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Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in *Poecilia* (Poeciliidae: Cyprinodontiformes)

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ABSTRACT

Members of Poeciliidae are used as model organisms for experimental studies on natural and sexual selection, and comparative studies of life-history evolution. The latter have demonstrated multiple origins of both superfetation and placentotrophy within Poeciliidae. Most recently, placentotrophy has been described in five species of *Poecilia* (*Pamphorichthys*), but only one of these (*P. hasemani*) shows evidence of superfetation. Here, we use a molecular phylogeny based on concatenated nuclear and mitochondrial gene sequences to test hypotheses of correlated evolution between superfetation and placentotrophy in *Poecilia*. Taxon sampling included all species in the subgenera *Micropoecilia* and *Pamphorichthys* for which the presence or absence of placentotrophy and superfetation have been determined, as well as representatives of all other *Poecilia* subgenera (*Acanthophacelus*, *Limia*, *Mollienesia*, *Poecilia*, *Pseudolimia*). Phylogenetic analyses were performed with maximum parsimony, maximum likelihood, and Bayesian methods; ancestral states for life-history characters were reconstructed with parsimony and SIMMAP; correlation analyses were performed with SIMMAP; and divergence times were estimated using a relaxed molecular clock. All subgenera in *Poecilia* were recovered as monophyletic. The basal split in *Poecilia* is between *P.* (*Acanthophacelus*) + *P.* (*Micropoecilia*) and the other five subgenera. In the latter clade, *P.* (*Poecilia*) is the sister-group to the remaining four subgenera. Within *P.* (*Pamphorichthys*), all analyses with the combined data set recovered *P.* (*Pamphorichthys*) *araguaiensis* as the sister taxon to *P.* (*Pamphorichthys*) *hollandi*, and *P.* (*Pamphorichthys*) *scalpridens* as the sister taxon to *P.* (*Pamphorichthys*) *minor*. *P.* (*Pamphorichthys*) *hasemani* was either the sister taxon to *P.* (*Pamphorichthys*) *hollandi* + *P.* (*Pamphorichthys*) *minor* (maximum likelihood, Bayesian) or the sister taxon to all other *Pamphorichthys* species (maximum parsimony). Ancestral state reconstructions suggest that placentotrophy and superfetation evolved on the same branch in *P.* (*Micropoecilia*), whereas placentotrophy evolved before superfetation in *P.* (*Pamphorichthys*). SIMMAP analyses indicate a statistically significant association between placentotrophy and superfetation. Within *P.* (*Micropoecilia*) both placentotrophy and superfetation evolved in ⩽4 million years. Within *P.* (*Pamphorichthys*), superfetation evolved in ⩽9 million years on the *P.* (*Pamphorichthys*) *hasemani* branch, and placentotrophy evolved in ⩽10 million years in the common ancestor of this subgenus.

1. Introduction

1.1. General background

The endemic New World cyprinodontiform family Poeciliidae (Rosen and Bailey, 1963: Poeciliinae *sensu* Parenti, 1981) is composed of ~220 species in 28 genera (Lucinda, 2003; Lucinda and Reis, 2005). All poeciliid fishes are characterized by the presence of a gonopodium (modified anal-fin rays 3, 4, and 5; Hubbs, 1924; Lucinda and Reis, 2005) and internal fertilization, and all but *Tomeurus* are viviparous (Regan, 1913; Rosen and Gordon, 1953; Rosen and Bailey, 1963). Members of this family are commonly found in the pet trade (e.g., swordtails, guppies, and mollies) and are model organisms for experimental studies on natural and sexual selection (Endler, 1983; Houde, 1997; Schluter et al., 1998; Hamilton, 2001). Comparative studies on life-history evolution in Poeciliidae have demonstrated multiple origins of superfetation (the ability to carry multiple broods at different developmental stages) and placentotrophy (the post-fertilization provisioning of developing embryos by the mother through a placenta) (Grove and Wourms, 1991, 1994; Arias and Reznick, 2000; Reznick et al., 2002, 2007; Pires, 2007; Meredith et al., 2010; Pires et al., 2010). In *Poecilia*, placentotrophy and superfetation evolved once in the subgenus *Micropoecilia* in the common ancestor of *P. bifurca*, *P. branneri*, and *P. parae* (Meredith et al., 2010; Pires et al., 2010). More recently, Pires and Reznick (submitted for publication) reported placentotrophy in all species of *Poecilia* (*Pamphorichthys*) that were investigated, whereas

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superfetation was limited to a single species, *P. hasemani*. Further, superfetation was only evident in two of 22 *P. hasemani* females, which suggests that superfetation is a "rare phenomenon", not only in *Pamphorichthys* but also in *P. hasemani*. The combination of placentotrophy without superfetation that occurs in most species of *Poecilia* (*Pamphorichthys*) is unique among poeciliid fishes that have so far been studied (Pires, 2007; Pires and Reznick, submitted for publication).

1.2. Systematics

Pamphorichthys was originally proposed as a new genus by Regan (1913), and has subsequently been recognized as a subgenus (e.g., Rosen and Bailey, 1963; Meredith et al., 2010) or genus (e.g., Costa, 1991; Breden et al., 1999; Hamilton, 2001; Lucinda and Reis, 2005). Costa (1991) recognized five species in *Pamphorichthys* (*P. araguaiensis*, *P. hasemani*, *P. scalpridens*, *P. hollandi*, and *P. minor*) and identified five synapomorphies for this clade. Figueiredo (2008) described a new species of *Pamphorichthys*, *P. pertapeh*, and suggested a sister-group relationship between this taxon and its congeners. Most species of *Pamphorichthys* are restricted to northern South America, and are found predominantly in the main river drainages of Brazil (Lucinda and Reis, 2005). Exceptions are *P. hasemani*, which extends into Bolivia (Lucinda and Reis, 2005), and *P. pertapeh*, which is only known from Lake Perta-Pé in Brazil (Figueiredo, 2008). Previous molecular studies are consistent with *Pamphorichthys* monophyly (Breden et al., 1999; Hamilton, 2001; Meredith et al., 2010), but have included no more than two of the six species that were recognized by Costa (1991) and Figueiredo (2008).

In addition to the above-mentioned taxa, *Limia heterandria* was originally described by Regan (1913) and subsequently placed in the subgenus *Pamphorichthys* by Rosen and Bailey (1963). Costa (1991) excluded this taxon from *Pamphorichthys*, but refrained from allocating it elsewhere. Poeser (2002) proposed the new genus *Pseudolimia* for *Pamphorichthys heterandria*, and did not consider it "closely related to *Pamphorichthys* (p. 54)." Molecular data bearing on the phylogenetic placement of this taxon are limited to ND2 sequences (Breden et al., 1999; Hamilton, 2001), and leave open several possibilities, including a sister-group relationship to *Limia* or *Pamphorichthys*.

Several molecular studies have examined the relationship of *Pamphorichthys* to other subgenera in *Poecilia* (Breden et al., 1999; Hamilton, 2001; Hrbek et al., 2007; Meredith et al., 2010). Meredith et al. (2010) assembled the most comprehensive molecular data set for *Poecilia*, and recovered a basal split between *P.* (*Acanthophacelus*) + *P.* (*Micropoecilia*) and *P.* (*Mollienesia*) + *P.* (*Limia*) + *P.* (*Pamphorichthys*). Relationships among subgenera in the latter clade were not resolved. Meredith et al. (2010) only included two species of *Pamphorichthys* and were also missing *P.* (*Poecilia*) *vivipara* and *P.* (*Pseudolimia*) *heterandria* from their taxonomic sampling. Thus, additional species of *Pamphorichthys* are required to examine relationships in this subgenus, and *P. vivipara* and *P. heterandria* are required to examine relationships among the full complement of *Poecilia* subgenera.

1.3. Life-history evolution

Several authors have postulated contingent evolution, in which the evolution of one trait facilitates the evolution of a second trait (Emerson et al., 1990; Brodie, 1992; Huey et al., 2003; Organ et al., 2009). Within Poeciliidae, previous authors have recognized an association between superfetation and placentotrophy (e.g., Turner, 1937; Thibault and Schultz, 1978). Constantz (1989) suggested that placentotrophy and superfetation are two parts of the same adaptation. In agreement with this hypothesis, *Heterandria formosa* (Turner, 1937; Scrimshaw, 1944a,b; Schrader and Travis, 2005), *Xenodexia* (Reznick et al., 2007), and three *Poecilia* (*Micropoecilia*) species (Pires, 2007; Pires et al., 2010) are placentotrophic and superfetatious. However, there are also examples of poeciliid species that exhibit placentotrophy without superfetation (Arias and Reznick, 2000; Pires and Reznick, submitted for publication) or superfetation without placentotrophy (Thibault and Schultz, 1978; Reznick et al., 2002).

The decoupling of placentotrophy and superfetation was first noted in *Poeciliopsis*, where Thibault and Schultz (1978) demonstrated the presence of superfetation without placentotrophy in *P. monacha*. Subsequently, Reznick et al. (2002) suggested that the common ancestor of *Poeciliopsis* was superfetatious, but lacked placentotrophy, based on parsimony ancestral state reconstructions. Reznick et al. (2002) also demonstrated that extensive placentotrophy evolved independently on three separate occasions in *Poeciliopsis*. In contrast, some populations of *Phalloceros caudimaculatus* exhibit small to moderate amounts of placentotrophy without superfetation (Arias and Reznick, 2000). Within *Poecilia* (*Pamphorichthys*), as noted above, extensive placentotrophy occurs in all species of the subgenus that have been examined, but superfetation is limited to a minority of *P.* (*P.*) *hasemani* individuals (Pires, 2007; Pires and Reznick, submitted for publication). Lastly, *Poecilia* (*Mollienesia*) *latipinna* exhibits facultative placentotrophy with very low matrotrophy indices (Trexler, 1985, 1997).

Placentotrophy and superfetation clearly have some capacity for independent evolution, but this does not preclude that these traits have evolved in a contingent and/or correlated manner. Simulation studies suggest that the prior presence of superfetation may facilitate the evolution of placentotrophy in stable environments with high resource availability (Trexler and DeAngelis, in press).

The occurrence of superfetation and/or placentotrophy, in conjunction with a robust hypothesis for phylogenetic relationships within and between subgenera of *Poecilia*, provides an opportunity to test if superfetation and placentotrophy are significantly correlated with each other within a natural system. Here, we build upon the combined nuclear and mitochondrial DNA data set of Meredith et al. (2010) and (1) evaluate the monophyly of *Poecilia* (*Pamphorichthys*), as well as relationships within this subgenus, (2) examine phylogenetic relationships among all subgenera of *Poecilia*, (3) determine the timing and number of origins of placentotrophy and superfetation in *Poecilia*, and (4) test for correlated evolution of placentotrophy and superfetation within *Poecilia*.

2. Methods and materials

2.1. Taxon sampling

Taxonomic sampling included all recognized species of *Poecilia* (*Pamphorichthys*) and *P.* (*Micropoecilia*) excepting *P.* (*P.*) *pertapeh* and *P.* (*M.*) *minima*, respectively. Our sampling for *Pamphorichthys* comprised all species that were examined by Pires and Reznick (submitted for publication). We also included representatives of all other presumed subgenera (*Limia*, *Poecilia*, *Mollienesia*, *Acanthophacelus*, *Pseudolimia*) in the genus *Poecilia*. Sampling for *Poecilia vivipara* consisted of one specimen from Brazil and one specimen from Trinidad. Two species of *Cnesterodon* were chosen as outgroup taxa based on a previous molecular study that identified this genus as the closest relative to *Poecilia* (Hrbek et al., 2007). A list of taxa that were included in this study, along with locality information for each specimen, is provided in Supplementary Information Table 1.

2.2. Gene sampling

Genomic DNA was extracted following Meredith et al. (2010). Portions of two mitochondrial and seven nuclear gene regions

were chosen based on their utility in previous phylogenetic studies (e.g., Hrbek et al., 2007; Li et al., 2007; Meredith et al., 2010). Two mitochondrial amplicons included the following gene segments: (1) 3' end of $tRNA^{Glu}$, complete cytochrome *b* (cytb), and 5' end of $tRNA^{Thr}$, and (2) 3' end of $tRNA^{Gln}$, complete $tRNA^{Met}$, complete NADH dehydrogenase subunit 2 (ND2), complete $tRNA^{Trp}$, complete $tRNA^{Ala}$, and 5' end of $tRNA^{Asn}$. Seven nuclear amplicons were as follows: (1) two partial exons (8 and 10), all of exon 9, and two introns (8 and 9) of the tyrosine kinase gene (X-src); (2) exon 1 of myosin, heavy polypeptide 6 (myh6); (3) exon 2 of ectodermal-neural cortex 1 like protein (ENC1); (4) exon 2 of glycosyltransferase (Glyt); (5) exon 1 of SH3 and PX domain containing 3 (SH3PX3); (6) a portion of the 7 transmembrane receptor region of rhodopsin (Rh); and (7) exon 3 of recombination activating gene-1 (Rag1). We used PCR primers, amplification conditions, and sequencing protocols that were previously described by Meredith et al. (2010). Sequencing reactions for all genes excepting myh6 were performed after an initial round of amplification with Meredith et al.'s (2010) outer primers, and a second round of amplification with nested, internal primers (Meredith et al., 2010). Sequencing reactions for myh6 were performed after amplification with Meredith et al.'s (2010) outer pair of primers. Accession numbers for the 54 new and 153 previously published sequences are given in Supplementary Information Table 1.

2.3. DNA alignments and data compatibility

New sequences were manually aligned to alignments from Meredith et al. (2010). Gaps were opened as necessary to accommodate insertions in newly added sequences. Twenty-four base pairs (bp) from introns and six bp from tRNAs were identified as alignment-ambiguous, as in Meredith et al. (2010), and were excluded from phylogenetic and molecular dating analyses. Exclusion of these 30 bp resulted in a combined alignment length of 8670 bp. Data set compatibility was tested using the bootstrap compatibility method (de Queiroz, 1993; Teeling et al., 2000) with each combination of partitions outlined below. The bootstrap compatibility method employed 500 bootstrap replicates and a 90% bootstrap support criterion. Each segment in the bootstrap compatibility test was given its own model of evolution as suggested by the Akaike Information Criterion implemented in Modeltest 3.06 (see below; Posada and Crandall, 1998).

Three different schemes were used to define data partitions as follows: (1) two nuclear partitions (exons, introns) and two mitochondrial partitions (protein-coding genes, tRNAs); (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, tRNAs); and (3) eight nuclear partitions (one for coding sequences from each of the seven different genes and one for X-src introns) and three mitochondrial partitions (NADH2, cytb, tRNA genes). Partition models were as follows: K80+I+Γ (ENC1, X-src exons); K81uf+Γ (X-src introns); TVM+I (2nd nuclear codon positions); TVM+I+Γ (cytb, 1st nuclear codon positions, 3rd nuclear codon positions); TrN+I+Γ (exons, NADH2); TrN+I (Glyt, Rh); TrNef+I+Γ (SH3PX3); GTR+I+Γ (1st, 2nd, and 3rd mitochondrial codon positions, mitochondrial protein-coding, myh6, Rag1, tRNA genes).

Bootstrap compatibility tests with maximum likelihood indicated that there were no conflicts within or between the mitochondrial and nuclear partitions with $\geqslant$90% bootstrap support. By contrast, Bayesian results suggested a possible conflict between the mitochondrial and nuclear partitions for the placement of *Poecilia* (*Pamphorichthys*) *minor*. Specifically, analyses with the mitochondrial data set recovered *P.* (*P.*) *minor* + *P.* (*P.*) *scalpridens* with posterior probabilities of 1.00, whereas analyses with the nuclear data set recovered *P.* (*P.*) *minor* + *P.* (*P.*) *hollandi* + *P.* (*P.*) *araguaiensis* with posterior probabilities that ranged from 0.89 to 0.95. The *P.* (*P.*) minor mitochondrial protein-coding sequences for cytb and ND2 translate into intact proteins, which suggests that they are not numts (i.e., nuclear mitochondrial DNAs; Lopez et al., 1994), unless they have recently been incorporated into the nuclear genome. Given these results, and also because Bayesian posterior probabilities may be inflated owing to model misspecification (Ronquist and Deans, 2010), we analyzed three different data sets: (1) Combined: concatenated alignment (seven nuclear segments and two mitochondrial segments; 8670 bp) for all taxa; (2) Nuclear: concatenated nuclear data set (6158 bp) for all taxa; and (3) Mitochondrial: concatenated mitochondrial data set (2512 bp) for all taxa.

2.4. Phylogenetic analyses

Maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses were performed on the three data sets with PAUP* 4.0b10 (Swofford, 2002), RAxML 7.0.4 (Stamatakis, 2006), and MrBayesV3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), respectively. Gaps were treated as missing data in all analyses. The branch and bound algorithm was used to find the shortest tree(s) with MP. MP bootstrap analyses included 1000 pseudoreplicate data sets and employed heuristic searches with 1000 randomized addition sequences with tree-bisection and reconnection branch swapping. ML and Bayesian analyses were performed with models from Modeltest (see above). For the RA$\times$ML analyses, the model selected by ModelTest3.06 (Posada and Crandall, 1998) was used to inform whether or not to include invariant sites. For the Bayesian analyses, the next most complex model was used if the model suggested by Modeltest 3.06 (Posada and Crandall, 1998) was not available in MrBayes. RAxML analyses employed 500 bootstrap pseudoreplicates, randomized MP starting trees, and the fast hill-climbing algorithm with all other free parameters estimated. Bayesian posterior probabilities were calculated with MrBayes v3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using eight Markov chains (seven hot, one cold), random starting trees, default priors, and chain sampling every 1000 generations; analyses were terminated when the average standard deviation of split frequencies for the simultaneous analyses fell below 0.01 ($\sim$2–7 million generations in different analyses).

2.5. Molecular dating analyses

We used Nuclear with eight partitions, Mitochondrial with three partitions, and Combined with 11 partitions for molecular dating analyses. The likelihood ratio statistic rejected the molecular clock ($P < 0.05$) for six of the 11 partitions (ENC1, Rag1, X-src introns, X-src exons, cytb, NADH2), and we chose to use the relaxed molecular clock method implemented in BEAST ver1.5.3 (Drummond et al., 2006; Drummond and Rambaut, 2007). BEAST allows for complex models of evolution and "soft" node constraints (Hedges and Kumar, 2004; Yang and Rannala, 2006). We implemented the uncorrelated lognormal distribution (UCLN) model, which draws the rate of each lineage independently from a lognormal distribution. Results from ModelTest3.06 (Posada and Crandall, 1998) were used but if more than two substitution rates were suggested, then the GTR model was implemented. Individual runs for each data set consisted of 30 million generations per run and were subsequently combined using Log-Combiner. Tracer 1.5 (Rambaut and Drummond, 2003) was used to inspect for stationarity/mixing and to verify that the estimated sample size for each parameter was $\geqslant$200. BEAST analyses were calibrated with a minimum of 19.92 mya and a maximum of 24.39 mya for the most recent common ancestor of *Pamphorichthys*, *Limia*, and *Mollienesia*

(Meredith et al., 2010). The prior distribution for this constraint followed a normal distribution with 95% of the distribution between the specified minimum (19.92) and maximum (24.39) and 2.5% in each tail.

2.6. Ancestral state reconstructions

Parsimony (MacClade 4.1: Maddison and Maddison, 2005) and SIMMAP Version 1.5 (Bollback, 2006) were used to estimate ancestral states for maternal provisioning (lecithotrophy, placentotrophy) and superfetation (absent, polymorphic, present). Analyses were performed with taxonomic sampling that matched each of the three data sets (Mitochondrial, Nuclear, Combined). Superfetation and the relative amounts of pre-and post-fertilization maternal provisioning to embryos have previously been quantified (see Pires, 2007; Pires et al., 2010; Pires and Reznick, submitted for publication); these results were used for the determination of the coding for terminal taxa used here (Table 1). *Poecilia* (*Pamphorichthys*) *hasemani* was coded as polymorphic given that only two individuals, from two separate collection sites near each other, were observed to exhibit superfetation (out of 22 individuals dissected, from three collection sites). We followed Hunt (2007) and coded the polymorphic state as intermediate between absent and present in an ordered series (absent ↔ polymorphic ↔ present).

SIMMAP (Bollback, 2006) implements a Bayesian MCMC approach for stochastic mutational mapping (Nielsen, 2002; Huelsenbeck et al., 2003). The posterior probability distribution accommodates uncertainties in ancestral states, evolutionary rates, and the phylogeny. Ancestral state reconstructions used all post-burnin trees from the Mitochondrial (3 partitions: 2471 trees), Nuclear (8 partitions: 1744 trees) and Combined (11 partitions: 4094 trees) MrBayes analyses.

The overall substitution rate of each morphological character (i.e., overall rate prior) was modeled with a gamma distribution whose parameters α and β describe the mean (α/β) and variance (α/β^2) of the distribution. For two-state morphological characters, SIMMAP also requires a bias parameter, which follows a symmetrical beta distribution and is described by the single parameter α. Priors were estimated using the two-step procedure implemented in SIMMAP 1.5. First, MCMC analyses with default settings (100,000 cycles, sampling frequency = 200; 10% burnin; rate upper bound = 1000) were used to sample overall rate parameter and bias parameters values. Next, the results of these analyses were analyzed with the R Statistical Package and the sumprmcmc.r script provided with SIMMAP 1.5 to find the best fitting gamma and beta distributions. Based on these analyses, we obtained the priors used in all subsequent ancestral state reconstruction analyses (Table 2). Rate and bias parameter priors were approximated with 60 and 31 categories, respectively.

Table 1
Coding of terminal taxa used in this study for maternal provisioning (lecithotrophy, placentotrophy) and superfetation (absent, polymorphic, present).

Taxon	Maternal provisioning	Superfetation
Cnesterodon decemmaculatus	Lecithotrophy	Absent
Cnesterodon hypselurus	Lecithotrophy	Absent
Poecilia (Limia) dominicensis	Lecithotrophy	Absent
P. (Limia) melanogaster	Lecithotrophy	Absent
P. (Pseudolimia) heterandria	Lecithotrophy	Absent
P. (Micropoecilia) bifurca	Placentotrophy	Present
P. (Micropoecilia) branneri	Placentotrophy	Present
P. (Micropoecilia) parae Surinam	Placentotrophy	Present
P. (Micropoecilia) parae French Guyana	Placentotrophy	Present
P. (Micropoecilia) picta Venezuela	Lecithotrophy	Absent
P. (Micropoecilia) picta Trinidad	Lecithotrophy	Absent
P. (Acanthophacelus) reticulata	Lecithotrophy	Absent
P. (Acanthophacelus) wingei	Lecithotrophy	Absent
P. (Pamphorichthys) araguaiensis	Placentotrophy	Absent
P. (Pamphorichthys) hasemani	Placentotrophy	Polymorphic
P. (Pamphorichthys) hollandi	Placentotrophy	Absent
P. (Pamphorichthys) minor	Placentotrophy	Absent
P. (Pamphorichthys) scalpridens	Placentotrophy	Absent
P. (Mollienesia) caucana	Lecithotrophy	Absent
P. (Mollienesia) latipunctata	Lecithotrophy	Absent
P. (Poecilia) vivipara Brazil	Lecithotrophy	Absent
P. (Poecilia) vivipara Trinidad	Lecithotrophy	Absent

2.7. Correlation analyses

SIMMAP Version 1.5 (Bollback, 2006) implements Bayesian mutational mapping (Nielsen, 2001, 2002; Huelsenbeck et al., 2003) and was used to determine if placentotrophy and superfetation covary with each other. As for ancestral state reconstructions, this approach accommodates uncertainties in ancestral states, evolutionary rates, and phylogeny. SIMMAP Version 1.5 (Bollback, 2006) uses two different statistics, d_{ij} and m_{ij}, to calculate the covariation between two different character states (i and j). The first statistic, d_{ij}, is the difference between the observed coincidence of states i and j and the expected coincidence of states i and j under independence. The second statistic is m_{ij} and is also known as the mutual historical information content (MHIC) statistic because of its relationship to the classical mutual information content statistic (Bollback et al., 2007).

Correlation analyses were performed with 10% of the post-burnin trees from the Mitochondrial (248 trees), Nuclear (174 trees) and Combined (409 trees) analyses with MrBayes. Post-burnin trees were sampled at evenly spaced intervals from the entire population of post-burnin trees. Each correlation analysis was configured to have an observed sample size (Obs N) > 2000 and a predictive sample size (Pred N) > 1000. SIMMAP 1.5 settings that achieved these target values were as follows: (1) sample size (=the number of mutational maps that were simulated for each tree and each character): Mitochondrial = 9; Nuclear = 12; Combined = 5; (2) number of prior draws: Mitochondrial = 1, Nuclear = 1, Combined = 1; and (3) number of predictive samples: Mitochondrial = 5; Nuclear = 6; Combined = 3. The predictive sample size was used to determine the p-values for the association between placentotrophy and superfetation. The null hypothesis is that characters evolve independently of each other and that associations are the result of chance rather than correlated evolution.

3. Results

3.1. Phylogenetic analyses

Maximum likelihood phylograms for sequences from each of the nine different amplicons are shown in Supplementary Material Fig. S1. Fig. 1 shows the ML tree for Combined with 11 partitions. ML bootstrap support percentages (BSPs) are shown above branches and mean Bayesian posterior probabilities (BPPs) based on two independent runs are shown below branches. ML and Bayesian results based on different partitioning schemes for each of the three data sets (Mitochondrial, Nuclear, Combined) are shown in Supplementary Information Fig. 2. Supplementary Information Fig. 3A and C shows the single most parsimonious trees for Mitochondrial (3238 steps) and Combined (4245 steps), respectively. Supplementary Information Fig. 3B shows one of the two most parsimonious trees recovered with Nuclear (987 steps). BSPs based on MP and ML analyses and BPPs based on MrBayes analyses are given in Table 3 and Supplementary Information Tables 2–4.

Table 2
Overall rate priors and bias parameters that were used in ancestral state reconstruction analyses.

Prior	Character	
	Placentotrophy	Superfetation
Overall rate prior	Mitochondrial: $\alpha = 1.543$, $\beta = 0.350$ Nuclear: $\alpha = 1.974$, $\beta = 0.451$ Combined: $\alpha = 1.885$, $\beta = 0.428$	Mitochondrial: $\alpha = 3.02$, $\beta = 0.396$ Nuclear: $\alpha = 3.764$, $\beta = 0.461$ Combined: $\alpha = 3.475$, $\beta = 0.408$
Bias parameter	Mitochondrial: $\alpha = 3.642$ Nuclear: $\alpha = 3.473$ Combined: $\alpha = 4.067$	Not applicable

Maximum parsimony, maximum likelihood, and Bayesian analyses with Combined resulted in the monophyly of all polytypic subgenera in *Poecilia* with BSPs of 100 and BPPs of 1.00 (Fig. 1, Supplementary Information Fig. 2, Supplementary Information Table 4). The basal split in *Poecilia* is between *Poecilia* (*Micropoecilia*) + *Poecilia* (*Acanthophacelus*) and *Poecilia* (*Poecilia*) + *Poecilia* (*Mollienesia*) + *Poecilia* (*Pamphorichthys*) + *Poecilia* (*Pseudolimia*) + *Poecilia* (*Limia*). In the latter clade, there is robust support for an association of *Poecilia* (*Mollienesia*) + *Poecilia* (*Pamphorichthys*) + *Poecilia* (*Pseudolimia*) + *Poecilia* (*Limia*) to the exclusion of *Poecilia* (*Poecilia*). In addition, *Poecilia* (*Pseudolimia*) + *Poecilia* (*Limia*) clustered together in analyses with Combined (BSP = 96–100, BPP = 1.00). Relationships between *Poecilia* (*Mollienesia*), *Poecilia* (*Pamphorichthys*), and *Poecilia* (*Pseudolimia*) + *Poecilia* (*Limia*) were not well resolved.

Within *Poecilia* (*Pamphorichthys*), robust support for a sister-group relationship between *P.* (*Pamphorichthys*) *araguaiensis* and *P.* (*P.*) *hollandi* was recovered in analyses with Mitochondrial (BSP = 100, BPP = 1.00), Nuclear (BSP = 93–96, BPP = 1.00), and Combined (BSP = 100, BPP = 1.00). Analyses with Mitochondrial provided further support for an association of these two with *P.* (*P.*) *hasemani* (BSP = 91–98, BPP = 1.00), whereas analyses with Nuclear grouped *P.* (*P.*) *araguaiensis*, *P.* (*P.*) *hollandi*, *P.* (*P.*) *scalpridens*, and *P.* (*P.*) *minor* to the exclusion of *P.* (*P.*) *hasemani* (BSP = 87–97, BPP = 1.00). Five of the six maximum likelihood or Bayesian analyses with Combined agreed with the Mitochondrial results and favored an association of *P.* (*P*). *hasemani* with *P.* (*P.*) *araguaiensis* and *P.* (*P.*) *hollandi*, albeit with weaker support (BSP = 61, BPP = 0.62–1.00), whereas maximum parsimony and maximum likelihood with the 8-partition version of Combined were in better agreement with the Nuclear results and supported a clade

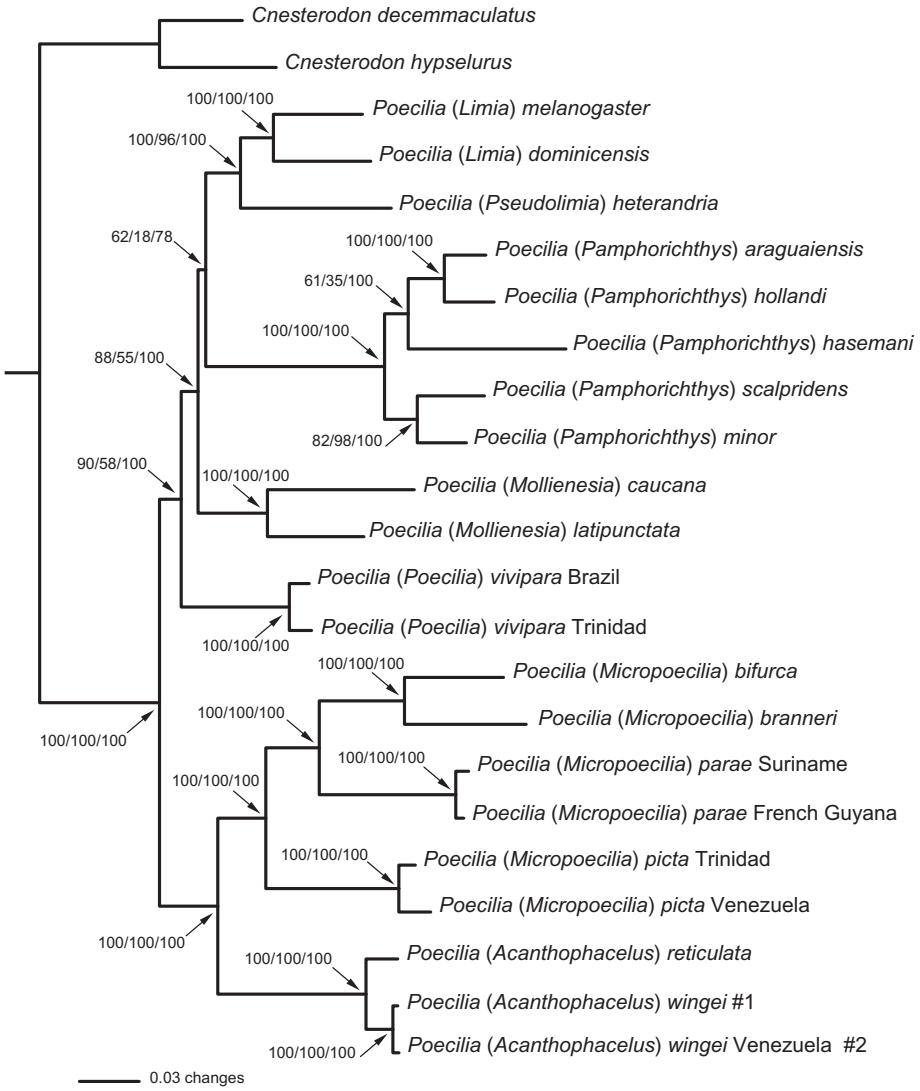

Fig. 1. ML phylogram (−ln likelihood = 32253.663488) obtained with RaxML for the combined data set with 11 partitions. Values above branches correspond to ML bootstrap support percentages (before first slash), MP bootstrap support percentages (between slashes), and Bayesian posterior probabilities (after second slash), respectively.

Table 3
Bootstrap support percentages (MP and ML) and Bayesian posterior probabilities for analyses with the Mitochondrial (3 partitions), Nuclear (8 partitions), and Combined (11 partitions) data sets. 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	Mitochondrial – 3 partitions		Nuclear – 8 partitions		Combined – 11 partitions	
	MP/ML	Bayesian	MP/ML	Bayesian	MP/ML	Bayesian
P. (*Limia*) + *P.* (*Pseudolimia*)	9/10	0.01	100/100	1.00	96/100	1.00
P. (*Pamphorichthys*) *hollandi* + *P.* (*P.*) *araguaiensis*	100/100	1.00	96/94	1.00	100/100	1.00
P. (*Pamphorichthys*) *scalpridens* + *P.* (*P.*) *minor*	99/98	1.00	26/15	0.04	98/82	1.00
P. (*Pamphorichthys*) *hollandi* + *P.* (*P.*) *araguaiensis* + *P.* (*P.*) *hasemani*	91/98	1.00	1/2	0.00	35/61	1.00
P. (*Limia*) + *P.* (*Pseudolimia*) + *P.* (*Pamphorichthys*)	2/33	0.15	32/25	0.20	18/62	0.78
P. (*Limia*) + *P.* (*Pseudolimia*) + *P.* (*Mollienesia*) + *P.* (*Pamphorichthys*)	10/42	0.17	47/46	0.37	55/88	1.00
All Poecilia species except P. (*Acanthophacelus*) + *P.* (*Micropoecilia*)	15/19	0.11	83/92	1.00	58/90	1.00
P. (*Micropoecilia*) *parae* + *P.* (*Micropoecilia*) *branneri* + *P.* (*Micropoecilia*) *bifurca*	99/100	1.00	97/99	1.00	100/100	1.00
P. (*Acanthophacelus*) + *P.* (*Micropoecilia*)	100/100	1.00	80/77	0.92	100/100	1.00
P. (*Micropoecilia*)	33/36	0.39	100/100	1.00	100/100	1.00
P. (*Limia*)	98/100	1.00	94/85	1.00	100/100	1.00

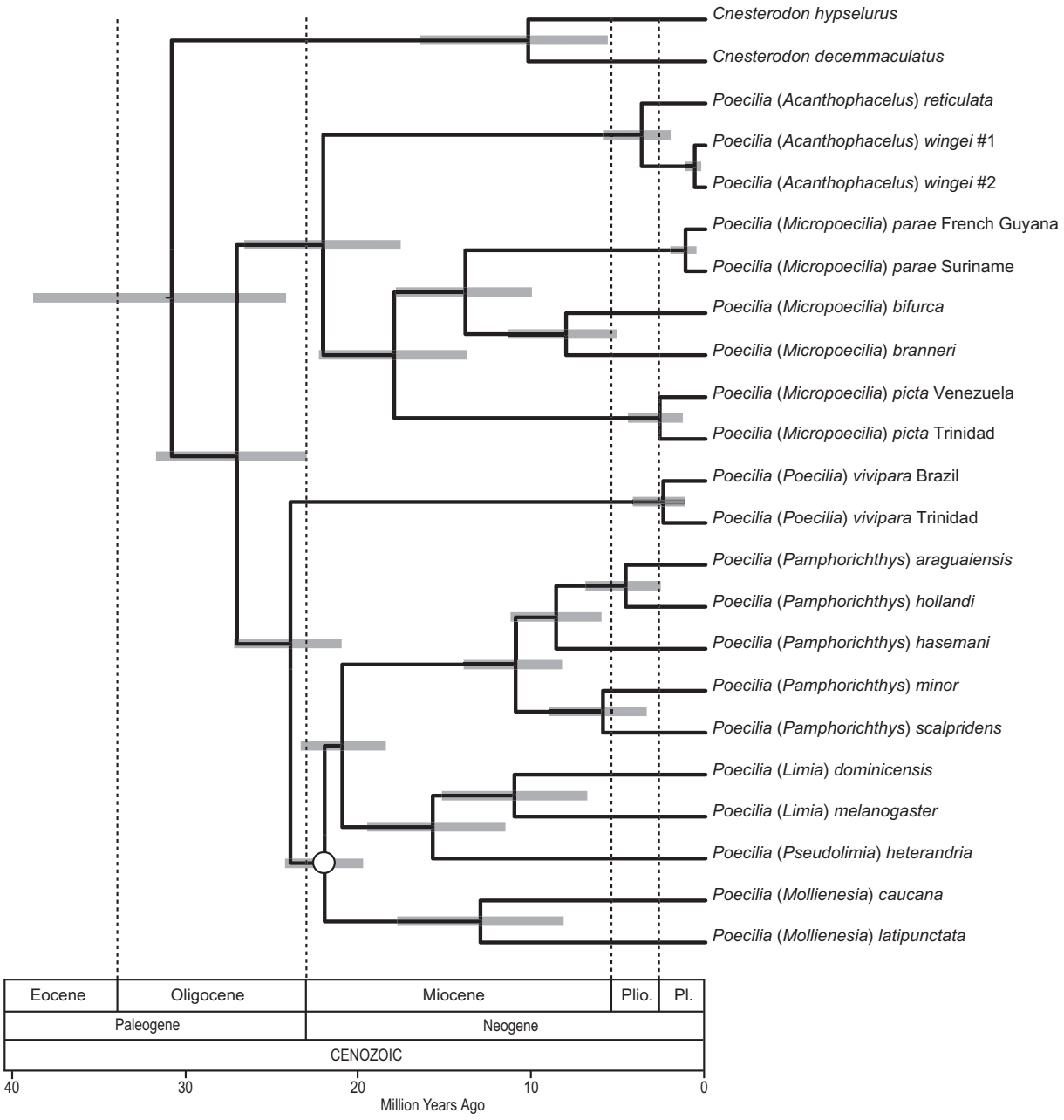

Fig. 2. Timeline in million of years before the present for *Poecilia* evolution based on the combined data set with 11 partitions using BEAST. Gray bars indicate 95% highest posterior densities (HPDs). The open circle indicates the node used to calibrate the divergence times. Plio. = Pliocene, Pl. = Pleistocene.

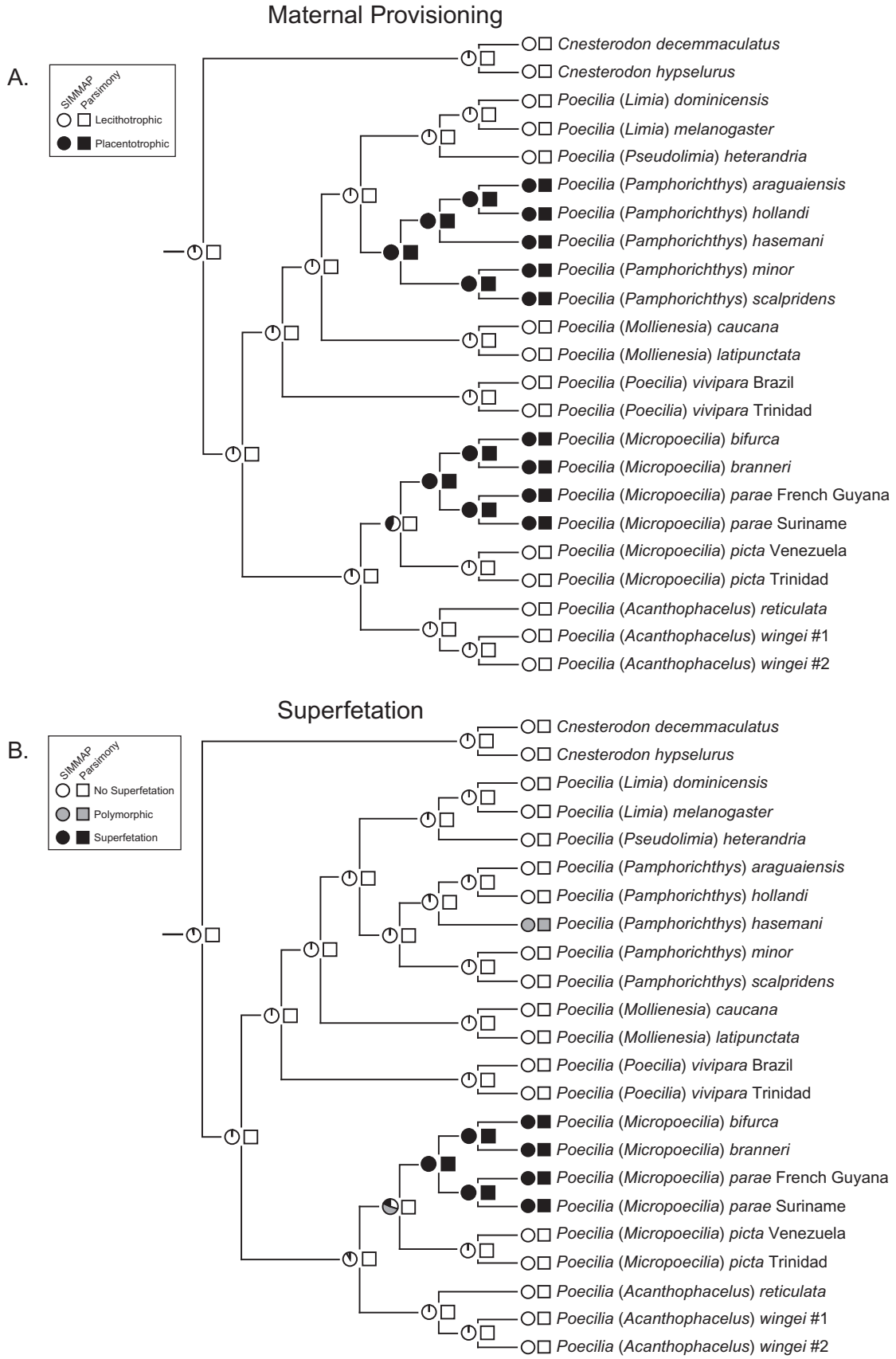

Fig. 3. Parsimony (squares) and SIMMAP (circles) ancestral state reconstructions for the evolution of placentotrophy and superfetation based on phylogenetic analyses with the Combined data set (11 partitions).

comprised of *P.* (*P.*) *araguaiensis*, *P.* (*P.*) *hollandi*, *P.* (*P.*) *scalpridens*, and *P.* (*P.*) *minor* to the exclusion of *P.* (*P.*) *hasemani* (BSP = 53–65).

3.2. Molecular dating analyses

Fig. 2 shows a chronogram based on molecular dating analysis performed with BEAST v1.5.3 (Combined, 11 partitions). Supplementary Information Fig. 4 shows chronograms based on analyses with Mitochondrial and Nuclear. Point estimates of divergence times and 95% highest posterior densities for all nodes and data sets are summarized in Supplementary Information Table 5. The base of *Poecilia* was dated at ~27 mya based on Combined, and all subgenera were distinct by ~16 mya when *Pseudolimia* diverged from *Limia*. The most recent common ancestor of *Pamphorichthys* was dated at ~11 mya (Fig. 2). *P.* (*Pamphorichthys*) *hasemani* diverged from *P.* (*P.*) *hollandi* + *P.* (*P.*) *araguaiensis* at ~9 mya, and the latter two species diverged ~5 mya (Fig. 2). *P.* (*Pamphorichthys*) *minor* diverged from *P.* (*P.*) *scalpridens* at ~6 mya (Fig. 2).

3.3. Ancestral state reconstructions

Parsimony and SIMMAP ancestral state reconstructions for maternal provisioning (lecithotrophy, placentotrophy) and superfetation (absent, polymorphic, present) based on Combined are shown in Fig. 3. Parsimony reconstructions were identical with accelerated transformation (acctran) and delayed transformation (deltran). Supplementary Information Fig. 5 shows reconstructions based on Mitochondrial and Nuclear. Posterior probabilities of the reconstructed ancestral states for placentotrophy and superfetation are given in Supplementary Information Table 6. Parsimony ancestral state reconstructions indicate two independent origins of placentotrophy within *Poecilia* – one in the common ancestor of *Poecilia* (*Pamphorichthys*) and the other in the common ancestor of *P.* (*Micropoecilia*) *bifurca*, *P.* (*M.*) *branneri*, and *P.* (*M.*) *parae*. SIMMAP ancestral state reconstructions also suggest two independent origins of placentotrophy. The common ancestor of *P.* (*Micropoecilia*) was reconstructed as lecithotrophic in parsimony analyses, but SIMMAP suggested approximately equal posterior probabilities for placentotrophy (0.41–0.50) and lecithotrophy (0.50–0.59).

Parsimony ancestral state reconstructions suggest that superfetation evolved in the common ancestor of *P.* (*Micropoecilia*) *bifurca*, *P.* (*M.*) *branneri*, and *P.* (*M.*) *parae*, and that polymorphism for superfetation evolved on the branch leading to *P.* (*Pamphorichthys*) *hasemani*. SIMMAP posterior probabilities likewise supported one independent origin of superfetation in the common ancestor of *P.* (*Micropoecilia*) *bifurca*, *P.* (*M.*) *branneri*, and *P.* (*M.*) *parae* (0.96–0.98), and the evolution of polymorphism for superfetation on the branch leading to *P.* (*Pamphorichthys*) *hasemani*. The ancestral state for the subgenus *P.* (*Micropoecilia*) was reconstructed as lacking superfetation in the parsimony analysis, whereas the highest posterior probabilities were for a polymorphic ancestor (0.44–0.48) in SIMMAP analyses.

3.4. Correlation analyses

Table 4 shows the results of the correlation analyses for placentotrophy and superfetation based on MrBayes trees for Combined, Nuclear, and Mitochondrial. The results of correlation analyses with these three data sets were concordant. In every case placentotrophy and superfetation were positively associated with each other ($P < 0.05$).

4. Discussion

4.1. Phylogenetic relationships

Our molecular phylogeny for *Poecilia* (Fig. 1) is the first to include nuclear sequences for representatives of *P.* (*Acanthophacelus*), *P.* (*Micropoecilia*), *P.* (*Poecilia*), *P.* (*Pamphorichthys*), *P.* (*Limia*), *P.* (*Pseudolimia*), and *P.* (*Mollienesia*). With the exception of a few nodes, analyses of the combined data set with maximum parsimony, maximum likelihood, and MrBayes provide support for a well-resolved phylogenetic tree for the genus *Poecilia*. The basal split in *Poecilia* is between *P.* (*Acanthophacelus*) + *P.* (*Micropoecilia*) and the other five subgenera. This result was strongly supported by maximum parsimony, maximum likelihood, and Bayesian analyses (Hamilton (2001) obtained similar results with mitochondrial ND2 sequences. In the latter group, *P.* (*Poecilia*) *vivipara* is distinct from other lineages and is the sister taxon to the remaining four subgenera (*Limia*, *Pseudolimia*, *Pamphorichthys*, *Mollienesia*) based on analyses with our concatenated sequence data. Again, this result agrees with Hamilton's (2001) ND2 phylogeny. Within this clade there was robust support for an association of *Pseudolimia* with *Limia*, but relationships between this pair, *Pamphorichthys*, and *Mollienesia* were not resolved. By contrast, Hrbek et al. (2007) found strong support (posterior probability = 0.97) for an association of *Pamphorichthys* and *Limia* to the exclusion of *Mollienesia* (*Pseudolimia* was not included in Hrbek et al.'s (2007) study). Our finding that *Limia* and *Pseudolimia* are closely related agrees with the morphological assessment of Poeser (2002). By contrast, Hamilton (2001) proposed a sister-group relationship between *Pseudolimia* and *Pamphorichthys* based on analyses of ND2 sequences, and Figueiredo (2008) suggested that *Pseudolimia* and *Pamphorichthys* share at least five derived characters. Our mitochondrial data set included ND2 and provided poor resolution for the placement of *P.* (*Pseudolimia*). Instead, firm resolution for the placement of this taxon was only achieved with nuclear sequences or concatenated mitochondrial + nuclear sequences.

In addition to informing higher-level relationships within *Poecilia*, our results provide the most comprehensive molecular

Table 4
Correlation results for placentotrophy and superfetation.

Data set	Statistic	Observed value	*P*-value	Observed sample size	Predictive sample size
Mitochondrial					
	$m(1,2)$	0.135371	0.008065*	2232	1240
	$d(1,2)$	0.086175	0.008871*	2232	1240
Nuclear					
	$m(1,2)$	0.103516	0.019157*	2088	1044
	$d(1,2)$	0.062675	0.028736*	2088	1044
Combined					
	$m(1,2)$	0.127136	0.008965*	2045	1227
	$d(1,2)$	0.080500	0.009780*	2045	1227

* Significant at $P \leqslant 0.05$ with a sequential Bonferroni test. The first number found within each set of parentheses is placentotrophy (present = 1) and the second number is superfetation (present = 2). Values for m_{ij} and d_{ij} are positive when they occur together more frequently than expected under independence.

phylogeny for *P.* (*Pamphorichthys*) and includes five of the six described species. Regan (1913) first erected the genera *Pamphorichthys* and *Pamphoria* for *Heterandria minor* (Garman, 1895) and *Cnesterodon scalpridens* (Garman, 1895), respectively. Subsequently, Rosen and Bailey (1963) demoted *Pamphorichthys* to a subgenus of *Poecilia* comprising *P. minor, P. hollandi, P. hasemani*, and *P. heterandria*. *Pamphoria scalpridens* was provisionally placed in *Poecilia* (*Lebistes*) by Rosen and Bailey (1963). Costa (1991) reinstated *Pamphorichthys*, placed *Poecilia* (*Acanthophacelus*) *scalpridens* in *Pamphorichthys*, and removed *Pamphorichthys heterandria* from *Pamphorichthys*, although he did not formally place this taxon into any genus or subgenus. Soon afterwards, Poeser (2002) reallocated *Pamphorichthys heterandria* to *Pseudolimia*. Our study confirms the monophyly of *P.* (*Pamphorichthys*) *sensu* Costa (1991) and Figueiredo (2008), and suggests that the basal split in this subgenus is between *P. scalpridens* + *P. minor* and *P. araguaiensis* + *P. hollandi* + *P. hasemani* (Combined, Mitochondrial) or *P. hasemani* and all other species (Nuclear).

4.2. The evolution of placentotrophy and superfetation in Poeciliidae

The evolution of placentotrophy is well documented in Poeciliidae. This feature occurs in *Heterandria formosa*, (Grove and Wourms, 1991, 1994), *Phalloceros caudimaculatus* (Arias and Reznick, 2000), *Xenodexia ctenolepis* (Reznick et al., 2007), *Poecilia* (*Micropoecilia*) (Meredith et al., 2010; Pires et al., 2010), *Poecilia* (*Pamphorichthys*) (Pires, 2007; Pires and Reznick, submitted for publication), and has evolved on three occasions within *Poeciliopsis* (Reznick et al., 2002). Superfetation, or the ability to carry embryos in more than one stage of development, is sometimes associated with placentotrophy. Constantz (1989) suggested that superfetation and placentotrophy are part of the same adaptation, but it is now generally accepted that these traits have the capacity to evolve independently of one another (Pires and Reznick, submitted for publication).

Most recently, Pires and Reznick (submitted for publication) examined the life histories of all species of *Poecilia* (*Pamphorichthys*) except for *P.* (*P.*) *pertapeh* and showed that four species are not superfetatious, whereas superfetation is rare in the fifth species, *P.* (*P.*) *hasemani*, with only 10% of sampled individuals showing signs of this trait. Pires and Reznick (submitted for publication) also noted that *P. hasemani* has a higher matrotrophy index than other *P.* (*Pamphorichthys*) species. Superfetation has previously been reported in *P.* (*P.*) *hollandi* (Casatti et al., 2006), but Pires and Reznick (submitted for publication) dissected more than 125 individuals from eight populations and found no evidence for this trait. This discrepancy is thus likely the result of Casatti et al.'s (2006) inclusion of "clutch overlap", or the simultaneous investment in two or more clutches (only one of which, in this case, including fertilized eggs), as evidence of superfetation. The distinction is that they scored eggs with no embryonic development as being a separate litter. The definition of superfetation is that there must be more than one litter of young in discretely different stages of development present at the same time. The presence of eggs without embryonic development is known from many species that lack superfetation; they represent the provisioning of the subsequent litter and will not be fertilized until after the birth of the current litter of embryos. The occurrence of extensive placentotrophy without superfetation, which occurs in most *Pamphorichthys* species, is unique among the poeciliid species that have been investigated.

Ancestral state reconstructions support the hypothesis that superfetation arose independently within *P.* (*Pamphorichthys*) *hasemani* and in the common ancestor of *P.* (*Micropoecilia*) *branneri*, *P.* (*M.*) *bifurca*, and *P.* (*M.*) *parae*. Furthermore, our results suggest that placentotrophy in *Poecilia* (*Pamphorichthys*) arose prior to the acquisition of superfetation in this subgenus. By contrast, Reznick et al. (2002) found that superfetation arose prior to placentotrophy in *Poeciliopsis*, and Trexler and DeAngelis' (in press) simulation study suggested that the presence of superfetation facilitates the evolution of placentotrophy.

Given that there are now known occurrences of poeciliids that are lecithotrophic and non-superfetatious, lecithotrophic and superfetatious, placentotrophic and non-superfetatious, and placentotrophic and superfetatious, it is clear that there is some capacity for independent evolution of these traits. At the same time, statistical tests with SIMMAP provide support for a significant association between placentotrophy and superfetation in Poeciliidae. This result agrees with Trexler and DeAngelis' (in press) simulation studies.

Molecular dates in Fig. 2 provide a framework for estimating maximum time spans for the evolution of life-history characters. Placentotrophy evolved on the common ancestral branch for *P.* (*Pamphorichthys*), which has a duration of ~10 million years and extends from ~21 mya to ~11 mya (Fig. 2). Superfetation has evolved on the branch leading to *P.* (*Pamphorichthys*) *hasemani*, which extends from ~9 mya to the present. Within *Poecilia* (*Micropoecilia*) both placentotrophy and superfetation evolved on the branch leading from the common ancestor of *Micropoecilia* to the common ancestor of *P.* (*Micropoecilia*) *branneri* + *P.* (*M.*) *bifurca* + *P.* (*M.*) *parae*. This branch has a duration of ~4 million years and extends from ~18 mya to ~14 mya. Meredith et al. (2010) suggested a maximum time interval of ~3–4 million years for the evolution of placentotrophy in the common ancestor of *P.* (*Micropoecilia*) *branneri*, *P.* (*M.*) *bifurca*, and *P.* (*M.*) *parae*. Within *Poeciliopsis*, placentotrophy evolved on branches with shorter durations (2.36 and 0.75 million years; Reznick et al., 2002) than in *Poecilia*. In every case placentotrophy and/or superfetation may have evolved over a shorter time window, but taxonomic sampling limits our ability to refine these estimates. A shortcoming of our divergence time estimates is that they are based on a single secondary calibration point from Hrbek et al. (2007). Hrbek et al. (2007), in turn, employed a partially overlapping set of molecular markers, different taxon sampling, different relaxed clock methods, and secondary calibration points from Reznick et al. (2002).

4.3. Taxonomic recommendations

A contentious issue in poeciliid systematics is whether or not to treat *Poecilia* as a genus with multiple subgenera (e.g., Rosen and Bailey, 1963; Meredith et al., 2010) or elevate each subgenus to the rank of genus (e.g., Hubbs, 1924, 1926; Lucinda and Reis, 2005). Meredith et al. (2010) recommended a single genus with multiple subgenera to maintain nomenclatorial stability for the common guppy, *Poecilia reticulata*, given its model organism status in scientific studies. Meredith et al. (2010) noted that if *Poecilia* subgenera are instead recognized as genera, *P. reticulata* would then be placed in the genus *Acanthophacelus* given that the type species of *Poecilia* is *P. vivipara,* which represents a separate and distinct lineage (Breden et al., 1999; Hamilton, 2001). Meredith et al.'s (2010) taxon sampling lacked *P. vivipara*, but the inclusion of this species in the present study confirms that *P. vivipara* is a distinct lineage, and that *P. reticulata* can only be maintained in the genus *Poecilia* if we follow Rosen and Bailey's (1963) approach and recognize a single genus with multiple subgenera. Whereas Rosen and Bailey (1963) recognized four subgenera in *Poecilia* (*Poecilia*, *Lebistes*, *Pamphorichthys*, *Limia*), we endorse a classification that recognizes seven subgenera (*Poecilia*, *Pamphorichthys*, *Limia*, *Pseudolimia*, *Mollienesia*, *Acanthophacelus*, *Micropoecilia*) in this genus based on our phylogenetic results. *P.* (*Pseudolimia*) *heterandria* could be subsumed within in the subgenus *Limia* based on its sister-group relationship to this subgenus, but *P.* (*Pseudolimia*)

heterandria's gonopodial features and pigmentation pattern are distinct and separate from *Limia* and other subgenera in *Poecilia* (Poeser, 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.01.014.

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