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HISTORICAL DEMOGRAPHY, SELECTION, AND COALESCENCE OF MITOCHONDRIAL AND NUCLEAR GENES IN *PROCHILODUS* SPECIES OF NORTHERN SOUTH AMERICA

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Abstract.—Fishes of the genus *Prochilodus* are ecologically and commercially important, ubiquitous constituents of large river biota in South America. Recent ecologic and demographic studies indicate that these fishes exist in large, stable populations with adult census numbers exceeding one million individuals. Abundance data present a stark contrast to very low levels of genetic diversity (Θ) and small effective population sizes (N_e) observed in a mitochondrial (mt) DNA dataset obtained for two species, *Prochilodus mariae*, and its putative sister taxon, *Prochilodus rubrotaeniatus*. Both species occupy major river drainages (Orinoco, Essequibo, and Negro) of northeastern South America. Disparity between expectations based on current abundance and life history information and observed genetic data in these lineages could result from historical demographic bottlenecks, or alternatively, natural selection (i.e., a mtDNA selective sweep). To ascertain underlying processes that affect mtDNA diversity in these species we compared Θ and N_e estimates obtained from two, unlinked nuclear loci (calmodulin intron-4 and elongation factor-1α intron-6) using an approach based on coalescent theory. Genetic diversity and N_e estimated from mtDNA and nuclear sequences were uniformly low in *P. rubrotaeniatus* from the Rio Negro, suggesting that this population has encountered a historical bottleneck. For all *P. mariae* populations, Θ and N_e based on nuclear sequences were comparable to expectations based on current adult census numbers and were significantly greater than mtDNA estimates, suggesting that a selective mtDNA sweep has occurred in this species. Comparative genetic analysis indicates that a suite of evolutionary processes involving historical demography and natural selection have influenced patterns of genetic variation and speciation in this important Neotropical fish group.

Key words.—Calmodulin, effective population size, elongation factor-1, intron, LAMARC, purifying selection.

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The estimated freshwater ichthyofaunal diversity in the Neotropics is approximately 8000 species (Schaefer 1998) and is nearly 28% of global fish diversity. This spectacular ichthyofaunal diversity has been shaped by various geomorphologic and demographic events over the last 90–115 million years (Lundberg et al. 1998). As systematists study Neotropical ichthyofauna, they are confronted with complex sets of processes that may have shaped fish species distributions (Vari and Malabarba 1998; Schaefer 1991, 1997). Using morphological and molecular data, Neotropical ichthyologists have recognized vicariance speciation as a primary factor in the diversification of Neotropical fishes (Vari and Weitzman 1990; Schaefer 1997; Lovejoy and de Araújo 2000; Lovejoy and Collette 2001; Sivasundar et al. 2001; Montoya-Burgos 2003). However, few studies have explored how past demographic processes have shaped present Neotropical fish distributions. One such study by Turner et al. (2004) concluded that demography and potentially natural selection have played critical roles in the diversification of two prochilodontid fishes of northeastern South America.

Prochilodontids are abundant, large-bodied, Neotropical freshwater fishes that constitute 50–60% of the fish biomass in many Neotropical rivers (Taphorn 1992). Because of their great abundances, these fishes are dominant constituents of many Neotropical fish assemblages and important components of commercial and subsistence fisheries in the Neotropics (Welcomme 1990; Barbarino-Duque et al. 1998). As detritivores, they also play a fundamental role in the transfer of energy and nutrients throughout the ecosystem (Flecker 1996). All *Prochilodus* species undergo mass (100–1000 km) cyclical migrations where juveniles and adults move from dry-season habitats to wet-season spawning and foraging grounds (Goulding 1988).

Our study focuses on two prochilodontid species, *Prochilodus mariae* Eigenmann, 1922 and *P. rubrotaeniatus* Jardine and Schomburgk, 1841. These fishes have broad, but disjunct distributions in northern South America east of the Andes Mountains, with *P. mariae* endemic to white-water rivers of the Orinoco Basin and *P. rubrotaeniatus* inhabiting clear- and black-water rivers of Orinoco, Essequibo, and upper Rio Negro Basins (see Fig. 1). The migratory life history and large census size (e.g., *P. mariae* $\sim 7.76 \times 10^6$, Barbarino-Duque et al. 1998) of each *Prochilodus* species provide ample opportunity for dispersal and gene flow (Sivasundar et al. 2001, Turner et al. 2004); thus, large panmictic populations should translate to large effective population size(s) (N_e; Wright 1931).

Turner et al. (2004) surveyed mitochondrial (mt) DNA nucleotide diversity for populations of *Prochilodus mariae*

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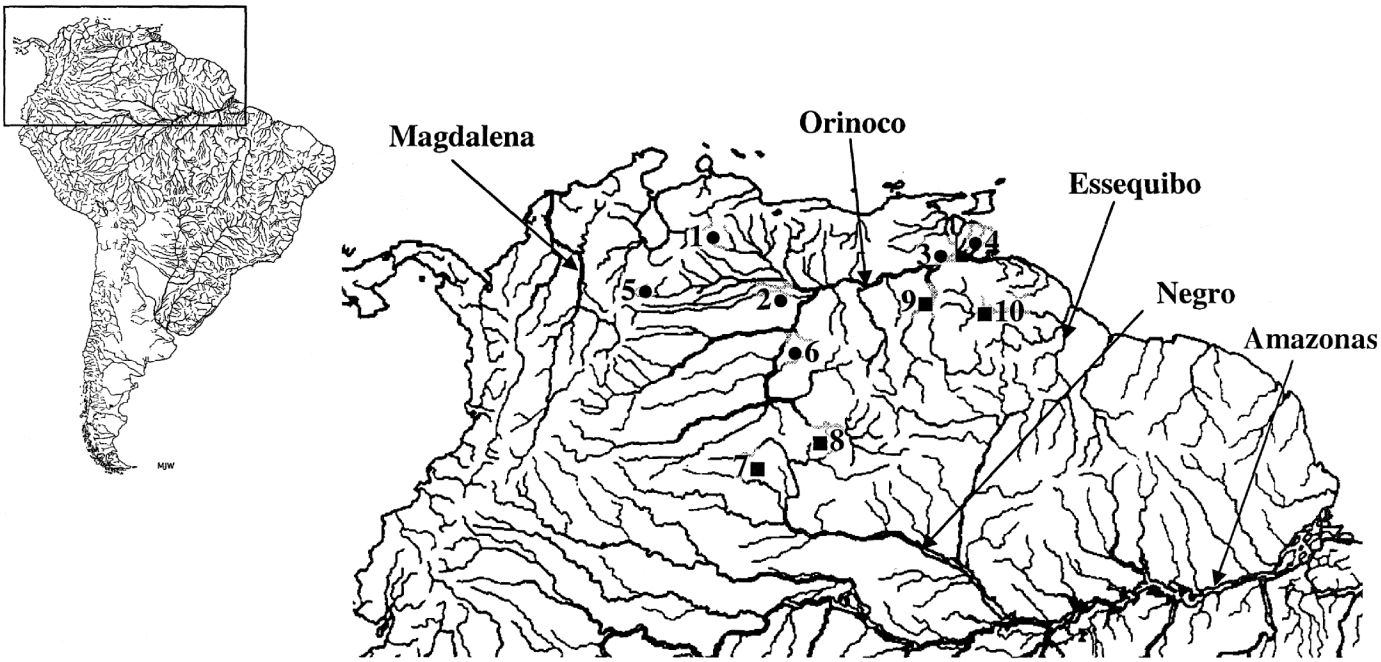

FIG. 1. Geographic distribution of *Prochilodus rubrotaeniatus* and *P. mariae* specimens examined. See Table 1 for exact coordinates. Numeric codes indicate survey locations as follows: 1, Portuguesa; 2, Chirere; 3, Barrancas; 4, Tucupita; 5, Caparo; 6, Ayacucho; 7, Casiquiare; 8, Siapa; 9, Paragua; and 10, Cuyuni. Populations of *P. mariae* and *P. rubrotaeniatus* are distinguished by closed circles and squares, respectively.

and *P. rubrotaeniatus* throughout northern South America (see Table 1, Fig. 1). In contrast to predictions based on abundance data, very low levels of genetic diversity were observed in *P. rubrotaeniatus* and *P. mariae* mtDNA datasets (see Table 2). For example, *P. rubrotaeniatus* Rio Negro localities (Casiquiare and Siapa) are fixed for a common mtDNA haplotype, and five of six *P. mariae* localities surveyed from the Orinoco Basin are fixed for a common mtDNA haplotype. These findings also contrast Sivasundar et al. (2001) who reported high values of mtDNA diversity in *P. lineatus* from the Parana basin. Turner et al. (2004) concluded that past demographic events best explained the paucity of observed variation in the Rio Negro populations of *P. rubrotaeniatus*. Yet, due to a significant result for Fu and Li's (1993) test of neutrality and the observation of two rare haplotypes with single, nonsynonymous substitutions, Turner et al. (2004) could not eliminate purifying selection on the mtDNA as a possible explanation for the observed paucity of genetic diversity in *P. mariae* populations.

In this paper, we assess the findings of Turner et al. (2004) using two nuclear loci that are unlinked to the mtDNA genome. If a lineage has encountered a genetic bottleneck, then mtDNA and nuclear loci should exhibit similarly low N_e estimates. In contrast, if purifying selection is acting on the mtDNA and nuclear loci are evolving in a near neutral fashion, then estimates of N_e at mtDNA loci should be significantly lower than estimates based on nuclear loci. Furthermore, nuclear loci should not differ significantly in levels of genetic diversity or N_e provided they evolve at similar rates.

MATERIALS AND METHODS

Taxon Sampling

Specimens, including voucher information, for this study are referenced in Table 1. Samples of *P. mariae* ($n = 55$) were collected from six localities in the Orinoco River (see Fig. 1), and *P. rubrotaeniatus* ($n = 42$) from four localities representing three major river basins the Essequibo, Orinoco, and Amazonas (see Table 1, Fig. 1). We also included *P. magdalenae* ($n = 3$) as an outgroup for phylogenetic comparisons and to provide estimates of mutation rates for each locus. DNA was extracted from EtOH preserved tissue using standard organic extraction procedures (Sambrook et al. 1989).

Characterization of Genetic Diversity

We used single-strand conformational polymorphism (SSCP) to characterize *Prochilodus* genetic diversity. We targeted two unlinked nuclear loci, calmodulin intron-4 (*Cal-4*) and elongation factor-1α intron-6 (*EF1α-6*). *Cal-4* was amplified via polymerase chain reaction (PCR) using forward calmex4f (5′-CCCAGAAAGATGAAGGACAC-3′) and reverse calmex5r (5′-CCCCAGGTTTGTCATCACAT-3′) primers (modified from Chow 1998) and *EF1α-6* using forward EF16cF (5′-GGAAACKTGGCTGGAGAC-3′) and reverse EF17bR (5′-AATGGCAGCATCTCCAGACT-3′) primers (based on sequences of Moyer et al. 2004). Each forward primer (0.03mM) was end-labeled with γ-^{33}P using T4 kinase in 40 μL volumes containing 1U T4 Polynucleotide Kinase (Bioline), 4.0 μL

TABLE 1. Species, sample sizes (*n*), and sampling localities of fishes surveyed for variation in *Cal-4* and *EF1α-6* loci. Asterisk indicates location not verified with GPS. Numbers in parentheses refer to each population's approximate position in Figure 1. See table I from Turner et al. (2004) for voucher information.

Species	*n*	Locality	Latitude, longitude	Drainage
Prochilodus mariae	10	Rio Portuguesa, VZ	09°07.50′N, 67°45.93′W*	Orinoco (1)
	10	Rio Chirere, VZ	07°47.11′N, 67°13.87′W	Orinoco (2)
	10	Barrancas, VZ	08°39.38′N, 62°12.19′W*	Orinoco (3)
	10	Tucupita, VZ	09°05.63′N, 62°04.68′W*	Orinoco (4)
	05	Rio Caparo, VZ	07°36.92′N, 71°29.75′W	Orinoco (5)
	10	Puerto Ayacucho, VZ	05°39.70′N, 67°37.68′W*	Orinoco (6)
P. rubrotaeniatus	11	Rio Casiquiare, VZ	01°57.73′N, 67°06.30′W	Amazonas (R. Negro) (7)
	05	Rio Siapa, VZ	02°04.69′N, 66°08.44′W*	Amazonas (R. Negro) (8)
	10	Rio Paragua, VZ	06°49.86′N, 63°19.80′W	Orinoco (9)
	14	Rio Cuyuni, VZ	06°42.93′N, 61°36.49′W	Essequibo (10)
P. magdalenae	04	Rio Magdalena, COL	10°43.78′N, 73°23.33′W*	Magdalena

10X T4 polynucleotide kinase reaction buffer (Bioline), and 0.12 mM [γ-^{33}P]ATP (sp. act. 3000 Ci/mmole; 10 μCi/μL). Kinase reactions incubated at 37°C for 30 min followed by 120 sec at 95°C. Single-strand conformational polymorphism PCR procedures were performed in 10 μL reactions as follows: 1.0 μL 10X PCR Buffer (Promega, Madison, WI), 2.0 mM $MgCl_2$, 0.2 mM each of dNTP, 1.0 μM reverse primer, 0.35 μL of labeled forward primer, 0.3 μM unlabeled forward primer, and 0.375 U *Taq* polymerase (Promega). Polymerase chain reaction conditions were an initial denaturation at 94°C, followed by 25 cycles of 94°C, 50°C for *Cal-4* or 56°C for *EF1α-6* for 30 sec, and 72°C for 30 sec. Completed reactions were diluted in loading buffer (1X TE, glycerol, bromophenol blue) to a final volume of 20 μL. Samples were denatured at 94°C for 5 min, and immediately quenched in ice slurry to prevent renaturation of double-stranded DNA. Cold samples were loaded into a 5% nondenaturing polyacrylamide (37.5:1 acrylamide:bis-acrylimade) gel with 5% glycerol. Electrophoresis was conducted on a sequencing gel apparatus at 10 W for 19 hr at room temperature. Fragments were visualized by autoradiography.

Allelic designations for each *Cal-4* and *EF1α-6* allele were confirmed by cloning and sequencing all observed allelic variants from each SSCP gel. We adhere to the human nomenclature committee's criteria for defining alleles, which state that there must be at least three identical sequenced clones, identified in either two separate PCRs from the same individual or from PCRs of at least two different individuals (Marsh et al. 2001). Cloning individual PCR products and sequencing representative clones followed the protocol of Moyer et al. (2004). We established codon positions for translation of *EF1α-6* and *Cal-4* exons using alignments from previous studies (Langenau et al. 1999, Moyer et al. 2004).

TABLE 2. Distribution of composite mtDNA haplotypes redrawn from Turner et al. (2004). The abbreviation *n* refers to the total number of individuals surveyed. Numbers in parentheses correspond to each population's approximate position in Figure 1. Haplotypes 2 and 3 result from single, nonsynonymous mutations in the *ND4* region.

		Haplotype designation								
Species/population	*n*	1	2	3	4	5	6	7	8	9
Prochilodus mariae										
Portuguesa (1)	10	10								
Chirere (2)	10	8	1	1						
Barrancas (3)	10	10								
Tucupita (4)	10	10								
Caparo (5)	5	5								
Ayacucho (6)	10	10								
P. rubrotaeniatus										
Casiquiare (7)	11				11					
Siapa (8)	5				5					
Paragua (9)	10							7	2	1
Cuyuni (10)	14					1	13			

Estimation of Gene Genealogies and Population Parameters

Gene genealogies for each locus were constructed using statistical parsimony (Templeton et al. 1992) as implemented by TCS version 1.13 (Clement et al. 2000). We tested for among-lineage mutational rate variation by comparing likelihoods of trees generated with and without enforcement of a molecular clock. Model choice and parameter estimates for phylogenetic reconstruction were obtained using hierarchical likelihood ratio tests (LRT) implemented with MODELTEST version 3.06 (Posada and Crandall 1998). Once a model and associated parameter-values were identified, we employed the following ML heuristic search strategy implemented using PAUP* version 4.0b10 (Swofford 1998): tree bisection reconstruction branch swapping on 100 random addition replicates with the MULTREES option in effect.

Nei (1987) showed that the expected value of the net number of nucleotide substitutions (d_A) between populations is:

$$d_A = 2\mu T, \tag{1}$$

where μ is the persite mutation rate and T is the time to most recent common ancestor (MRCA) between two populations. We estimated d_A ($\hat{d}_A$), correcting for ancestral polymorphism, using the equation

$$\hat{d}_A = \hat{d}_{XY} - \frac{\hat{d}_X + \hat{d}_Y}{2} \tag{2}$$

(Nei 1987), where $\hat{d}_{XY}$ is the number of nucleotide substitutions between lineages X and Y, and $\hat{d}_X$ and $\hat{d}_Y$ are the average number of nucleotide substitutions within lineage X and Y, respectively. To calculate each parameter, we first estimated ML branch lengths (for each locus) using the ''describe trees'' feature in PAUP*. Because we were interested

in $\hat{d}_A$ between *P. magdalanae* and *P. mariae* + *P. rubrotaeniatus* clades, $\hat{d}_{XY}$ was the ML branch length separating these clades, and $\hat{d}_X$ and $\hat{d}_Y$ was the average branch length estimate for alleles within *P. magdalanae* and *P. mariae* + *P. rubrotaeniatus* clades, respectively.

We therefore estimated μ by substituting $\hat{d}_A$ and time to MRCA (10 mya; Lundberg et al. 1998) into the equation $d_A = 2\mu T$. The estimated time to MRCA is based on the well documented (Vari 1988; Schaefer 1997) separation of the Magdalena and Orinoco ichthyofauna after the final rise of the Eastern Cordillera 10 mya (Lundberg et al. 1998).

To characterize intraspecific genetic diversity and divergence patterns, the parameter Θ ($\Theta_{\text{autosomal}} = 4N_e\mu$ and $\Theta_{\text{mtDNA}} = N_e\mu$) was estimated using a ML approach based on coalescent theory (Kingman 1982) as implemented by LAMARC version 1.2.2 (Kuhner et al. 1995; Beerli and Felsenstein 2001). LAMARC simultaneously estimates Θ, population growth, migration, and recombination parameters. We initially considered *P. mariae* localities as separate populations; however, LAMARC migration matrices indicated that localities comprise a larger panmictic population (as expected from ecological and life history data). Subsequently, we combined *P. mariae* localities and reevaluated Θ. Localities surveyed for *P. rubrotaeniatus* correspond to three different drainages as follows: Negro (Rio Casiquiare and Rio Siapa), Essiquibo (Rio Cuyuni), and Orinoco (Rio Paragua). The Orinoco (Rio Paragua) population of *P. rubrotaeniatus* is restricted to the Rio Caroní Basin (*P. mariae* does not occur in the Caroní system) (Turner et al. 2004).

LAMARC searches were initiated for each locus and drainage (each drainage was run independently) with a starting value of Θ based on Waterson's (1975) estimate. All analyses used the F84 model (Felsenstein 1989) of sequence evolution with empirical base frequencies and transistion/transversion ratios. Default values were used for initial growth and recombination parameters. Migration matrices were not estimated for runs because we assume no migration occurs among basins. The Metropolis Monte Carlo sampling technique (Metropolis et al. 1953) implemented by LAMARC is starting point dependent; to check for more than one local optimum, two separate analyses were performed for each population and results compared. Runs used the following search strategy for each replicate: 20 short chains with 2500 steps and 10 long chains with 25,000 steps (initial 1000 genealogies were discarded/chain). We reevaluated the mtDNA *ND4* data (Turner et al. 2004) using the same approach; however, recombination was not estimated. Once LAMARC generated an estimate of Θ per population, we determined N_e for each population by substituting the parameters Θ and μ into the equation $N_e = \Theta/4\mu$ for autosomal loci and $N_e = \Theta/\mu$ for the mtDNA locus.

Effective Population Size and Nucleotide Diversity Comparisons

MtDNA has a lower expected N_e than autosomal genes (Avise 2000), thus fewer mtDNA polymorphisms are expected simply due to shorter coalescent times. To address this point, we first tested the null hypothesis that the observed and expected mtDNA Θ ($\Theta_{\text{mtDNA}} = N_e\mu$) estimates for each *Prochilodus* population are equal, using *Cal-4* and *EF1α-6* estimates of N_e. The expected mtDNA value of Θ is

$$E(\hat{\Theta}) = \frac{M}{1 + (4/3)M} \tag{3}$$

(Nei 1987) where $M = N_e\mu$. The parameter M was calculated by multiplying each autosomal estimate of N_e (obtained from Table 5) by the estimated mtDNA μ of 0.52×10^{-8} (Turner et al. 2004). In doing so, we provide an expected mtDNA estimate of Θ for each population conditioned on the autosomal estimate of N_e for that population.

The estimated error variance of $E(\hat{\Theta})$ is

$$V(\hat{\Theta}) = \frac{n + 1}{3(n - 1)m_T}M + \frac{2(n^2 + n + 3)}{9n(n - 1)}(M)^2 \tag{4}$$

(Nei 1987) where n is the number of sequences examined (see *ND4* data in Table 2) and m_T is the number of nucleotides examined (m_T = 264bp fragment of *ND4*). Knowing the variance allows us to calculate the standard error (SE) of the estimate ($\text{SE} = V(\hat{\Theta})^{1/2}$) for the mtDNA $E(\hat{\Theta})$.

In addition to the above approach, we also evaluated whether confidence intervals (CI) produced by LAMARC for mtDNA and autosomal N_e estimates overlap. If the mtDNA and autosomal loci are evolving in a near neutral fashion, then our null hypothesis is that autosomal and mtDNA N_e estimates should be equal.

RESULTS

Molecular Dynamics

The *EF1α-6* alignment (333 bp) consists of partial sequences of exon 6 (49 bp) and 7 (175 bp), and complete intron 6 (109 bp). The position of intron 6 within the codon is phase 0 (Li 1997; Moyer et al. 2004) and flanked by symmetrical (i.e., the exon is flanked by introns of the same phase) exons 6 and 7. Translation of exon sequence reveals a conserved reading frame of 74 amino acids. There are two insertion-deletion (indel) events associated with intron 6 at positions 54–55 and 93–95 in the alignment.

The partial *Cal-4* alignment (209 bp) includes complete intron 4 (87 bp) (phase 0) flanked by partial sequences of symmetrical exons 4 (65 bp) and 5 (57 bp). A conserved reading frame of 40 amino acids is apparent after translation. We observed one indel in intron 4 at position 98 in the alignment.

Estimation of Gene Genealogies and Population Parameters

Using SSCP, we successfully amplified and screened the *Cal-4* locus for 39 *P. rubrotaeniatus* and 51 *P. mariae* samples (see Table 3). Eight of 18 *Cal-4* alleles are found in surveyed *P. rubrotaeniatus* populations; the Rio Paragua population has the most diversity (six alleles), and the Rio Casiquiare and Rio Siapa populations are fixed for allele 8. In comparison, *P. mariae* populations have a greater number of *Cal-4* alleles (15 alleles) and share five alleles (alleles 1, 2, 3, 5, and 7) with *P. rubrotaeniatus*. We successfully amplified and screened the *EF1α-6* locus for 40 *P. rubrotaeniatus* and 54 *P. mariae* samples (see Table 4). Eleven of 31 *EF1α-6* alleles are found in surveyed *P. rubrotaeniatus* populations, 24 in

TABLE 3. Distribution of *Cal-4* alleles among *Prochilodus mariae* and *P. rubrotaeniatus* populations. The abbreviation *n* refers to the total number of individuals surveyed. Numbers in parentheses correspond to each population's approximate position in Figure 1.

		Allele designation																	
Species/population	*n*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
P. mariae																			
Portuguesa (1)	10	6	3			3		2		6									
Chirere (2)	10		7			4				1	1	6						1	
Barrancas (3)	10	9				6		1										3	1
Tucupita (4)	8	7	1	1		2				2						1	2		
Caparo (5)	4	2	2			4													
Ayacucho (6)	9	7	1			2		1		2		1	1	2	1				
P. rubrotaeniatus																			
Casiquiare (7)	11								22										
Siapa (8)	5								10										
Paragua (9)	10	7	2	3	2	5	1												
Cuyuni (10)	13					24		2											
Total	90	38	16	4	2	50	1	6	32	11	1	7	1	2	1	1	2	4	1

P. mariae populations, and four (1, 2, 4, and 14) shared between species.

The number of polymorphic sites for *Cal-4* is 18 of 209 sites (8.6%). Similarly, the estimate of variable sites for *EF1α-6* is 31 of 333 sites (9.3%). The *Cal-4* gene geneology (see Fig. 2) supports a *P. mariae* + *P. rubrotaeniatus* clade, but fails to support the monophyly of either *P. mariae* or *P. rubrotaeniatus*. Likewise, the *EF1α-6* gene network (see Fig. 3) recovers a paraphyletic *P. mariae*, *P. rubrotaeniatus*, and *P. magdalenae*. Among populations of *P. mariae* and *P. rubrotaeniatus*, there is no apparent relationship among *Cal-4* or *EF1α-6* alleles and rivers surveyed (see Figs. 2 and 3, Tables 3 and 4).

Likelihood ratio tests chose the JC + Γ + I ($\alpha = 0.7291$, $I = 0.7333$) and K80 + Γ ($\alpha = 0.1303$; Tratio = 2.0960) models of sequence evolution for *Cal-4* and *EF1α-6*, respectively. The estimated −lnL values for constrained (i.e., molecular clock enforced) and unconstrained *Cal-4* ML topologies are 501 and 482, respectively. The LRT statistic, which is twice the difference in −lnL values, is 38 with 26 degrees of freedom, and the critical value ($\chi_{0.05,26}$) is 38.89. The LRT fails to reject the null hypothesis of *Cal-4* rate homogeneity among *Prochilodus* lineages. Evaluation of corrected branch lengths based on the *Cal-4* ML tree (JC + Γ + I model) indicates *P. magdalanae* is 0.49 % divergent from a *P. mariae* + *P. rubrotaeniatus* clade. Assuming that speciation between these two clades occurred roughly 10 mya (i.e., the separation of the Magdalena and Orinoco basins after the final rise of the Eastern Cordillera [Lundberg et al. 1998]), then nucleotide divergences between these clades imply that $\mu \approx 0.25 \times 10^{-9}$ substitutions/site for *Cal-4*. This rate is similar to estimates obtained from introns of select vertebrates (0.74×10^{-9}; Yu et al. 2001). As shown in Figure 3, the divergences of *EF1α-6* alleles predate the *P. magdalanae* and *P. rubrotaeniatus* + *P. mariae* divergence. Consequently, we could not calculate μ from the *EF1α-6* ML topology.

Estimates of Θ for each population are summarized in Table 5 (no comparable differences were observed between independent LAMARC runs). Population estimates of Θ are similar between nuclear and mtDNA loci (i.e., confidence intervals overlap), except for the *P. mariae* population. The autosomal Θ estimate for the *P. mariae* population is significantly greater than values obtained from mtDNA data.

Knowing μ and Θ, we solved for N_e ($N_{e\ \text{autosomal}} = \Theta/4\mu$, $N_{e\ \text{mtDNA}} = \Theta/\mu$) for each locality. Unable to calculate μ for the *EF1α-6* dataset, we used the *Cal-4* value of μ for calculating N_e based on *EF1α-6* sequence data. The ranges in estimated N_e values for the three loci are as follows: *Cal-4*, $3.3 \times 10^6 - 3.1 \times 10^7$; *EF1α-6*, $1.4 \times 10^6 - 7.8 \times 10^7$; and *ND-4*, $3.1 \times 10^5 - 9.4 \times 10^5$ (see Table 5). Long-term N_e for *Cal-4* and *ND4* was unattainable for the *P. rubrotaeniatus* Rio Negro (Siapo + Casiquiare) locality that was monomorphic.

Effective Population Size and Nucleotide Diversity Comparisons

The null hypothesis that observed and expected mtDNA Θ estimates are equal (given the nuclear estimate of N_e) is rejected in the *P. mariae* population (see Table 6); the observed mtDNA Θ values are significantly less than expectations. Furthermore, the LAMARC estimate of mtDNA N_e for *P. mariae* is significantly (i.e., 99.5% CI values do not overlap) less than *Cal-4* and *EF1α-6* N_e estimates (see Table 5). MtDNA N_e is lower than observed for nuclear genes despite higher estimated μ for mtDNA *ND4* in these prochilodontid fishes ($\mu = 0.52 \times 10^{-8}$; Turner et al. 2004).

Observed and expected *P. rubrotaeniatus* mtDNA Θ estimates are not significantly different (i.e., 99.5% CI values overlap), and LAMARC mtDNA N_e for *P. rubrotaeniatus* populations are not significantly (i.e., 99.5% CI values overlap) less than *Cal-4* and *EF1α-6* N_e estimates (see Table 5).

DISCUSSION

Phylogeography of Prochilodus spp.

Our *Cal-4* and *EF1α-6* gene genealogies do not resolve relationships among *Prochilodus* species examined. With the retention of alleles among *P. mariae* and *P. rubrotaeniatus* and the outgroup, this result is not surprising. There is an expected fourfold difference in coalescent times between neu-

TABLE 4. Distribution of *EF1α-6* alleles among *Prochilodus mariae* and *P. rubrotaeniatus* populations. The abbreviation *n* refers to the total number of individuals surveyed. Numbers in parentheses correspond to each population's approximate position in Figure 1.

Species/ population	*n*	Allele designation																												
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
P. mariae																																
Portuguesa (1)	10	2	5	1	6	3	1	1	1																							
Chirere (2)	10	6	1		5		2			1	1	2	1	1																		
Barrancas (3)	10	4	4	4	3					1					4																	
Tucupita (4)	10	6	4	1	3							1			2				1		1	1										
Caparo (5)	4	2	1												1								1	1	1	1						
Ayacucho (6)	10		1	1	6		1		2						4		1	1	1	2												
P. rubrotaeniatus																																
Casiquiare (7)	11														22																	
Siapo (8)	7														12																	2
Paragua (9)	8	7													5												1	3				
Cuyuni (10)	14		2		1										10	2													8	3	2	
Total	94	27	18	7	24	3	4	1	3	2	1	3	1	1	60	2	1	1	2	2	1	1	1	1	1	1	1	3	8	3	2	2

trally evolving diploid autosomal and haploid mtDNA genes (Kingman 1982). This difference translates into a fourfold decrease in the rate of lineage sorting for autosomal genes (Avise 2000). Therefore, based on the magnitude of N_e for *P. rubrotaeniatus* and *P. mariae* populations and the slower mutation rate for each autosomal gene, we would expect the maintenance of ancestral polymorphisms. The end result of this continuance is *P. mariae* and *P. rubrotaeniatus* autosomal genes have not attained reciprocal monophyly.

Turner et al. (2004) observed a paraphyletic *P. rubrotaeniatus* (i.e., *P. rubrotaeniatus* from the Orinoco is sister to *P. mariae*, see Fig. 4). Although the maintenance of ancestral polymorphisms in each autosomal network precludes us from evaluating the monophyly of each species, two explanations exist for the observed paraphyly: mtDNA introgression from *P. rubrotaeniatus* to *P. mariae*, or *P. mariae* shared a recent common ancestry with *P. rubrotaeniatus* in the Orinoco Basin. Based on our data, we can not eliminate either of these scenarios. However, *P. rubrotaeniatus* (Rio Paragua, Orinoco Basin) has allele and haplotype differences that are qualitatively different from other *P. rubrotaeniatus* populations. The allelic and haplotypic distributions of *P. rubrotaeniatus* (Orinoco) and the observed affinities of *P. rubrotaeniatus* (Orinoco) and *P. mariae* haplotypes suggest *P. rubrotaeniatus* from the Orinoco may be a separate species. Further morphological and molecular studies are necessary before confirmation of this conclusion.

Retention of ancestral polymorphisms also indicates that the MRCA of *P. magdalenae*, *P. mariae*, and *P. rubrotaeniatus* maintained a large ancestral effective population size. Theory predicts that the expected coalescent time for autosomal diploid gene copies is on average $4N_e$ generations (Kingman 1982). The time of MRCA for *P. magdalenae* and *P. mariae* + *P. rubrotaeniatus* is ~10 mya, which suggest the effective size of the ancestral population was $> 2.5 \times 10^6$ and is consistent with our empirical findings. Assuming that the population size of these *Prochilodus* species has remained constant through time, why then do the mtDNA data of Turner et al. (2004) suggest relatively small N_e estimates for *P. mariae* populations? Studying historical demographics of these populations with independent nuclear loci in a coalescent framework provide insight into this question.

Genetic Diversity in P. rubrotaeniatus

Turner et al. (2004) observed a single mtDNA haplotype in *P. rubrotaeniatus* samples ($n = 16$) surveyed from the Rio Negro Basin (i.e., the Casiquiare and Siapa populations) (see Table 2). To explain the distribution of genetic variation within this population, they proposed that either a demographic bottleneck resulting from a recent founding event, or purifying selection lowered the observed genetic diversity. We found a pattern of genetic variation in the Rio Negro population of *P. rubrotaeniatus* consistent with the mtDNA data: autosomal loci are nearly monomorphic for these populations, and observed and expected values of mtDNA Θ are not significantly different. These findings indicate that the distribution of haplotypes and alleles for *P. rubrotaeniatus* in the Rio Negro are a result of a past bottleneck in genetic variation presumably from a founder event. Although Turner et al.

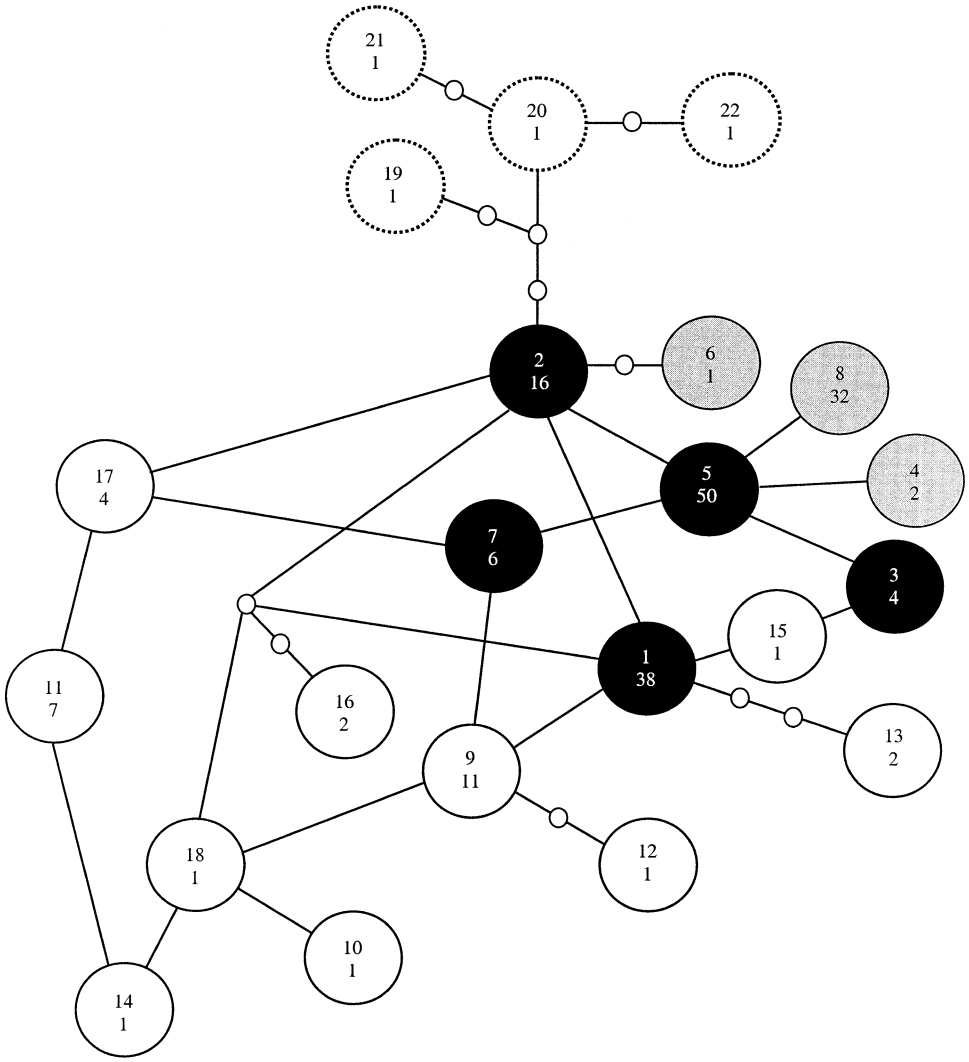

FIG. 2. Statistical parsimony network of *Prochilodus Cal-4* alleles. The top number inside each circle refers to the allelic designation (see Table 3) and the bottom number represents the number of surveyed individuals sharing the respective allele. Colored circles represent the following: black, alleles shared between species (see Table 3 for relative allele frequency in each species); white with solid line, alleles common only to *P. mariae*; gray, alleles common only to *P. rubrotaeniatus*; and white with dashed line, alleles surveyed from *P. magdalanae*. Smaller circles along branches designate missing intermediate alleles.

(2004) and Lovejoy and Araujo (2000) have proposed a relatively recent connection between the Orinoco and Amazonas through the Essequibo Basin, our findings are inconclusive because no association of alleles exists among drainages).

Genetic Diversity in P. mariae

Turner et al. (2004) observed that mtDNA *ND4* and *COI* regions were monomorphic for *P. mariae* ($n = 53$), except in one population, which exhibited two rare haplotypes in two individuals (see Table 2). Paucity of genetic variation in these regions translates to low levels of nucleotide diversity and estimates of N_e. Turner et al. (2004) indicated that the lowered observed genetic diversity within *P. mariae* populations resulted from either a demographic bottleneck or purifying selection. If a bottleneck occurred, we would expect mtDNA and autosomal estimates of N_e to be similar. Based on our finding we can eliminate the possibility of a bottleneck in genetic diversity because mtDNA and autosomal N_e estimates are statistically different. Numerous explanations exist for the observed lower values of nucleotide diversity between mtDNA and the nuclear loci and include population structure in mtDNA but not nuclear loci (caused by reduced movement of females relative to males), among-lineage rate variation, skewed sex ratios, recombination, increased mtDNA coalescent times, and natural selection.

Unaccounted population structure in autosomal or mtDNA loci tends to underestimate values of Θ (Knowles and Maddison 2002); however, no appreciable genetic substructure exists in *P. mariae* populations surveyed (a common mtDNA haplotype is shared in high frequencies across six sampling localities in the Orinoco Basin). Second, the LRT of *Cal-4* ML topologies indicated conformity to a molecular clock, suggesting that there is no among-lineage rate variation. Third, if our nuclear estimates of N_e are accurate (see below),

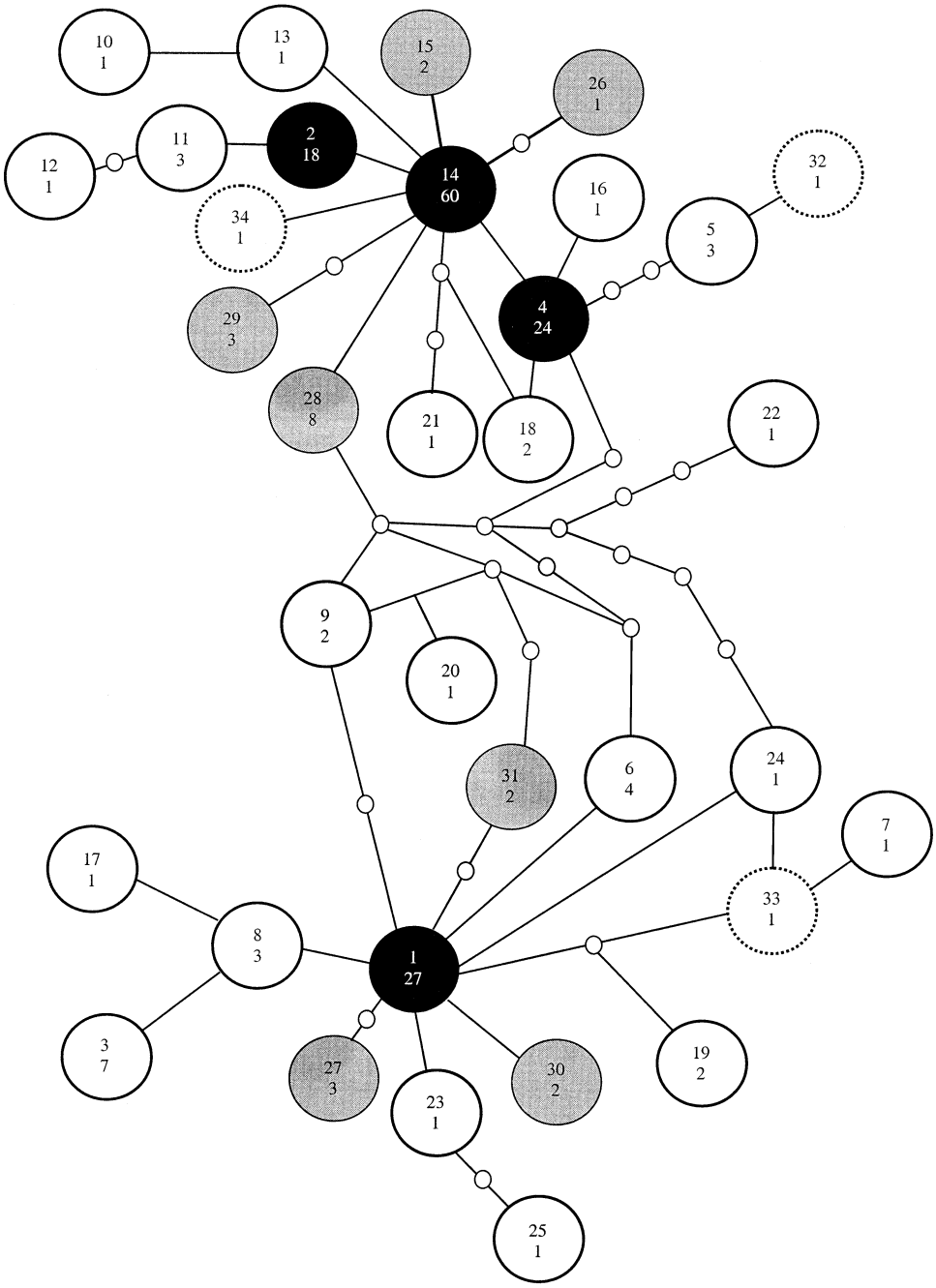

FIG. 3. Statistical parsimony network of *Prochilodus EF1α-6* alleles. The top number inside each circle refers to the allelic designation (see Table 4) and the bottom number represents the number of surveyed individuals sharing the respective allele. Colored circles represent the following: black, alleles shared between species (see Table 4 for relative allele frequency in each species); white with solid line, alleles common only to *P. mariae*; gray, alleles common only to *P. rubrotaeniatus*; and white with dashed line, alleles survey from *P. magdalanae*. Smaller circles along branches designate missing intermediate alleles.

then a skewed sex ratio (in either direction) of 1:199 is needed to produce the mtDNA estimate of Θ for the *P. mariae* (ratio determined using eq. 1 [Nunney 1993] with N_e value of 3.1×10^7 obtained from Table 5). This value is biologically implausible. Finally, recombination is not contributing to the disparity in N_e estimates because the recombination rate is incorporated into LAMARC's estimate of Θ.

We are left with two explanations for the differences in mtDNA and nuclear estimates of N_e. First, the neutral theory predicts that mtDNA loci will tend to coalesce about four times faster than nuclear DNA, which can result in fewer intraspecific mtDNA polymorphisms compared to autosomal loci. Second, natural selection, in the form of a selective sweep on the mitochondrion, can eliminate all neutral mtDNA genetic variation (Gillespie 2000). If the discrepancy between mtDNA and nuclear estimates of N_e are a result of

TABLE 5. *Prochilodus rubrotaeniatus* and *P. mariae* population parameter estimates from mtDNA-*ND4*, *Cal-4*, and *EF1α-6* sequences. Abbreviations Θ and N_e correspond to nucleotide diversity and long-term effective population size, respectively. Numbers in parentheses are 99.5% confidence intervals for Θ and N_e estimates. MtDNA estimate of N_e is Θ/μ and autosomal estimates of N_e are Θ/4μ. The mutation rates for calculating *ND4* and autosomal N_e are $\mu = 0.52 \times 10^{-8}$ and 0.25×10^{-9}, respectively. N_e could not be estimated for monomorphic populations.

Parameter	Locus	*P. mariae* Combined	*P. rubrotaeniatus* Paragua (Orinoco)	*P. rubrotaeniatus* Siapo + Casiquiare (Negro)	*P. rubrotaeniatus* Cuyuní (Essequibo)
Θ					
	ND4	0.0016 (0.0009–0.0046)	0.0049 (0.0006–0.6610)	0.0000	0.0018 (0.0003–0.0414)
	EF1α-6	0.0446 (0.0194–0.0592)	0.0067 (0.0012–0.0555)	0.0014 (0.0003–0.0056)	0.0077 (0.0018–0.0345)
	Cal-4	0.0307 (0.0145–0.0448)	0.0187 (0.0020–0.5676)	0.0000	0.0033 (0.0013–0.2192)
N_e					
	ND4	3.1×10^5 ($1.7 \times 10^5 - 8.8 \times 10^5$)	9.4×10^5 ($1.2 \times 10^5 - 5.7 \times 10^7$)	N/A	3.5×10^5 ($5.8 \times 10^4 - 8.0 \times 10^6$)
	EF1α-6	4.5×10^7 ($2.0 \times 10^7 - 6.0 \times 10^7$)	6.8×10^6 ($1.2 \times 10^6 - 1.9 \times 10^7$)	1.4×10^6 ($3.0 \times 10^5 - 5.7 \times 10^6$)	7.8×10^6 ($1.8 \times 10^6 - 3.5 \times 10^7$)
	Cal-4	3.1×10^7 ($1.5 \times 10^7 - 4.5 \times 10^7$)	1.9×10^7 ($2.0 \times 10^6 - 5.7 \times 10^8$)	N/A	3.3×10^6 ($1.3 \times 10^6 - 2.2 \times 10^8$)

differences in times to coalescence or historical bottlenecks, then each observed estimate of mtDNA Θ should be equal to its expectation. Observed values of Θ for *P. mariae* are significantly lower than expected values in all localities. Furthermore, the mtDNA estimate of N_e, which should be similar to autosomal estimates, is statistically less than estimates calculated from *Cal-4* and *EF1α-6* data (i.e., the 99.5% CIs do not overlap). These findings suggest that differences in coalescent times between nuclear and mtDNA loci or historical bottlenecks can not explain the paucity of mtDNA genetic diversity in *P. mariae*.

Turner et al. (2004) argued that purifying selection acting on the mitochondrion (i.e., a selective sweep) could explain the lack of genetic diversity in *P. mariae* populations; however, they could not definitively rule out the effects of past bottlenecks. Their argument for natural selection was based on a significant result for Fu and Li's (1993) test of neutrality and the observation of two rare haplotypes having single nonsynonymous mutations. To document selection acting on the mitochondrion, Gerber et al. (2001) suggest eliminating alternative explanations for observed patterns of mtDNA diversity. We follow their suggestion by assessing and eliminating various alternative demographic, ecological, and molecular explanations for the mtDNA diversity in *P. mariae* populations. Our findings, based on two nuclear loci, further suggest that purifying selection is acting on the mitochondrion of *P. mariae*.

Recent theoretical (Gillespie 2000, 2001) and empirical studies (Ballard and Kreitman 1994; Kennedy and Nachman 1998; Nachman 1998; Ballard 2000; Weinreich and Rand 2000; Gerber et al. 2001; Moilanen et al. 2003; Shoemaker et al. 2003; Zhan et al. 2003) suggest selection can and does frequently affect patterns of mtDNA diversity. Various environmental and physiological processes influence mtDNA haplotype performance and affect organismal fitness (reviewed by Gerber et al. 2001). The direct cause of a mtDNA selective sweep in *P. mariae* is difficult to discern, but like Turner et al. (2004), we suggest that it is in association with the substantial environmental and presumably physiological differences between Orinoco populations of *P. mariae* and *P. rubrotaeniatus*. *Prochilodus rubrotaeniatus* is broadly distributed throughout the Essequibo and Amazonas River systems in northeastern South America. However, in the Orinoco system, *P. rubrotaeniatus* is restricted to the Rio Caroní Basin—a black-water system characterized by relatively low pH (4.5–6.0) and low nutrient levels. In contrast, *P. mariae* is restricted to white-water rivers of the Orinoco Basin that are characterized by neutral pH, high conductivity, and high suspended solids. Turner et al. (2004) suggested that white-water habitats may impose purifying selection on the mitochondrion, which may explain the observed rare, nonsynonymous mtDNA substitutions. Although the exact mechanism imposing purifying selection on the mitochondrion is difficult to determine, hydrological and physiological differences in river systems have also been suggested to play a role in the evolution and diversification of other Neotropical fishes such as doradid catfishes (Sabaj and Ferraris 2003) and callichthyid catfishes (Mol 1994).

The study of Sivasundar et al. (2001) included white- and black-water habitats in a phylogenetic survey of mtDNA var-

TABLE 6. Observed and expected values of mtDNA Θ for *P. mariae* populations. MtDNA expectations of Θ were derived using autosomal estimates of N_e (see Table 5). The confidence interval (CI) is reported as two standard errors around the expectation of Θ.

Gene	Population	N_e	Θ (obs.)	Θ (exp.)	lower CI	upper CI
Cal-4						
	Orinoco	3.1×10^7	0.0016	0.1329	0.0025	0.2633
	Paragua	1.9×10^7	0.0049	0.0870	0.0000	0.1786
	Siapo + Casiquiare (Negro)	0.0000	0.0000	0.0000	0.0000	0.0000
	Cuyuni (Essequibo)	3.4×10^6	0.0077	0.0170	0.0000	0.0370
EF1α-6						
	Orinoco	4.5×10^7	0.0016	0.1788	0.0043	0.3532
	Paragua	6.8×10^6	0.0049	0.0337	0.0000	0.0708
	Siapo + Casiquiare (Negro)	1.4×10^6	0.0000	0.0073	0.0000	0.0171
	Cuyuni (Essequibo)	7.8×10^6	0.0077	0.0384	0.0000	0.0804

iation in *P. lineatus*, and contrary to our results, reported greater mtDNA variation throughout the Parana basin. Perhaps the selective hypothesis may be constrained to cases where the ancestral species lives in black-water systems (such as the Caroní and Negro basins) and the descendant populations colonized a neighboring white-water river. Although our data and data from Turner et al. (2004) can not unequivocally establish that the ancestor of *P. mariae* was a black-water form, further studies could specifically address this idea.

Though systematists (e.g., Vari and Weitzman 1990; Schaefer 1997; Lovejoy and de Araújo 2000; Lovejoy and Collette 2001; Sivasundar et al. 2001; Montoya-Burgos 2003) have emphasized the association of phylogeographic patterns of fishes with orogenic patterns of river systems, allopatric models can not single handedly explain the alpha diversity of Neotropical freshwater fishes. In contrast, ecologists have documented differences in fish community structure (Mol 1994; Saint-Paul 2000), life history (Winemiller 1989), and form and function (Marrero and Winemiller 1993; Winemiller et al. 1995) of various Neotropical freshwater fish species. To understand the processes that have lead to the alpha diversity and substantial phenotypic diversity of Neotropical fishes requires explicit testing of not only allopatric models but other models (e.g., the gradient model of Moritz et al. 2000) that incorporate selection as a mechanism for speciation. To do so involves the integration of molecular, ecological, and biogeographical data with data describing phenotypic variation and reproductive divergence (Moritz et al. 2000). Although our study did not attempt to test various alternative models of speciation as outlined in Moritz et al. (2000), we do incorporate molecular, ecological, and biogeographical data to propose that both demography and natural selection have played roles in the diversification of two *Prochilodus* species in northern South America.

Conclusions

Although the use of mtDNA and autosomal genes for phylogenetic and population studies have been thoroughly discussed at length (Moore 1995; Avise 2000; Hare 2001), studies based solely on mtDNA data for phylogeny and/or parameter estimation have limited resources to detect whether random genetic drift or selection is operating on mtDNA variation. The use of statistical tests of neutrality (e.g., Tajima 1989; Fu and Li 1993; Fu 1997) provide powerful methods to test whether the observed patterns of polymorphism are consistent with the neutral model of evolution; yet, these tests

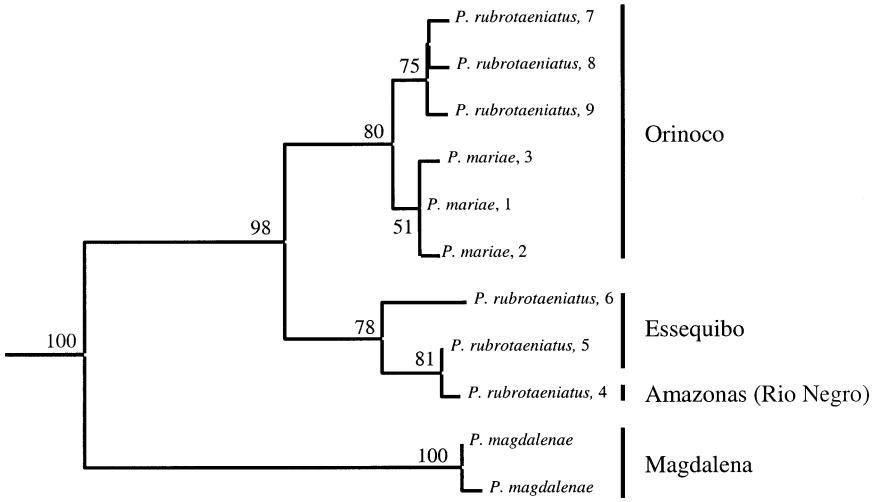

FIG. 4. Redrawn mtDNA parsimony tree from Turner et al. (2004). This tree is based on partial *COI* and *ND4* sequence data (564 bp). Bootstrap values >50% are reported on branches, and branch lengths are proportional to the number of substitutions. Numbers behind OTUs represent unique haplotypes corresponding to Table 2. Sample drainages for haplotypes are indicated.

may yield different conclusions (Turner et al. 2004), especially when the total number of mutations inferred among sequences is low (Akashi 1999). Because of potential problems associated with mtDNA, studies should attempt to at least address whether selection is affecting mtDNA polymorphism by applying statistical tests of neutrality, testing for conformance to a molecular clock, and corroborating phylogenies and genetic parameters with data from multiple independent loci. This is not to say that noncompliance of one of these tests is direct evidence of natural selection, but these tests in conjunction with data from independent sources provide a more accurate understanding of dynamics affecting genetic diversity. Furthermore, species-level phylogenies and phylogeography studies should aim to capture more than allelic patterns among geographic areas; these studies should go a step further and explore inter- and intraspecific population dynamics that are ascribed to these allelic patterns. To do so requires data from multiple independent loci, broad geographic sampling, and an understanding of the organism's ecology, life history, and demography.

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