Molecular Phylogenetics and Evolution 55 (2010) 631–639

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetic relationships and the evolution of the placenta in *Poecilia* (*Micropoecilia*) (Poeciliidae: Cyprinodontiformes)

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ARTICLE INFO

Article history:
Received 12 August 2009
Revised 6 November 2009
Accepted 9 November 2009
Available online 14 November 2009

Keywords:
Cyprinodontiformes
Poeciliidae
Poecilia
Micropoecilia
Acanthophacelus
Placentotrophy

ABSTRACT

Poeciliids are one of the most intensively studied groups within Cyprinodontiformes owing to their use as model organisms for experimental studies on natural and sexual selection, and comparative studies of life-history evolution. Life-history studies have demonstrated multiple origins of placentotrophy and superfetation in poeciliids, including the recent description of placentotrophy in three species of *Poecilia* (*Micropoecilia*): *P. bifurca*, *P. branneri*, and *P. parae*. Here, we use a concatenation of seven nuclear gene segments and two mitochondrial segments to examine relationships within *Micropoecilia* and between this subgenus and other subgenera in *Poecilia* (*Mollienesia*, *Limia*, *Pamphorichthys*, *Acanthophacelus*). The combined molecular data set (8668 bp) was analyzed with maximum parsimony, maximum likelihood, and Bayesian methods. We also employed a relaxed molecular clock method to estimate divergence times within *Poecilia*. All phylogenetic analyses with the combined DNA data set supported the monophyly of *Poecilia* and recovered a basal split between *Poecilia* (*Acanthophacelus*) + *Poecilia* (*Micropoecilia*) and the other three subgenera. Within *Micropoecilia*, *P. bifurca* grouped with *P. branneri*, and these joined *P. parae* to the exclusion of *P. picta*. Ancestral reconstructions based on parsimony and Bayesian methods suggest that placentotrophy evolved once in *Micropoecilia* in the common ancestor of *P. bifurca*, *P. branneri*, and *P. parae*. Divergence time estimates suggest that placentotrophy in *Micropoecilia* evolved in $\leqslant$4 million years.

1. Introduction

The family Poeciliidae (Rosen and Bailey, 1963), equivalent to the subfamily Poeciliinae of Parenti (1981), is a diverse group of freshwater fishes in the order Cyprinodontiformes that comprises ~220 species in 28 genera (Lucinda, 2003; Lucinda and Reis, 2005). The group includes well-known aquarium fishes such as guppies, mollies, and swordtails (Lucinda and Reis, 2005). Poeciliids are endemic to the New World and are widely distributed throughout the Americas and Caribbean. All poeciliids have internal fertilization and all except *Tomeurus* are viviparous (Regan, 1913; Rosen and Gordon, 1953; Rosen and Bailey, 1963). Poeciliids are characterized by the presence of a gonopodium that is formed by modified anal-fin rays 3, 4, and 5 (Lucinda and Reis, 2005). Members of the group are frequently used as model organisms for experimental studies on natural and sexual selection (Endler, 1983; Houde, 1997; Schluter et al., 1998; Hamilton, 2001), and comparative studies of life-history evolution that have demonstrated multiple origins of superfetation and placentotrophy (Grove and Wourms, 1991, 1994; Arias and Reznick, 2000; Reznick et al., 2002, 2007). Most recently, placentotrophy was reported in all populations of *P. parae*, *P. bifurca* and *P. branneri* that were investigated (Pires, 2007; Pires et al., in press). In every case, placentotrophy was documented by a significant increase in embryo dry mass during development, and accompanied by a thickened maternal follicle and enlarged and externalized pericardial membrane in the embryo (Pires et al., in press).

Micropoecilia was proposed as a new genus by Hubbs (1926), who included *M. bifurca*, *M. parae*, *M. picta*, *M. melanozona*, and *M. branneri*, but also indicated that he was not convinced of the distinctness of any of the species and suggested that all may eventually be united with *M. parae*. Rosen and Bailey (1963), in their classic monograph on the family Poeciliidae (=Poeciliinae of Parenti, 1981), described the genus *Poecilia* and its constituent subgenera: *Poecilia*, *Lebistes*, *Pamphorichthys*, and *Limia* (Table 1). Rosen and Bailey (1963) included *Poecilia reticulata* (common guppy), the genus *Micropoecilia* of Hubbs (1926), *Poecilia amazonica*, and *Poecilia scalpridens* in the subgenus *Lebistes*. *Micropoecilia parae*, *M. bifurca*, and *M. melanzona* of Hubbs (1926) were treated as three polymorphs of *Poecilia* (*Lebistes*) *parae* by Rosen and Bailey (1963).

Costa (1991) reallocated *Poecilia* (*Lebistes*) *scalpridens* to the subgenus *Pamphorichthys*. Meyer (1993) reinstated the genus *Micropoecilia* of Hubbs (1926) for *Poecilia parae*, *P. bifurca*, *P. picta*,

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doi:10.1016/j.ympev.2009.11.006

Table 1
Previous classifications of *Poecilia* species relevant to this study.

Hubbs (1924, 1926)	Rosen and Bailey (1963)	Meyer, (1993)	Poeser et al. (2005)	Lucinda and Reis (2005)
Family Poeciliidae	Family Poeciliidae	Family Poeciliidae	Family Poeciliidae	Family Poeciliidae
Subfamily Poeciliinae	Subfamily Poeciliinae	Subfamily Poeciliinae	Subfamily Poeciliinae	Subfamily Poeciliinae
Tribe Poeciliini	Tribe Poeciliini	Tribe Poeciliini	Tribe Poeciliini	Tribe Poeciliini
Genus *Limia*	Genus *Poecilia*	Genus *Poecilia*	Genus *Poecilia*	Genus *Poecilia*
Genus *Micropoecilia*	*P.* (*Poecilia*) *caucana*	*P.* (*Lebistes*) *reticulata*	*P.* (*Acanthophacelus*) *reticulata*	Genus *Limia*
M. bifurca	*P.* (*Poecilia*) *latipunctata*	*P.* (*Pamphorichthys*)	*P.* (*Acanthophacelus*) *wingei*	Genus *Micropoecilia*
M. picta	*P.* (*Lebistes*) reticulata	Genus *Micropoecilia*	*P.* (*Micropoecilia*) *parae*	Genus *Pamphorichthys*
M. branneri	*P.* (*Lebistes*) *parae* [included *Micropoecilia bifurca*]	*M. bifurca*	*P.* (*Micropoecilia*) *branneri*	
M. parae	*P.* (*Lebistes*) *picta*	*M. picta*	*P.* (*Micropoecilia*) *bifurca*	
M. melanzona	*P.* (*Lebistes*) *branneri*	*M. branneri*	*P.* (*Micropoecilia*) *picta*	
Genus *Lebistes*	*P.* (*Pamphorichthys*) minor	*M. parae*	*P.* (*Micropoecilia*) *minima*	
L. reticulatus	*P.* (*Limia*) *dominicensis*			
Genus *Mollienisia*[a]	*P.* (*Limia*) *melanogaster*			
Genus *Allopoecilia*				
A. caucana				
Tribe Pamphoriini				
Genus *Pamphorichthys*				

[a] Hubbs (1924, 1926) used *Mollienisia* rather than *Mollienesia*. Bailey and Miller (1950) subsequently provided justification for using *Mollienesia* instead of *Mollienisia*.

P. amazonica and *P. branneri* based on three derived morphological characters: (1) variegated males and females; most specimens with a relatively well-developed dark humeral spot on the side of the body; (2) males with anal-fin rays 6 and 7 and ventral fins extending to the base of the gonopodium palp; and (3) ray 3 of the gonopodium with 10–16 rose thorn-like spines. Within *Micropoecilia*, Meyer (1993) also proposed synapomorphic characters for *M. bifurca* and *M. picta*, these two plus *M. parae*, and these three plus *M. amazonica*. Meyer (1993) also defined *Poecilia* (*Lebistes*) as a monotypic subgenus that included only *P. reticulata*. Meyer's (1993) *M. amazonica* is of questionable taxonomic value because its type series is composed of a combination of *M. branneri* and *M. parae* individuals (C.A. Figueiredo, pers. comm. to M. Pires).

Subsequent to Meyer (1993), Costa and Sarraf (1997) described *Poecilia* (*Lebistes*) *minima* and hypothesized a close relationship of this taxon to *P. branneri* based on five morphological synapomorphies. Costa and Sarraf (1997) also challenged Meyer's (1993) putative synapomorphies for *Micropoecilia* and argued that (1) the variegated pattern does not occur in both sexes, (2) the humeral spot is not present in all species, and (3) the morphological features of the anal-fin rays and third gonopodial ray are also present in other groups. Given these differences with Meyer (1993), Costa and Sarraf (1997) did not concur with Meyer's (1993) reinstatement of *Micropoecilia* and questioned the monophyly of this genus.

Breden et al. (1999) examined relationships among representatives of the major divisions of the genus *Poecilia* using mitochondrial ND2 sequences. Breden et al. (1999) included two species of *Micropoecilia* (*sensu* Meyer, 1993), *M. picta* and M. *parae*, which grouped together and were the sister taxon to *Poecilia reticulata*. Similarly, Hrbek et al. (2007) and Lucinda and Reis (2005) found support for an association of *P. reticulata* with representative species of *Micropoecilia* based on a multigene concatenation and a matrix of osteological and soft anatomical characters, respectively.

Poeser et al. (2005) described *Poecilia wingei* (the Endler's livebearer, Campoma guppy, or Cumaná guppy), a close relative of *Poecilia* (*Lebistes*) *reticulata*, and placed both species in the subgenus *Acanthophacelus* rather than *Lebistes*. Poeser et al. (2005) argued that apparent autapomorphies of *P. reticulata* were instead synapomorphies of *P. reticulata* and *P. wingei*, and grouped these species in the subgenus *Acanthophacelus*. Poeser et al. (2005) chose *Acanthophacelus* rather than *Lebistes* because the latter name is tied to a molly, not the guppy, and is not available as a subgenus name for *P. reticulata* (Poeser and Isbrücker, 2002). *Acanthophacelus* Eigenmann, 1907 then becomes the first available subgenus name for *P. reticulata* (Poeser and Isbrücker, 2002). Shories et al. (2009) described *Poecilia* (*Acanthophacelus*) *obscura*, which represents a third species in the subgenus *Acanthophacelus*. Poeser et al. (2005) also placed all of Meyer's (1993) *Micropoecilia* species and the newly described *Poecilia* (*Lebistes*) *minima* of Costa and Sarraf (1997) into *Poecilia* (*Micropoecilia*) (see Table 1).

In the present study we use a combined mitochondrial and nuclear DNA data set to examine relationships among species in Poeser et al.'s (2005) subgenus *Micropoecilia* (*bifurca*, *branneri*, *parae*, *picta*). Our study includes all of the placentotrophic *Micropoecilia* species reported by Pires et al. (in press), as well as representatives of most of the other major subdivisions of *Poecilia*. This taxon sampling provides a basis for (1) evaluating the monophyly of *Poecilia* (*Micropoecilia*), (2) determining the number of origins of placentotrophy in *Poecilia* (*Micropoecilia*), and (3) examining relationships among the major lineages within the genus *Poecilia*.

2. Methods and materials

2.1. Taxon sampling

We included all recognized species of *Poecilia* (*Micropoecilia*) except for *P. minima*. We also included two representatives each from the *Poecilia* subgenera *Acanthophacelus*, *Limia*, *Mollienesia*, and *Pamphorichthys*. Two species of *Cnesterodon* were included as outgroups. A previous molecular study that included both mitochondrial and nuclear loci demonstrated that *Cnesterodon* is the closest outgroup to *Poecilia* (Hrbek et al., 2007). All ingroup and outgroup taxa are listed in Supplementary Information Table 1.

2.2. Gene sequences

QUIAGEN DNeasy Tissue extraction kits were used to extract genomic DNA from the skeletal muscle of 95% ethanol-preserved specimens. Two mitochondrial and seven nuclear gene regions were amplified. The first mitochondrial segment included the 3′

end of $tRNA^{Glu}$, the complete cytochrome *b* (cytb) gene, and the 5′ end of $tRNA^{Thr}$. The second segment consisted of the 3′ end of $tRNA^{Gln}$, all of $tRNA^{Met}$, the complete NADH dehydrogenase subunit 2 gene (NADH2), all of $tRNA^{Trp}$, all of $tRNA^{Ala}$, and the 5′ end of $tRNA^{Asn}$. The nuclear regions included two partial exons (8 and 10), all of exon 9, and two introns (8 and 9) of the tyrosine kinase gene (X-src); exon 1 of myosin, heavy polypeptide 6 (myh6); exon 2 of ectodermal-neural cortex 1 like protein (ENC1); exon 2 of glycosyltransferase (Glyt); exon 1 of SH3 and PX domain containing 3 (SH3PX3); a portion of the 7 transmembrane receptor region of rhodopsin (Rh); and exon 3 of recombination activating gene-1 (Rag1). The nuclear markers Rh, X-src, myh6, ENC1, Glyt, SH3PX3, and Rag1 are located on chromosomes 8, 17(?), 20, 5, 16, 25, and 25, respectively, of the zebrafish *Danio rerio*.

Sequences for most PCR primers used in this study have previously been published. New internal and external primers were designed as necessary to amplify problematic taxa. Sequences for new primers, previously used primers, and primer combinations used in amplifications are provided in Supplementary Information Table 2. Previously described primers used in this study include those for X-src (Meyer and Lydeard, 1993); MYH6, ENC1, Glyt, and SH3PX3 (Li et al., 2007); Rh (Chen et al., 2003); Rag1 and NADH2 (Hrbek et al., 2007; Kocher et al., 1995; Ptacek and Breden, 1998; Breden et al., 1999); and cytb (Schmidt et al., 1998). Nested PCRs were carried out following the protocols outlined in Li et al. (2007) for myh6, ENC1, Glyt, and SH3PX3. All other genes were amplified using the following protocol: initial denaturation at 94 °C for 2 min; 35 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing), and 1 min at 72 °C (extension); and a final extension of 10 min at 72 °C. In all cases PCR products were run out on a 1% agarose gel and the product of interest was then excised and cleaned using QIAGEN QIAquick PCR purification kits. Products were then sequenced in both directions at the University of California Riverside's Core Genetics Institute, which uses an automated DNA sequencer (ABI 3730xl). Sequencing primers were designed as necessary. All accession numbers for both the 136 new and 17 previously published sequences are given in Supplementary Information Table 1.

2.3. DNA alignments and data compatibility

We used the program SOAP v1.2a4 (Löytynoja and Milinkovitch, 2001), with gap opening (11–19) and gap extension (3–11) penalties in steps of two, to identify alignment-ambiguous regions. These settings correspond to those that were used by Gatesy et al. (1993). Alignment-ambiguous regions were excluded from phylogenetic analyses. The appropriateness of combining the individual gene segments into a combined data set was tested using (1) the partition homogeneity test (Farris et al., 1994; Swofford, 2002) with each combination of partitions outlined below, 1000 replications, and 100 taxon input orders per replicates, and (2) the bootstrap compatibility method (de Queiroz, 1993; Teeling et al., 2000) with 500 bootstrap replicates and a 90% bootstrap support criterion. ML bootstrap compatibility tests allowed each segment to have its own model of sequence evolution as suggested by the Akaike Information Criterion of Modeltest 3.06 (Posada and Crandall, 1998). We used three different schemes to partition the data as follows: (1) two nuclear partitions (exons and introns) and two mitochondrial partitions (protein-coding and tRNA); (2) four nuclear partitions (1st codon positions, 2nd codon positions, 3rd codon positions, introns) and four mitochondrial partitions (1st codon positions, 2nd codon positions, 3rd codon positions, tRNA); and (3) eight nuclear partitions (one for coding sequences from each of seven different genes and one for X-src introns) and three mitochondrial partitions (NADH2, cytb, tRNA genes). Models for individuals partitions were as follows: K80+I (ENC1); HKY+Γ (Glyt); TrN+I (SH3PX3, myh6, Rh, 2nd mitochondrial codon positions); GTR+I+Γ (Rag1, NADH2, 3rd mitochondrial codon positions); K80+I+Γ (X-src exons); TVM+I (X-src introns, 1st and 2nd nuclear codon positions); TVM+I+Γ (cytb); TrN+I+Γ (exons); TVM+I+Γ (mitochondrial protein-coding); K81uf+I+Γ (tRNAs); TVM+Γ (3rd nuclear codon positions); GTR+Γ (1st mitochondrial codon positions). Both the bootstrap compatibility method and the partition homogeneity test (four partitions, $p = 0.673$; eight partitions, $p = 0.500$; 11 partitions, $p = 0.820$) suggested that it was appropriate to combine the individual segments into one multigene data set.

2.4. Phylogenetic analyses

PAUP 4.0b10 (Swofford, 2002), RAxML 7.0.4 (Stamatakis, 2006), and MrBayes V3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) were used to perform maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses, respectively, on three data sets: (1) the combined data set that included seven nuclear segments and two mitochondrial segments (length = 8668 bp); (2) the combined nuclear data set (length = 6159 bp); and (3) the combined mitochondrial data set (length = 2509 bp). Gaps were treated as missing data in all analyses. Branch and bound analyses were employed to find the shortest tree(s) in MP analyses. MP bootstrap analyses (1000 replications) employed heuristic searches with 1000 randomized taxon addition sequences and tree-bisection and reconnection branch swapping. ML and Bayesian analyses were performed with the three partitioning schemes outlined above with models suggested by Modeltest 3.06 (Posada and Crandall, 1998). ML analyses with RAxML employed 500 replicates, randomized MP starting trees, and the fast hill-climbing algorithm; all other free parameters were estimated. MrBayes v3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used to calculate Bayesian posterior probabilities. MrBayes carries out Metropolis-coupled Markov chain Monte Carlo sampling. Bayesian analyses employed eight Markov chains (seven hot, one cold), with random starting trees, default priors, and chain sampling every 1000 generations. Analyses were terminated once the average standard deviation of split frequencies for the simultaneous analyses fell below 0.01. If the model suggested by Modeltest 3.06 was not available in MrBayes, the next most complex model was used.

2.5. Molecular dating analyses

The likelihood ratio statistic rejected the molecular clock ($p < 0.05$) for 11 of the 19 possible partitions (cytb, NADH2, mitochondrial protein-coding genes, ENC1, Rag1, X-src exons, nuclear exons, nuclear 2nd codon positions, nuclear 3rd codon positions, mitochondrial 1st codon positions, mitochondrial 3rd codon positions). Therefore, we employed the relaxed molecular clock method implemented in BEAST ver 1.4.8 (Drummond et al., 2006; Drummond and Rambaut, 2007), which allows for complex models of evolution and "soft" node constraints (Hedges and Kumar, 2004; Yang and Rannala, 2006). We used the uncorrelated lognormal distribution (UCLN) model, which draws the rate of each lineage independently from a lognormal distribution. Models of molecular evolution were from Modeltest 3.06 (Posada and Crandall, 1998). In cases where the model selected by Modeltest was not available in BEAST (i.e., models with three to five categories for substitution rates), we used the next most general model (i.e., GTR). For each partition scheme, three independent analyses were run for ten million generations. The three runs were combined using Log-Combiner; Tracer 1.4 (Rambaut and Drummond, 2003) was used to inspect for stationarity/mixing and to confirm that the estimated sample size for each parameter was greater than 200. Given the

lack of a fossil record for taxa included in our analyses, we used a secondary constraint taken from Hrbek et al. (2007), who reported a minimum of 19.92 mya and a maximum of 24.39 mya for most recent common ancestor of *Pamphorichthys*, *Limia*, and *Mollienesia*. We employed a soft constraint, which followed a normal distribution, with 95% of the normal distribution between the specified minimum and maximum given in Hrbek et al. (2007).

2.6. Ancestral state reconstructions

Parsimony and SIMMAP Version 1.0 B2.3.2 (Bollback, 2006) were used to estimate ancestral states for lecithotrophy versus placentotrophy. Terminal taxa included all species of *Acanthophacelus* and *Micropoecilia* that were included in our phylogenetic analyses. The relative amounts of pre- and post-fertilization maternal provisioning to embryos have been quantified for all of these taxa (Pires et al., in press) and indicate that *P. reticulata*, *P. wingei* and *P. picta* embryos lost weight during development and that all or most of the resources necessary for embryonic growth were provided by females in the form of yolk (lecithotrophy); in contrast, *P. parae*, *P. bifurca*, and *P. branneri* females exhibited extensive post-fertilization resource allocation to embryos via a follicular placenta (placentotrophy).

SIMMAP implements the procedures of Nielsen (2002) and Huelsenbeck et al. (2003) for stochastically mapping mutations on phylogenies. SIMMAP uses a fully Bayesian approach with Markov chain Monte Carlo sampling to calculate a posterior probability distribution that accommodates uncertainty in ancestral states, evolutionary rates, and the phylogeny. We used all post burnin Bayesian trees (5957 trees) from the combined MrBayes analysis (11 partitions). SIMMAP employs a Γ distribution prior on the overall substitution rate of the morphological character. The parameters of the Γ distribution are α and β, and these parameters describe the mean (α/β) and variance (α/β^2) of the distribution. We used 60 discrete categories to describe the Γ distribution of the rate prior. For two-state morphological characters SIMMAP uses an additional prior—the bias parameter. The bias parameter is a symmetrical beta distribution that is described by a single parameter, α, and was approximated with 19 categories. Branch length proportionality was maintained by rescaling branch lengths before the priors were applied. Ancestral state reconstructions were performed with three different combinations of parameters for the Γ distribution prior: $\alpha = 1$, $\beta = 1$; $\alpha = 3$, $\beta = 2$; and $\alpha = 5$, $\beta = 5$. The bias parameter prior was specified with $\alpha = 1$, which specifies an uninformative prior with equal prior probabilities. The number of draws from each prior distribution was set at ten.

3. Results

3.1. Sequence alignments and sequence variability

The combined alignment included 8668 bp after excluding alignment-ambiguous regions of tRNA (6 bp) and X-src (15 bp). The nuclear and mitochondrial components of the combined data set were 6159 and 2509 bp in length, respectively. The nuclear data included 371 (=6.0%) parsimony-informative sites [52 (=2.6%) at 1st codon positions; 24 (1.2%) at 2nd codon positions; 266 (=13.4%) at 3rd codon positions; and 29 (=12.8%) at intronic positions]. The mitochondrial data included 774 (=30.8%) parsimony-informative sites [164 (=22.5%) at 1st codon positions; 41 (=5.6%) at second codon positions; 538 (=73.8%) at 3rd codon positions; and 32 (=9.8%) at tRNA positions]. Among coding sequences for individual nuclear genes, parsimony-informative sites ranged from 4.7% (Glyt and SH3PX3) to 7% (Rag1). Rescaled consistency (RC) and retention index (RI) values were both higher for the nuclear data (0.65, 0.81) than the mitochondrial data (0.30, 0.58). Table 2 summarizes information on partition length, the number of constant and variable (parsimony-uninformative and parsimony-informative) characters in each partition, the contribution to the length of the most-parsimonious tree for the combined data set (see below) that derives from each partition, RC, and RI. The number of changes per site was also calculated for different genes/partitions and ranged from 0.02 (2nd codon positions of nuclear genes) to 2.60 (3rd codon positions of mitochondrial genes) (Table 2).

3.2. Phylogenetic analyses

Fig. 1 shows the single most-parsimonious tree (3325 steps) that was recovered with the combined data set. RAxML analyses with four, eight, or 11 partitions resulted in ML trees that were topologically identical to each other (Fig. 2). The ML trees differed from the MP tree in grouping *Poecilia* (*Limia*) and *Poecilia* (*Pamphorichthys*) to the exclusion of *Poecilia* (*Mollienesia*). Fig. 2 also shows mean Bayesian posterior probabilities (BPP) based on two independent runs above the branches, and ML Bootstrap Support Percent-

Table 2
Parsimony statistics for different partitions.

Partition	Partition length	Number of constant characters	Number of variable, uninformative characters	Number of informative characters	Rescaled consistency index	Retention index	Number ofsteps on globally most-parsimonious tree	Number of changes per site on globally most-parsimonious tree
Nuclear	6159	5565	223	371	0.65	0.81	787	0.13
Nuclear 1st codon	1975	1893	30	52	0.69	0.85	106	0.05
Nuclear 2nd codon	1976	1943	9	24	0.73	0.88	41	0.02
Nuclear 3rd codon	1981	1544	171	266	0.64	0.80	579	0.29
Introns	227	185	13	29	0.57	0.76	61	0.27
ENC1	847	784	21	42	0.70	0.86	82	0.10
Glyt	886	808	36	42	0.75	0.85	91	0.10
SH3PX3	724	667	23	34	0.58	0.76	78	0.11
MYH6	767	692	30	45	0.68	0.83	97	0.13
Rag1	1561	1384	67	110	0.59	0.76	241	0.15
Rh	822	749	24	49	0.68	0.86	98	0.12
X-src	552	481	22	49	0.60	0.78	100	0.18
X-src exon	325	296	9	20	0.65	0.81	39	0.12
Mitochondrial	2509	1543	192	774	0.30	0.58	2538	1.01
Mitochondrial 1st codon	729	524	41	164	0.36	0.64	441	0.60
Mitochondrial 2nd codon	729	656	32	41	0.54	0.72	109	0.15
Mitochondrial 3rd codon	729	101	90	538	0.27	0.56	1892	2.60
Mitochondrial tRNAs	322	262	29	31	0.46	0.66	96	0.30
Cytb	1140	702	71	367	0.28	0.57	1210	1.06
NADH2	1047	579	92	376	0.31	0.59	1232	1.18

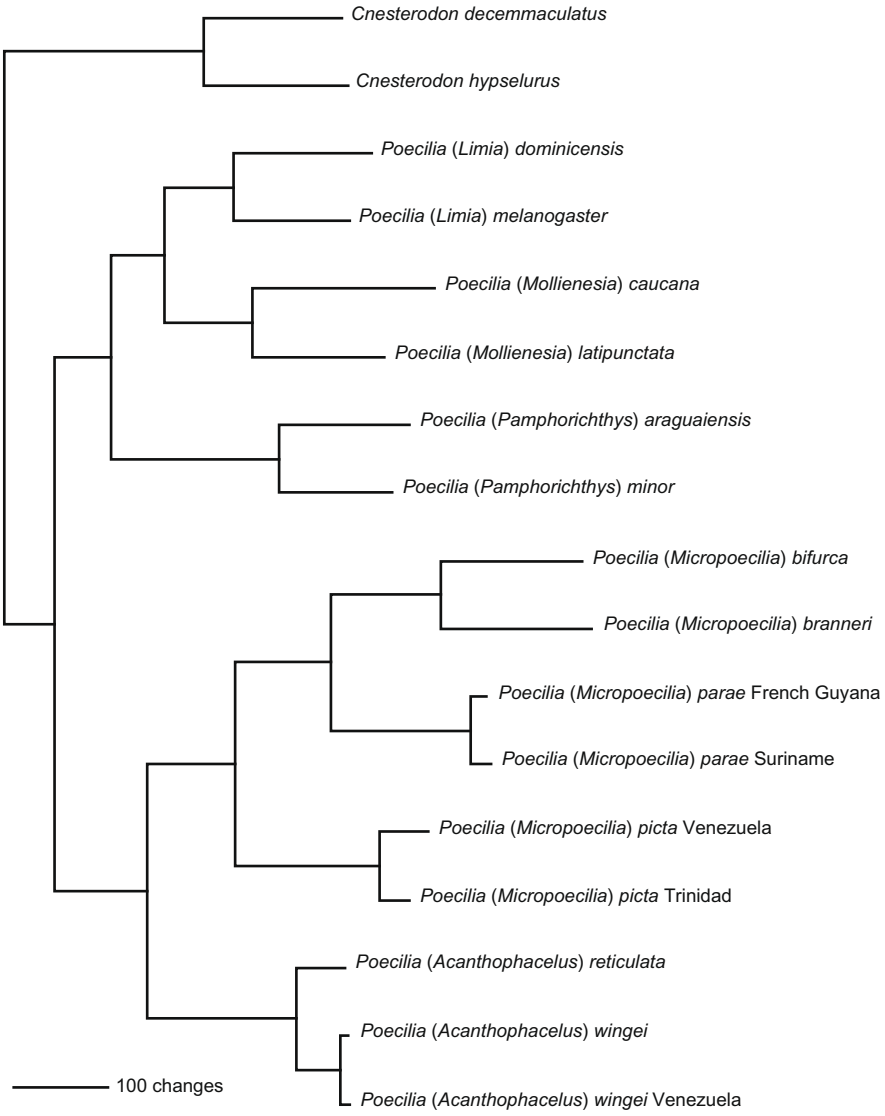

Fig. 1. The shortest tree (3326 steps) for the combined data set based on analyses with PAUP 4.0b10 (Swofford, 2002).

ages (BSPs) below the branches for analyses with 11 partitions. All of the topological relationships shown in Fig. 2 were also recovered in Bayesian analyses of the combined data set with four or eight partitions. Table 3 summarizes bootstrap support percentages based on MP and ML analyses, and BPP values based on Bayesian analyses with four, eight, and 11 partitions.

All species, subgenera, and genera were recovered as monophyletic (100% BSP and 1.00 BPP). Within the genus *Poecilia*, there was a basal split between *Limia* + *Mollienesia* + *Pamphorichthys* and *Micropoecilia* + *Acanthophacelus*. Within *Micropoecilia,* all clades were strongly supported (100% BSP and 1.00 BPP). *Poecilia* (*Micropoecilia*) *bifurca* grouped with *P.* (*M.*) *branneri*, these two were sister to *P.* (*M.*) *parae*, and this clade was the sister-group to *P.* (*M.*) *picta*. Bayesian trees supported an association of *Limia* and *Pamphorichthys* to the exclusion of *Mollienesia,* whereas MP and ML bootstrap analyses favored an association of *Limia* and *Mollienesia* to the exclusion of *Pamphorichthys*.

Analyses based on the nuclear and mitochondrial data sets are generally concordant with the combined data set, but support values for some nodes are less robust (Supplementary Information Figs. 1 and 2; Supplementary Information Tables 3 and 4). In contrast to analyses with the combined and nuclear data sets, analyses with the mitochondrial data set (MPP tree shown in Fig. 4) excluded *Poecilia* (*Micropoecilia*) *picta* from a clade containing all other *Poecilia* (*Micropoecilia*) species plus *Poecilia* (*Acanthophacelus*). The mitochondrial MP tree differed from the ML and MPP mitochondrial trees in recovering *Pamphorichthys* as the sister taxon to all other ingroup taxa.

Two synapomorphic indels were discovered. First, a six bp deletion in Glyt (3469–3474 in concatenation) is shared by all species belonging to *Micropoecilia* and *Acanthophacelus*. Second, a seven bp deletion (8514–8520 in concatenation) in intron 9 of X-src is shared by both *Poecilia* (*Micropoecilia*) *picta* individuals.

3.3. Molecular dating analyses

A chronogram based on molecular dating analyses with BEAST v1.4.8 is shown in Fig. 3. Point estimates of divergence times, along with 95% highest posterior densities, are given in Supplementary Information Table 5. The base of *Poecilia* was dated at ~26 mya. Within *Poecilia*, the split between *Acanthophacelus* and *Micropoecilia* was dated at ~21–22 mya.

The most recent common ancestor of *Poecilia* (*Micropoecilia*) was dated at ~18 mya. Within this subgenus, *P. parae* diverged from *P. bifurca* + *P. branneri* at ~14–15 mya, and the latter two species split at ~8–9 mya.

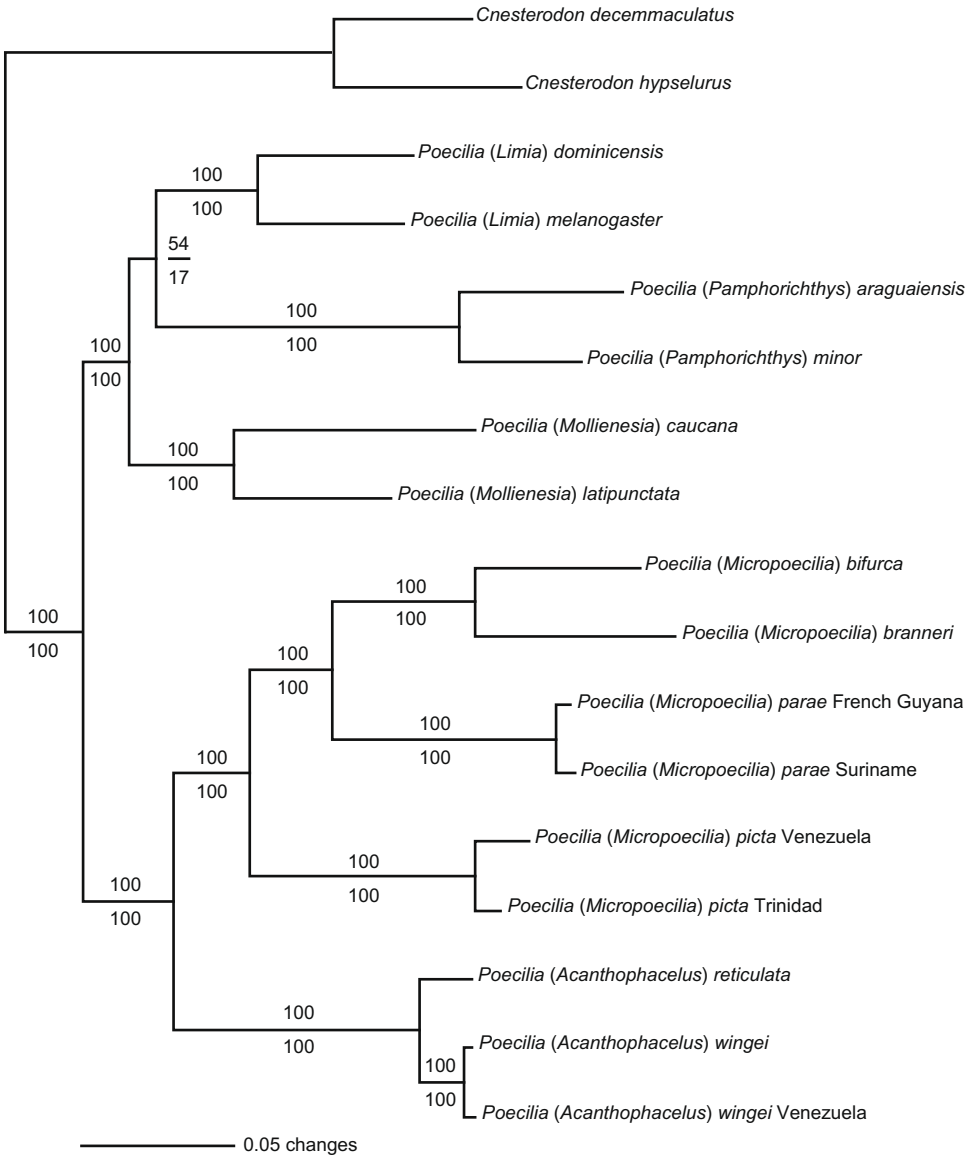

Fig. 2. ML phylogram (ln likelihood = −27849.8817) obtained with RAxML for the combined data set with 11 partitions. Mean Bayesian posterior probabilities (BPPs) based on two independent runs and ML bootstrap support percentages (BSPs) are shown above and below branches, respectively.

3.4. Ancestral state reconstructions

Fig. 4 shows the posterior probabilities of the reconstructed ancestral states for lecithotrophy and placentotrophy using rate parameter priors of $\alpha = 3$ and $\beta = 2$ for the Γ distribution, and an uninformative prior on the bias parameter ($\alpha = 1$). Parsimony ancestral state reconstructions are also shown in Fig. 4. Supplementary Information Table 6 gives the posterior probabilities of the reconstructed ancestral states (lecithotrophy, placentotrophy) for analyses with each combination of priors that were employed. The three different combinations of priors gave posterior probabilities that differed by no more than 0.0541 at a given node. The ancestral state reconstruction based on parsimony indicates that placentotrophy evolved once in *Poecilia* (*Micropoecilia*) in the common ancestor of *P. parae*, *P. bifurca*, and *P. branneri*. SIMMAP reconstructions also support a single origin of placentotrophy in the common ancestor of these species, but with less certainty.

Table 3
Bootstrap support percentages (ML and MP) and Bayesian posterior probabilities for analyses with the combined data set. Nodes in Fig. 1 that were supported by 100% bootstrap percentages in MP and ML analyses, and 1.00 posterior probabilities in Bayesian analyses, are not reported.

Node	MP	ML			Bayesian		
		Four Partitions	Eight Partitions	11 partitions	Four Partitions	Eight Partitions	11 partitions
P. (*Pamphorichthys*) + *P.* (*Limia*) + *P.* (*Mollienesia*)	76	99	100	100	1.00	1.00	1.00
P. (*Limia*) + *P.* (*Mollienesia*)	66	62	61	83	0.28	0.04	0.46
P. (*Pamphorichthys*) + *P.* (*Limia*)	29	38	39	17	0.72	0.96	0.54
P. (*Limia*) + *P.* (*Mollienesia*) + *P.* (*Micropoecilia*) + *P.* (*Acanthophacelus*)	17.5	0	0.2	0	0.00	0.00	0.00

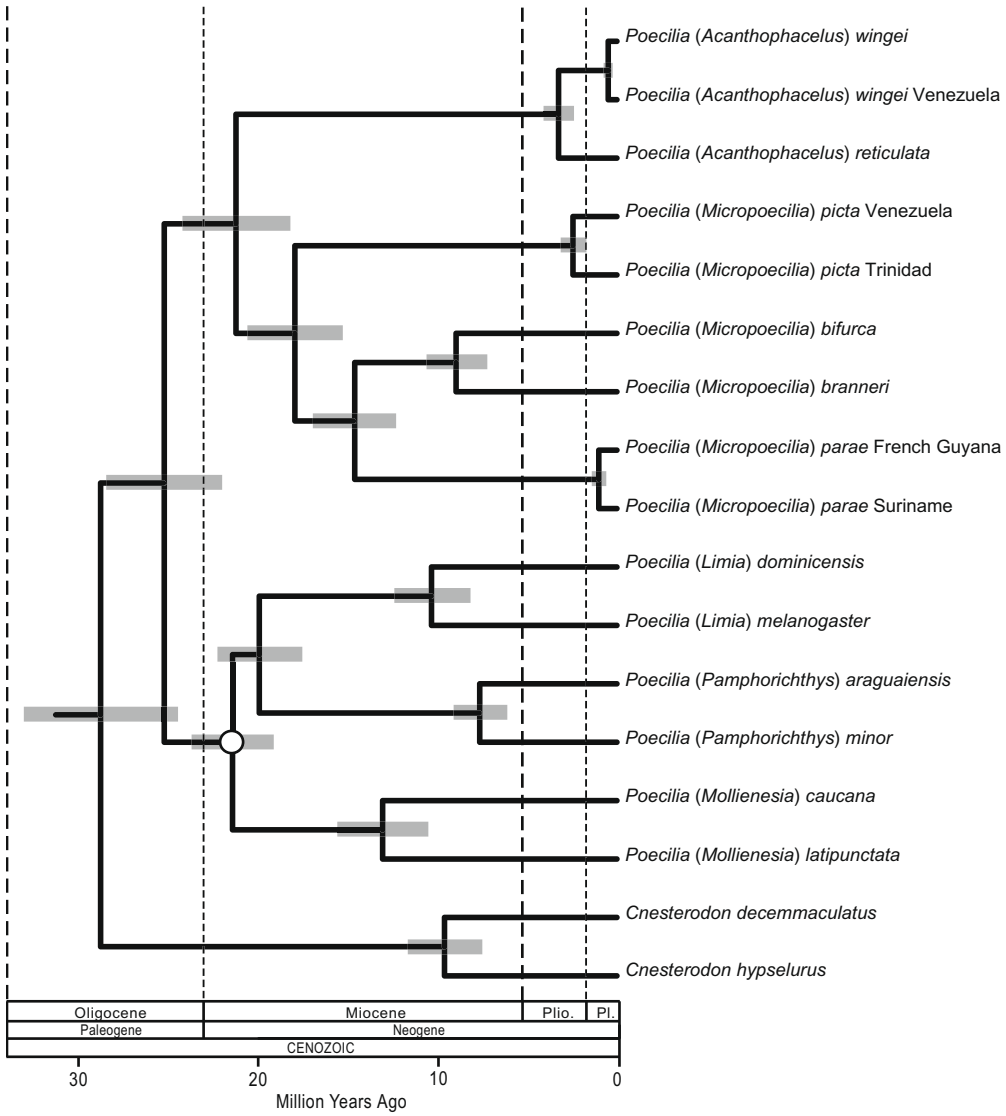

Fig. 3. A timeline in millions of years before present for *Poecilia* evolution based on analyses with BEAST and the combined data set (11 partitions). Grey bars indicate 95% highest posterior densities (HPDs). The open circle denotes the node that was used to calibrate divergence times (see text). Plio. = Pliocene; Pl. = Pleistocene.

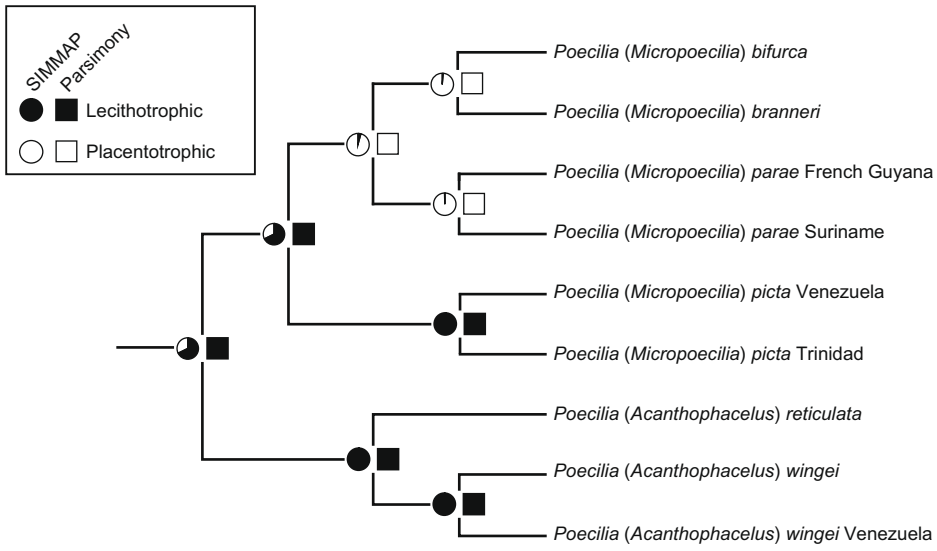

Fig. 4. SIMMAP (circles) and parsimony (squares) reconstructions for placentotrophy and lecithotrophy in *Poecilia* (*Acanthophacelus*) and *Poecilia* (*Micropoecilia*).

4. Discussion

4.1. Mitochondrial versus nuclear data

The mitochondrial data set exhibits much more variation than the nuclear data set. The average number of substitutions per site for the mitochondrial data set is 7.8-fold higher than for the nuclear data set (Table 2). The fastest-evolving partition of the mitochondrial data set (3rd codon positions) is 130-fold faster than the slowest-evolving partition of the nuclear data set (2nd codon positions) (Table 2). Whereas the mitochondrial sequences are evolving faster and contain a larger percentage of parsimony-informative sites than nuclear sequences, the nuclear data exhibit less homoplasy based on RC and RI values. Even with these differences, trees based on the mitochondrial and nuclear data sets provide robust support for many of the same clades (e.g., *Limia*, *Mollienesia*, *Pamphorichthys*, *Acanthophacelus*). There are also instances of congruence, but with one of the two data sets providing much stronger support than the other data set. Mitochondrial data provide robust support for the monophyly of *Micropoecilia* + *Acanthophacelus*, but only moderate support for *Mollienesia* + *Pamphorichthys* + *Limia*. In contrast, nuclear data provide stronger support for *Mollienesia* + *Pamphorichthys* + *Limia*, but weaker support for *Acanthophacelus* + *Micropoecilia*. Within *Micropoecilia* + *Acanthophacelus*, nuclear data provide stronger support for *Micropoecilia* monophyly than mitochondrial data. Together, the mitochondrial and nuclear data sets complement each other and yield a robust phylogeny for almost all nodes. The inclusion of gene regions that evolve at different evolutionary rates maximizes the likelihood of obtaining phylogenetic resolution at both shallow and deep levels in a phylogenetic tree (Hrbek et al., 2007).

4.2. Phylogenetic relationships

Hubbs (1926) erected the genus *Micropoecilia* and included five species (*M. bifurca*, *M. parae*, *M. picta*, *M. melanzona*, *M. branneri*) in this genus. Rosen and Bailey (1963) folded *Micropoecilia* Hubbs, 1926 into the subgenus *Poecilia* (*Lebistes*) along with *P. reticulata*, *P. amazonica*, and *P. scalpridens*. *Micropoecilia* was reinstated by Meyer (1993). Subsequently, the monophyly of this group has been challenged (Costa and Sarraf, 1997) and also supported (Figueiredo, 2003; pers. comm.) based on analyses of morphological characters. Most recently, Poeser et al. (2005) recognized five species (*P. parae*, *P. branneri*, *P. bifurca*, *P. picta*, *P. minima*) in the subgenus *Poecilia* (*Micropoecilia*).

The present study is the most comprehensive molecular phylogenetic study to date on *Micropoecilia*, both in terms of taxonomic representation and gene sampling. We included four of five *Micropoecilia* species recognized by Poeser et al. (2005), all of which were originally included in *Micropoecilia* when it was proposed as a new genus by Hubbs (1926). Our results corroborate Meyer's (1993) hypothesis that *Micropoecilia* is monophyletic.

Within *Micropoecilia*, our molecular phylogeny is discordant with Meyer's (1993) morphological phylogeny. Meyer (1993) proposed a sister-group relationship between *M. bifurca* and *M. picta*, a sister-group relationship between these two and *M. parae*, and a basal split between *M. branneri* and all other species of *Micropoecilia*. Each of these hypotheses is supported by one or more putative gonopodial synapomorphies (Meyer, 1993). In contrast, our results support a sister-group relationship between *M. bifurca* and *M. branneri*, a sister-group relationship between these two and *M. parae*, and a basal split between *M. picta* and all other species of *Micropoecilia* (Figs. 1 and 2).

Our results also provide support for a sister-group relationship between *Micropoecilia* and *Acanthophacelus* and corroborate previous morphological (Lucinda and Reis, 2005) and molecular (Breden et al., 1999; Hamilton, 2001; Hrbek et al., 2007) studies that have supported this hypothesis. In addition, our results confirm Poeser et al.'s (2005) hypothesis that *Poecilia wingei*, which was described after Rosen and Bailey's (1963) publication, is the sister taxon to *Poecilia reticulata*.

Finally, our results are consistent with a clade comprising *Limia*, *Mollienesia*, and *Pamphorichthys* as in Hamilton (2001) and Hrbek et al. (2007). However, we interpret this result with caution given our incomplete taxonomic sampling for these lineages. Our analyses failed to resolve relationships among *Pamphorichthys*, *Limia*, and *Mollienesia*, whereas Hrbek et al. (2007) reported a high posterior probability (0.97) for a sister-group relationship between *Limia* and *Pamphorichthys*. In contrast to Hamilton (2001), Hrbek et al. (2007), and our own molecular results, Lucinda and Reis (2005) found that *Pamphorichthys* was more closely related to *Micropoecilia* and *Acanthophacelus* than to *Limia* based on morphological data. Lucinda and Reis (2005) listed seven characters that diagnose this clade, but also noted that all of these characters were "not uniquely derived and/or reversed features" (p. 46).

4.3. The evolution of placentotrophy in Micropoecilia

Within Poeciliidae, placentotrophy has evolved in *Heterandria formosa* (Grove and Wourms, 1991, 1994), *Phalloceros caudimaculatus* (Arias and Reznick, 2000), *Xenodexia ctenolepis* (Reznick et al., 2007), and three times within the genus *Poeciliopsis* (Reznick et al., 2002). Pires et al. (in press) documented yet another independent origin of placentotrophy in *Poecilia* (*Micropoecilia*). Specifically, placentotrophy occurs in *P. branneri*, *P. bifurca*, and *P. parae*, but not in *P. picta*. Our results provide robust support for the monophyly of *P. branneri*, *P. bifurca*, and *P. parae* within *Micropoecilia.* Ancestral state reconstructions support the hypothesis that placentotrophy evolved in the common ancestor of these taxa. The branch on which placentotrophy evolved extends from ~18 to ~14–15 mya, and establishes a maximum time interval of ~3–4 myr for the evolution of placentotrophy. Reznick et al. (2002) reported maximum time intervals of 2.36 and 0.75 million years for the evolution of placentotrophy in two different clades of *Poeciliopsis*.

4.4. Taxonomic recommendations

An important debate in poeciliid systematics is whether to treat *Poecilia* as a genus with multiple subgenera (e.g., Rosen and Bailey, 1963) or to elevate each subgenus to the level of genus. In our view, resolution of this debate hinges on maintaining the scientific name *Poecilia reticulata* for the common guppy, which has been and remains a model organism for scientific studies. This stability is maintained if we follow a modified version of Rosen and Bailey's (1963) taxonomic scheme that recognizes multiple subgenera in the genus *Poecilia*. Accordingly, we support a taxonomy that recognizes *Acanthophacelus*, *Micropoecilia*, *Limia*, *Pamphorichthys*, and *Mollienesia* as subgenera in the genus *Poecilia* for taxa that were included in our study. Whereas each of these subgenera has been recognized in one or more modern classifications [e.g., Rosen and Bailey (1963) recognized *Limia*, and *Pamphorichthys*; Meyer and Radda (2000) recognized *Mollienesia*; Poeser et al. (2005) recognized *Acanthophacelus* and *Micropoecilia*], they have never been recognized as monophyletic subgenera in the same classification. Our recognition of these subgenera is based on phylogenetic analyses of the most comprehensive molecular data set yet published for representative taxa in the genus *Poecilia*. Rosen and Bailey (1963) included mollies and *P. vivipara* in the subgenus *Poecilia* rather than *Mollienesia*, but molecular studies (Breden et al., 1999; Hamilton, 2001) suggest that Rosen and Bailey's (1963) subgenus *Poecilia* is polyphyletic, and that *P. vivipara* represents a distinct lineage within the genus *Poecilia* that is separate from mollies. We therefore recommend placing mollies in the subgenus *Mollienesia*, and retaining the subgenus

Poecilia for *Poecilia vivipara*. Breden et al. (1999) and Hamilton (2001) also found that *P. vivipara* is separate from *P. reticulata*. In contrast to the taxonomic approach that we recommend, which will maintain taxonomic stability for *P. reticulata*, elevation of subgenera to the generic level will necessitate moving *Poecilia reticulata* to a different genus (i.e., *Acanthophacelus*) because *P. vivipara* represents a distinct lineage based on other molecular studies (Breden et al., 1999; Hamilton, 2001) and is the type species for the genus *Poecilia*. If subgenera are elevated to genera, *P. vivipara* has priority and will retain the genus name *Poecilia*.

Acknowledgments

We thank F. Breden, Heather Alexander, J. de Greef, M. Schartl, and the São Paulo University Museum of Zoology, Brazil (MZUSP), in particular M. de Pinna, C.R. Moreira, and C.A. Figueiredo for providing some of the specimens used in this work. We also thank C. Li and G. Ortí for providing primer sequences for nuclear genes. J. Heraty provided helpful comments on nomenclature. This study was supported by NSF grant DEB0416085 to D.N.R. and M.S.S.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.11.006.

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