinct, monophyletic assemblages within *Poecilia*, we feel that their broad classification in fact masks many relationships, and therefore the taxonomic status of this clade, *Poecilia* (sensu Rosen and Bailey (1963)), should be suprageneric. However, the exact set of monophyletic assemblages within *Poecilia* and the relationships among them is still not clear. Rather than advocate the proper taxonomic rank for any of these clades or create new names, we feel that a systematic revision of this genus should await a more inclusive study (i.e., analyzing more species and more genera and combining more types of data) to avoid polyphyletic or paraphyletic assemblages.

Implications for Sexual Selection Studies

There was strong and consistent support for a clade formed by the members of the subgenus *Lebistes* that we examined and moderate support for a sister taxon relationship between the guppy and the species previously classified as *Micropoecilia*. Within this subgenus, the guppy, *P. reticulata*, is a model organism for the study of sexual selection, mainly because attractive male characters and female preferences vary between populations (Houde, 1997). In order to evaluate the direction of change within *P. reticulata* and to evaluate

the sensory exploitation model for the evolution of female preference, it is necessary to infer the ancestral states for these preferences and male traits. In terms of coloration, *P. parae* has three male morphs: one has nearly the entire body covered by a pigmented stripe, one has a dorsal caudal stripe, and one is colorless (Rosen and Bailey, 1963; Liley, 1966). Males of *P. picta* throughout the range have an orange stripe on the dorsum of the tail and yellow and black spots on the tail and body. *P. bifurca* and *P. branneri* have some male coloration, although these are not well described. Given that male coloration is widespread in both the *Micropoecilia* group and *P. reticulata*, conspicuous coloration is most likely ancestral in the subgenus. This would suggest that critical tests of the sensory exploitation model would have to be conducted on females from species from the sister taxon to *Lebistes* that do not exhibit male coloration (Basolo, 1996). The sequence variation in ND2 is not sufficient to determine the sister taxon relationship with the other monophyletic assemblages within *Poecilia*.

Results of the phylogenetic analyses strongly support the monophyly of the sailfin molly species. This finding implies a single origin of the sailfin phenotype and subsequent speciation of *P. latipinna, P. velifera,* and *P. petenensis*. A similar result was obtained from an analysis that examined both ND2 and D-loop sequences from more species within the *Mollienesia* group (Ptacek and Breden, 1998). Since males of all three sailfin species possess an enlarged dorsal fin and use this fin in a courtship display behavior directed toward females, the monophyly of the group argues that these traits evolved in the ancestor of this clade. Females of *P. latipinna* use intraspecific variation in dorsal fin morphology and courtship display rates to distinguish among males from different populations (Ptacek and Travis, 1997). These same behavioral and morphological traits also distinguish males of sailfin species from shortfin species (Ptacek, 1998) and may have been important in the speciation of this group.

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