Molecular Phylogenetics and Evolution 59 (2011) 725–735

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

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

Phylogenetic relationships of the North American cyprinid subgenus *Hydrophlox*

Mollie F. Cashner [a,b,*], Kyle R. Piller [b], Henry L. Bart [a]

[a] Department of Ecology and Evolutionary Biology, Tulane University, New Orleans, LA 70118, USA
[b] Department of Biological Sciences, Southeastern Louisiana University, Hammond, LA 70402, USA

ARTICLE INFO

Article history:
Received 31 October 2010
Revised 15 March 2011
Accepted 15 March 2011
Available online 21 March 2011

Keywords:
Notropis
Phylogeny
ND2
Rhodopsin
ITS1

ABSTRACT

Notropis is one of the largest genera of North American fishes and is composed of a number of morphologically diagnosed subgroups; however, the validity of many has not been tested in a phylogenetic framework. One such subgroup is the subgenus *Hydrophlox*, which is composed of brilliantly colored species that engage in the symbiotic reproductive behavior of nest association. Although they have long been recognized as a cohesive group due to their nuptial coloration and fin tuberculation, very little is known about the relationships of species within *Hydrophlox*. We tested the monophyly of *Hydrophlox* using a mitochondrial marker (ND2) and two nuclear markers (ITS1 and RH), with Maximum Parsimony and Bayesian inference approaches. A well supported clade of "core" *Hydrophlox* was recovered and is composed of five taxa: *Notropis chiliticus, Notropis rubricroceus, Notropis lutipinnis, Notropis chlorocephalus,* and *Notropis chrosomus*. *Hydrophlox s.l.* is paraphyletic with respect to three taxa: *Notropis baileyi, Notropis leuciodus* and *Notropis nubilus*. While there was some discordance among the individual marker topologies, a combined evidence analysis recovered a topology that incorporated elements from all single-gene trees. Our analyses suggest that *Hydrophlox* is composed of five nominal species and additional undescribed diversity exists within this clade.

1. Introduction

Cyprinidae is the largest family of freshwater fishes in North America, encompasing over 50 genera. Nearly all of the approximately 300 North American species are members of subfamily Leuciscinae (Mayden, 1991; Nelson, 1994; Berra, 2001; Simons et al., 2003). Fossil evidence indicates that cyprinids have inhabited North America for over 31 million years (Cavender, 1991), and due to their significant diversification, resolving North American cyprinid relationships has been difficult (Cunha et al., 2002; Simons et al., 2003).

North American cyprinids exhibit astonishing taxonomic, morphological, behavioral, and ecological diversity, and have only recently become the focus of phylogenetic studies (Simons and Mayden, 1998; Broughton and Gold, 2000; Cunha et al., 2002; Simons et al., 2003; Schonhuth et al., 2008; Bufalino and Mayden, 2010). Of particular phylogenetic interest is the genus *Notropis*, which has been construed to include at least six recently segregated genera, and contains nearly 100 species (Gilbert, 1978; Bortone, 1989; Mayden, 1991; Warren et al., 1994; Wood et al., 2002) divided into three subgenera (*Notropis* [20 species], *Alburnops* [8 species], *Hydrophlox* [8 species]), at least three species groups (*Notropis texanus* species group [8 members], *Notropis volucellus* species group [10 members], *Notropis dorsalis* [6 species]) and a group which contains approximately 21 species whose relationships within *Notropis* are currently unresolved (Swift, 1970; Bortone, 1989; Mayden, 1991; Warren et al., 1994; Raley and Wood, 2001; Wood et al., 2002). *Notropis* has been the focus of few published molecular phylogenetic analyses (Bielawski and Gold, 2001; Raley and Wood, 2001; Schonhuth and Doadrio, 2003; Berendzen et al., 2008), all but the most recent using only mtDNA sequences (cyt*b*) or allozymes. The monophyly of genus *Notropis* has not been resolved, and not surprisingly, there is taxonomic volatility within and among subgenera and species groups.

Many members of the subgenus *Hydrophlox* are noted for their intense nuptial coloration and are among the 30% of North American minnows that engage in nest association (Outten, 1961; Johnston and Page, 1992; Clayton, 2000). This reproductive strategy, considered a specialized version of broadcast spawning, occurs when one species spawns in the prepared substrate (nest) of a host species (Johnston and Page, 1992). Despite years of observations, (e.g. Raney, 1947; Wallin, 1992; Fletcher, 1993; Johnston, 1994a,b; Johnston and Kleiner, 1994; Cochran, 2000), many aspects of this symbiotic relationship remain unknown. Understanding phylogenetic relationships among a monophyletic clade of minnows that engage in nest association provides a solid framework from which to begin investigations of the evolution of nest association in North American minnows.

* Corresponding author at: Department of Biological Sciences, Southeastern Louisiana University, Hammond, LA 70402, USA.
E-mail addresses: pimephales1@yahoo.com (M.F. Cashner), kyle.piller@selu.edu (K.R. Piller), hank@museum.tulane.edu (H.L. Bart).

1055-7903/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2011.03.019

1.1. Taxonomic history and distribution

Since its description by David Starr Jordan (Jordan and Brayton, 1878), *Hydrophlox* has contained as many as 33 taxa (Swift, 1970). Characteristics such as nuptial coloration, size, and habitat were often used to include taxa within the subgenus. Swift (1970) redefined *Hydrophlox* and designated three species groups (Table 1) containing the following species: *Notropis rubricroceus* (Cope 1868), the type; *Notropis chiliticus* (Cope 1870); *Notropis chlorocephalus* (Cope 1870); *Notropis lutipinnis* (Jordan and Brayton 1878); *Notropis baileyi* Suttkus and Raney 1955; *Notropis leuciodus* (Cope 1816); *Notropis chrosomus* (Jordan 1877); *Notropis nubilus* (Forbs 1878); and *Notropis rubellus* (Agassiz 1850). *Hydrophlox* species were united by uniserial tubercles on pectoral fin rays, fine breeding tubercles over most of the body and head, and bright red, orange, and or yellow nuptial coloration. Although a cladistic analysis was not performed, Swift (1970) proposed relationships within and among his designated species groups of *Hydrophlox* based on distribution, ecology, and morphological characters (Fig. 1). Swift (1970) determined that members of the *texanus* species group were not closely related to *Hydrophlox* species; however, the placement of *Hydrophlox* within *Notropis* remains unknown as no sister-relationship hypotheses have been explicitly proposed.

Members of *Hydrophlox* are primarily found in fast-flowing streams of the southeastern United States (Fig. 2). Many are allopatric with a few notable exceptions: *N. baileyi* and *N. chrosomus* are sympatric in portions of the Alabama River system; *N. leuciodus* is sympatric with populations of *N. rubricroceus* in the French Broad River system and eastern portions of the Tennessee River drainage. Hybridization has been observed between *N. baileyi* and *N. chrosomus* (Boschung and Mayden, 2004) and also for *N. chiliticus* and *N. chlorocephalus* in areas where *N. chiliticus* populations have been introduced (Menhinick, 1991).

Notropis lutipinnisand *N. chlorocephalus* have been considered subspecies by some authors (Menhinick, 1991), based primarily on an apparent intergrade zone in the Broad River system in North Carolina. Wood and Mayden (1992), using allozyme data, found evidence for a polyphyletic *N. lutipinnis* in which Broad River system populations of *N. lutipinnis* are sister to *N. chlorocephalus*. They additionally found evidence for three diagnosable forms within *N. lutipinnis*, two of which remain undescribed.

Notropis nubilus was removed from the genus *Dionda* by Swift (1970) based on breeding coloration, tuberculation on body and head, large uniserial tubercles on pectoral rays, scalloped dorsolateral scales, low circumferential body scale count, and sharp predorsal line; however gut morphology, breeding biology, and distribution may not support this placement (Glazier and Taber, 1980; Fowler et al., 1984). In their examination of *Cyprinella*, Schonhuth and Mayden (2010) included *N. nubilus* in their analysis, the only such inclusion in a phylogenetic analysis of this species; however, the focus of their study was *Cyprinella* and placement of *N. nubilus* within *Hydrophlox* was not tested.

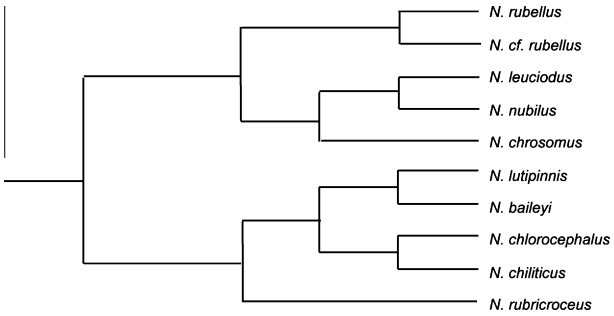

Fig. 1. Relationships of *Notropis* (*Hydrophlox*) inferred from Swift 1970.

Mayden and Matson (1988), Humphries and Cashner (1994), and Bielawski and Gold (2002) found *N. rubellus* to be more closely allied with subgenus *Notropis* based on allozymes, morphology, and mtDNA, respectively. Even in Swift's (1970) designation, *N. rubellus* is distinct from all other *Hydrophlox* (Table 1). Swift also noted that *N. rubellus* exhibited considerable variation throughout its range and he suggested further examination before definitive inclusion into *Hydrophlox*. Currently, the *N. rubellus* complex contains five taxa: *N. rubellus* (Agassiz, 1850), *Notropis percobromus* (Cope, 1871), *Notropis micropteryx* (Cope, 1868), *Notropis suttkusi* Humphries and Cashner, 1994, and three undescribed forms allied with subgenus *Notropis* (Humphries and Cashner, 1994; Wood et al., 2002; Berendzen, et al., 2008; Berendzen et al., 2009).

The objectives of this study were to: (1) test the monophyly of *Hydrophlox* with one mitochondrial DNA (mtDNA) and three nuclear DNA (nucDNA) markers by including all putative members of the subgenus with representatives from multiple populations; (2) determine possible sister relationships between *Hydrophlox* and other members of the *Notropis* genus by selecting outgroup taxa from a broad sample of members of *Notropis*; and (3) investigate the relationships within *Hydrophlox* to account for cryptic or undescribed diversity and establish a hypothesis from which to explore the evolution of nest association within this group of colorful minnows.

Table 1
Species groups and character designations from Swift (1970) for members of the subgenus *Hydrophlox*.

Species group	Member taxa	Uniting characters
rubricroceus	*N. rubricroceus* (Cope, 1868) *N. chiliticus* (Cope, 1870) N. chlorocephalus (Cope, 1870) *N. lutipinnis* (Jordan and Brayton, 1878) *N. baileyi* Suttkus and Raney, 1955	Specialization of small, cool streams, with a gradient and turbulent habitats. Long fins; deep compressed body; crowded predorsal scales; rounded dorsolateral scales; high body circumference scale counts (25–30); high circumference caudal peduncle scale counts (12–18); strong predorsal stripe; lateral band present above and below lateral line canal; bright red, orange or yellow breeding colors with no iridescence
leuciodus	*N. leuciodus* (Cope, 1868) *N. chrosomus* (Jordan, 1877) *N. nubilus* (Forbes, 1878)	Adapted to fast waters in small to medium sized streams and steady flow habitats. Lower predorsal, body circumference, and caudal peduncle circumference scale counts; scalloped dorsolateral scales; thin predorsal stripe; unpigmented area above lateral line; melanophores on lateral line scales above and below lateral line: *N. leuciodus* with strong dashes, *N. chrosomus* and *N. nubilus* with "squarish blotches"; iridescent breeding coloration (though absent in *N. nubilus*)
rubellus	*N. rubellus* (Agassiz, 1850) and others	Terete body with well marked lateral canal; shares black lateral stripe, strong predorsal stripe, crowded predorsal scales, and high body circumference scale counts with rubricroceus species group. Distinct characters are: high anal ray count (9–12); high vertebral count; dorsal origin far behind pelvic origin; sharp snout; no caudal spot; small fins

2. Materials and methods

2.1. Specimen collection, DNA extraction, amplification and sequencing

Twenty-five specimens of *Hydrophlox sensu* Swift (1970) were collected from populations dispersed throughout each species known range (Fig. 2, Table 2). An additional nine specimens from six outgroup taxa representing known species groups or subspecies of *Notropis* were also included in the analysis. Multiple specimens of *N. texanus* were included to examine relationships among *N. baileyi* and members of the *texanus* species group (Swift 1970). All specimens were preserved whole in 95% ethanol, and deposited in the Tulane Museum of Natural History. Tissue samples of *Notropis petersoni* and *Notropis blennius* were provided by the Florida Museum of Natural History and the Southeastern Louisiana University Vertebrate Museum, respectively.

DNA was extracted from fin clips of all preserved specimens using either Qiagen DNeasy Blood and Tissue kit or a modified Chelex 100 extraction protocol: 50–100 ng of Proteinase K and approximately 25 ng of fin tissue (Walsh et al., 1991). Four target genetic markers were amplified from whole genomic DNA template via PCR using primers from previously published studies: mitochondrially encoded ND2 (GLN and ASN from Kocher et al., 1995); nucDNA ITS1 (Presa, et al., 2002); nuclear encoding RH (Rod-F2w and Rod-R4n from Sevilla et al., 2007); and nuclear first intron S7 (Chow and Hazama, 1998). Thermal cycling protocols followed those previously published with above-mentioned primers, except ITS1: 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 56 °C for 15 s and 72 °C for 30 s, and a final extension of 72 °C for 1 min.

All PCR products were purified using ExoSAP-IT, and visualized on an 0.8% agarose gel to assess quality. Purified S7 and ITS1 products were sent to Macrogen Inc. (Seoul, South Korea) for direct sequencing. All ND2 (sequencing primers B-L and E-H from Broughton and Gold, 2000) and a subset of ITS1 products were prepared for cycle sequencing in ABI BigDye ¼-volume reactions, purified using gel filtration (Edge BioSystems), and sequenced on ABI 3100 or 3130 machines. Due to prevalence of heterozygotic indels and multiple ambiguous bases in many S7 and ITS1 sequences, problematic specimens were cloned using Topo TA Cloning kit (Invitrogen, Inc.), and purified products were sent to Macrogen Inc. for sequencing (three to five clones per individual). Sequences were visualized and edited using Sequencher v. 4.5 (Gene Codes Corp.).

2.2. Molecular markers

We intended to use four molecular markers with varying mutation rates to investigate realtionships within subgenus *Hydrophlox*

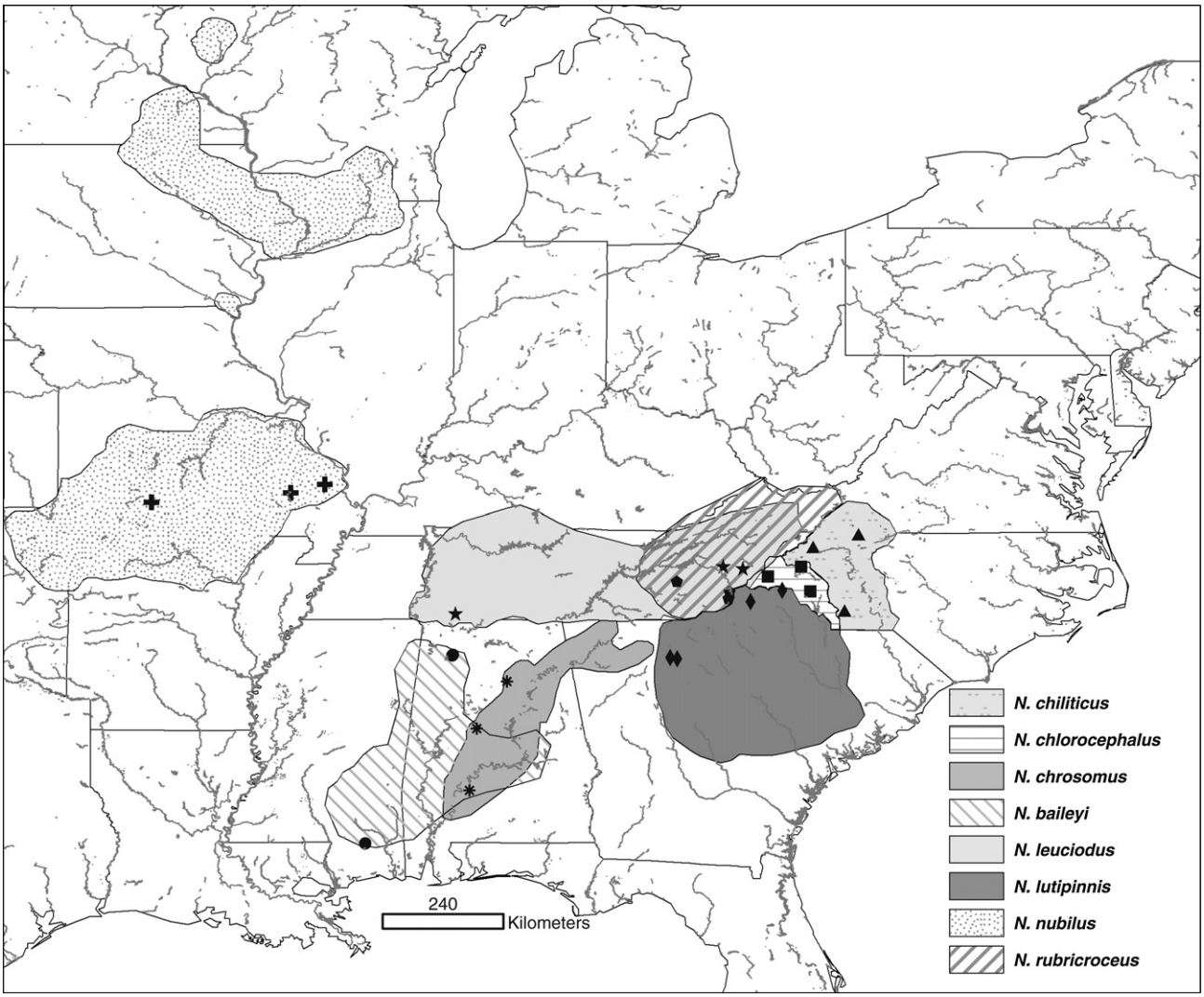

Fig. 2. Distribution of all putative members of *Notropis* (*Hydrophlox*) and collection localities. Symbols are unique for each species.

Table 2
Specimen information including specimen code, museum catalog number (TUMNH, Tulane Museum of Natural History; FMNH, Florida Museum of Natural History; and SLU-TC, Southeastern Louisiana University – Tissue Collection), locality, and NCBI GenBank aquisition numbers.

Species	Specimen code	Catalog number	Stream, county, state	GenBank ND2	GenBank ITS1	GenBank Rho
N. chiliticus	001.01	TUMNH 196712	West Prong & Roaring Fork, Wilkes, NC	JF523487	JF523487	JF523451
N. chiliticus	001.03	TUMNH 196711	Peters Creek, Stokes, NC	JF523488	JF523416	JF523452
N. chiliticus	001.06	TUMNH 196674	Goose Creek, Union, NC	JF523489	JF523417	JF523453
N. chlorocephalus	002.01	TUMNH 196715	Paddy Creek, Burke, NC	JF523491	JF523418	JF523454
N. chlorocephalus	002.02	TUMNH 198133	Lippard Creek, Lincoln, NC	JF523490	JF523419	JF523455
N. chlorocephalus	002.05	TUMNH 198179	Duck Creek, Alexander, NC	JF523492	JF523420	JF523456
N. chrosomus	003.01	TUMNH 196662	Little Schultz Creek, Bibb, AL	JF523493	JF523421	JF523457
N. chrosomus	003.05	TUMNH 196722	Crump Branch, Blount, AL	JF523494	JF523422	JF523458
N. chrosomus	003.07	TUMNH 200682	Shoal Creek, Wilcox, AL	JF523495	JF523423	JF523459
N. lutipinnis	004.01	TUMNH 196717	Ostin Creek, Co., NC	JF523496	JF523424	JF523460
N. lutipinnis	004.06	TUMNH 196693	Cox Creek, Co., NC	JF523497	JF523425	JF523461
N. lutipinnis	004.09	TUMNH 199678	Candler Creek, Co., GA	JF523498	JF523426	JF523462
N. lutipinnis	004.10	TUMNH 199661	Hickory Level Creek, Co., GA	JF523499	JF523427	JF523463
N. rubricroceus	005.01	TUMNH 196621	South Fork Mills, Co., NC	JF523500	JF523428	JF523464
N. rubricroceus	005.04	TUMNH 198154	Trib to Roaring Fork Creek, Co., TN	JF523501	JF523429	JF523465
N. rubricroceus	005.06	TUMNH 198157	Bent Creek, Co., NC	JF523502	JF523430	JF523466
N. baileyi	006.01	TUMNH 196719	Boiler Branch, Co., AL	JF523503	JF523431	JF523467
N. baileyi	006.03	TUMNH 196720	Chenault Spring Branch, Co., AL	JF523504	JF523432	JF523468
N. baileyi	006.10	TUMNH 200672	Griffin Branch, Co., MS	JF523505	JF523433	JF523469
N. leuciodus	007.01	TUMNH 196686	Shelton Laurel Creek, Co., TN	JF523506	JF523434	JF523470
N. leuciodus	007.03	TUMNH 196771	Clack Branch	JF523507	JF523435	JF523471
N. leuciodus	007.04	TUMNH 199695	Cane River, Co., NC	JF523508	JF523436	JF523472
N. nubilus	008.01	TUMNH 200796	Finley Creek, Co., MO	JF523509	JF523437	JF523473
N. nubilus	008.03	TUMNH 197466	Panther Creek, Co., MO	JF523510	JF523438	JF523474
N. nubilus	008.04	TUMNH 197468	Bennett Creek, Co., MO	JF523511	JF523439	JF523475
N. rubellus	009.01	TUMNH 196772	Clack Branch	JF523512	JF523440	JF523476
N. longirostris	040.03	TUMNH 199436	Bouie Creek, Co., MS	JF523513	JF523441	JF523477
N. texanus	041.10	TUMNH 199466	Bouie Creek, Co., MS	JF523514	JF523442	JF523478
N. texanus	041.11	MFC06-03	Pine Log Creek, Co., FL	JF523515	JF523443	JF523479
N. texanus	041.13	KRP41-1	Wolf River; Outagamie Co., WI	JF523516	JF523444	JF523480
N. texanus	045.01	MFC06-3	Pine Log Creek, Co., FL	JF523522	JF523448	JF523485
N. atherinoides	042.01	TUMNH 198149	Leaf River, Co., MS	JF523517	JF523445	JF523481
N. volucellus	043.02	TUMNH 199467	Leaf River, Co., MS	JF523518	JF523446	JF523482
N. spectrunculus	044.03	TUMNH 199472	Davidson River, Co., NC	JF523520	JF523447	JF523483
N. petersoni	045.51	FMNH 160403	Daisey Creek, Marion, FL	JF523521	JF523449	JF523484
N. blennius	046.01	SLU-TC 451	Wisconsin River, Crawford, WI	JF523519	JF523450	JF523486

and its placement within the genus *Notropis*: the mtDNA coding gene NADH dehydrogenase subunit 2 (ND2), which has been used to assess phylogenetic relationships within genera and species groups (Kocher et al., 1995; Breden et al., 1999; Broughton and Gold, 2000; Near et al., 2003); the intron-free, nuclear coding Rhodopsin gene (RH) has been used in phylogenetic analyses at the family and ordinal levels (Schonhuth et al., 2008; Mayden et al., 2009); the first intron of the nuclear coding S7 ribosomal protein gene (S71) has been utilized in many fish phylogeny studies (Lavoue et al., 2003; Near, 2004; Piller et al., 2008; Schonhuth, et al., 2008; Moyer et al., 2009; Bufalino and Mayden, 2010); and the first nuclear ribosomal internal transcribed spacer (ITS1), part of the ribosomal DNA (rDNA) transcription unit which is composed of tandomly repeating arrays of three genes (18S, 5.8S and 28S), which has been used to identify species, investigate hybrid populations, and in phylogenetic analyses of closely related taxa (Presa et al., 2002; Chow et al., 2006; Wyatt et al., 2006). As detailed in the results, we were only able to produce reliable data for three of these markers.

2.3. Sequence alignment and phylogenetic analyses

Alignments of ND2 and RH sequences were straightforward and performed in Sequencher v 4.5. Large numbers of indels required the use of Clustal W (Larkin et al., 2007) with default parameters for ITS1 and S7 alignments. Because RH primers amplified only a portion of the gene, alignment and codon position were based on a GenBank sequence of *Astyanax mexicanus* (GenBank U12328). Transitions and transversions were plotted against Jukes and Cantor 1969 genetic distance in DAMBE (Xia and Xie, 2001) to assess levels of saturation among each marker. All other genetic distances are expressed as uncorrected 'p' percentages.

Phylogenetic analyses were conducted on sequence alignments of 36 OTUs using maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony analyses were conducted in PAUP* v 4.01b (Swofford, 1991) using equal weights and TBR branch swapping. Nodal support was determined by 1000 bootstrap pseudoreplicates. Each marker was analyzed separately, and the test for homogeneity of variance (Huelsenbeck et al., 1996) was used to determine whether the data could be combined for MP analysis (Huelsenbeck et al., 1996). Bayesian inference analyses were implemented in MrBayes (v 3.1.2) (Ronquist and Huelsenbeck, 2003) for each marker and a combined dataset. For the BI analysis, the Akaike Information Criterion (AIC) was used to determine the best models of substitution as implemented in ModelTest 3.7 (Posada and Crandall, 1998). Coding genes (ND2 and RH) were partitioned by codon position. Non-coding regions were not partitioned. The Bayesian analysis was run for 5 million generations in two concurrent runs for each marker and the combined data set, with trees sampled every 100 generations. Burn-in was determined by Markov chain convergence for each analysis. Bayesian inference topologies were determined by generating 90% majority rule consensus trees in PAUP*.

Eight different hypotheses of relationships within and among *Hydrophlox s.l.* were generated in Mesquite (v2.72, Maddison and Maddison, 2009) based on Swift's (1970) work and that of subsequent authors in addition to the BI total evidence topology (Table 3). Both Shimodaira-Hasegawa (RELL test distribution,

Table 3
Hypotheses of relationships tested using Shimodaira-Hasagawa and Bayesian Filter.

Name	Description	S–H *p* value	BF compatibility
Monophyletic Hyd 1	Large polytomy with all *N.* (*Hydrophlox*) *s.l.* Swift (1970), excluding *N. rubellus*	<0.0005	0/50,000
Monophyletic Hyd 2	All *N.* (*Hydrophlox*) *s.l.* Swift (1970), excluding *N. rubellus* in large polytomy with each species designated as monophyletic	<0.0005	0/50,000
Swift Hyd	Species groups from Swift (1970) , with inclusion of *N. rubellus*	<0.0005	0/50,000
Swift Hyd x rub	Species groups from Swift (1970) , with exclusion of *N. rubellus*	<0.0005	0/50,000
Core Hyd + bail	*N. chiliticus, N. chlorocephalus, N. rubricroceus, N. chrosomus, N. lutipinnis, N. baileyi* as monophyletic polytomy	<0.0005	0/50,000
Core Hyd + bail + tex	*N. chiliticus, N. chlorocephalus, N. rubricroceus, N. chrosomus, N. lutipinnis, N. baileyi*, and *N. texanus* as monophyletic polytomy	<0.0005	0/50,000
Core Hyd sis bail + tex	*N. chiliticus, N. chlorocephalus, N. rubricroceus, N. chrosomus, N. lutipinnis* in monophyletic polytomy sister to a baileyi + texanus clade	<0.0005	0/50,000
Bayesian total evidence	BTE topology	Best	31,500/50,000 (63%)

1000 bootstrap replicates, one-tailed) and a Bayesian filter were used to determine which of the eight hypotheses best fit the molecular data, both processes were executed in PAUP* v 4.01b.

3. Results

3.1. Sequence analyses

Thirteen specimens were successfully sequenced for the S7 marker, however, five of those individuals had to be cloned. Surprisingly, of those five, two to five unique sequences were recovered per individual with an uncorrected 'p' molecular distance of 0.2–2.6%. A control cloning experiment, in which one sequence was cloned 10 times resulted in 10 identical sequences, so sequencing or cloning error cannot explain this result. The most likely cause of multiple S7 sequences within individuals is pseudogenes, and because we could not confidently identify a pseudogene versus the target, we abandoned this marker for further analysis.

All 36 OTUs were sequenced for ND2, ITS1, and RH. We sequenced the entire ND2 gene (1047 bp), for which there were 586 invariable sites, 461 variable sites and 414 phylogenetically informative sites: 69% and 22% of the variation occurred in the third and first codon positions, respectively. Plots of transitions and transversions against genetic distance suggested saturation at both the first and third codon positions. Additionally, at the third codon position, there was heterogeneity among taxa (X^2 test of homogeneity of base frequencies across taxa), as well as an adenine/cytosine bias in nucleotide frequency. Uncorrected 'p' genetic distances within putative *Hydrophlox* (*N. chiliticus, N. chlorocephalus, N. chrosomus, N. lutipinnis, N. rubricroceus, N. baileyi, N. leuciodus,* and *N. nubilus*) species ranged from 0.2 to 9.2%. Among putative members of *Hydrophlox*, genetic distances ranged from 7.0 to 20.0% and between *Hydrophlox* and outgroup taxa, distances ranged from 6.7 to 20.0%.

Unlike S7, ITS1 is known as a multi-copy locus, and for each of the nine individuals we cloned (due to difficulty in direct sequencing attempts) we recovered two to four unique sequences with 0.3–2.4% sequence divergence. Nearly all of the variation in the multiple copied sequences was due to single nucleotide repeat regions, as would be expected with this marker (Hillis and Davis, 1988). Consensus sequences were generated from each set of clone sequences resulting in a single sequence per individual for phylogenetic analysis. Complete sequences of ITS1 (exclusive of the flanking 18S and 5.8S genes) ranged from 272 to 305 bp with an alignment length of 369 bp. There were 233 invariable sites, 136 variable and 104 parsimony informative sites within ITS1. Plots of transitions and transversions against genetic distance suggested no saturation within this marker. There was a slight cytosine bias in the nucleotide frequency. Uncorrected 'p' genetic distances within species of *Hydrophlox* ranged from 0% to 3.8%. Among members of *Hydrophlox*, genetic distances ranged from 2.4% to 16.2%. Between *Hydrophlox* and the outgroup taxa, genetic distances ranged from 5.8% to 15.4%.

Partial sequences of the RH gene were obtained with a total length of 495 bp. Rhodopsin sequences were aligned with *Astyanax mexicanus* (GenBank U12328) to determine the location of the first codon position in our dataset. There were 459 invariable sites, with only 36 variable and 15 parsimony informative sites within RH, and plots of transitions and transversions were not interpretable due to too few data points. Saturation in this marker was unlikely due to the very low levels of variation. There was no evidence for heterogeneity among taxa in nucleotide frequencies, but there was a strong bias for cytosine in the third position, which also contained 75% of the variable sites.

3.2. Phylogenetic analyses

Both MP and BI analyses yielded similar topologies – less structure at deeper nodes in the MP analyses – with respect to the single-marker analyses, and we report the results for the BI analyses here. The test for homogeneity of variance was significant ($p < 0.05$) for the three markers, thus we did not run a combined MP analysis. A total evidence BI analysis was performed using ND2, ITS1, and RH, with seven partitions and mixed models of evolution. Partitions and models for each were: ITS1 (GTR + G); Rhodopsin 1st position (F81 + I), 2nd position (F81), 3rd position (HKY + G); ND2 1st position (k81uf + I + G), 2nd position (GTR + I), and 3rd position (GTR + I + G).

Hydrophlox s.l was not recovered as monophyletic in all three single-marker analyses (Figs. 3–5). *Notropis leuciodus* is nested within a clade of subgenus *Notropis* in all three topologies; *N. nubilus* is either basal to all taxa, exclusive of *N. longirostris* (ND2, Fig. 3), basal to a *Hydrophlox* + *N.*(*Notropis*) clade (ITS1, Fig. 4), or embedded within a large polytomy of *Hydrophlox* + *texanus* group. Additionally, *N. baileyi* is sister to a *texanus* group clade in both the ND2 and ITS1 topologies, while it is embedded in the large polytomy of *Hydrophlox* + *texanus* group clade with one individual allied with the *N.* (*Notropis*) + *volucellus* group clade in the RH topology (Fig. 5). None of the alternate hypotheses of relationships were a better fit to the data than the combined BI topology (Fig. 6) in the Shimodaira-Hasegawa test ($p < 0.05$), nor were any alternative hypotheses compatible in the Bayesian filter (0/50,000 for all alternate hypotheses).

In the ND2, ITS1, and total evidence topologies, a well supported clade of "core" *Hydrophlox* that included five currently recognized taxa (*N. chiliticus, N. rubricroceus, N. chlorocephalus, N. lutipinnis* and *N. chrosomus*) was recovered. Although the sister clade to this core *Hydrophlox* clade was incongruent between the ITS1 (polytomy with *N.* (*Notropis*) and *N. blennius*) and ND2 (*N. baileyi* + *texanus* group + *N. petersoni*) topologies, the total evidence topology

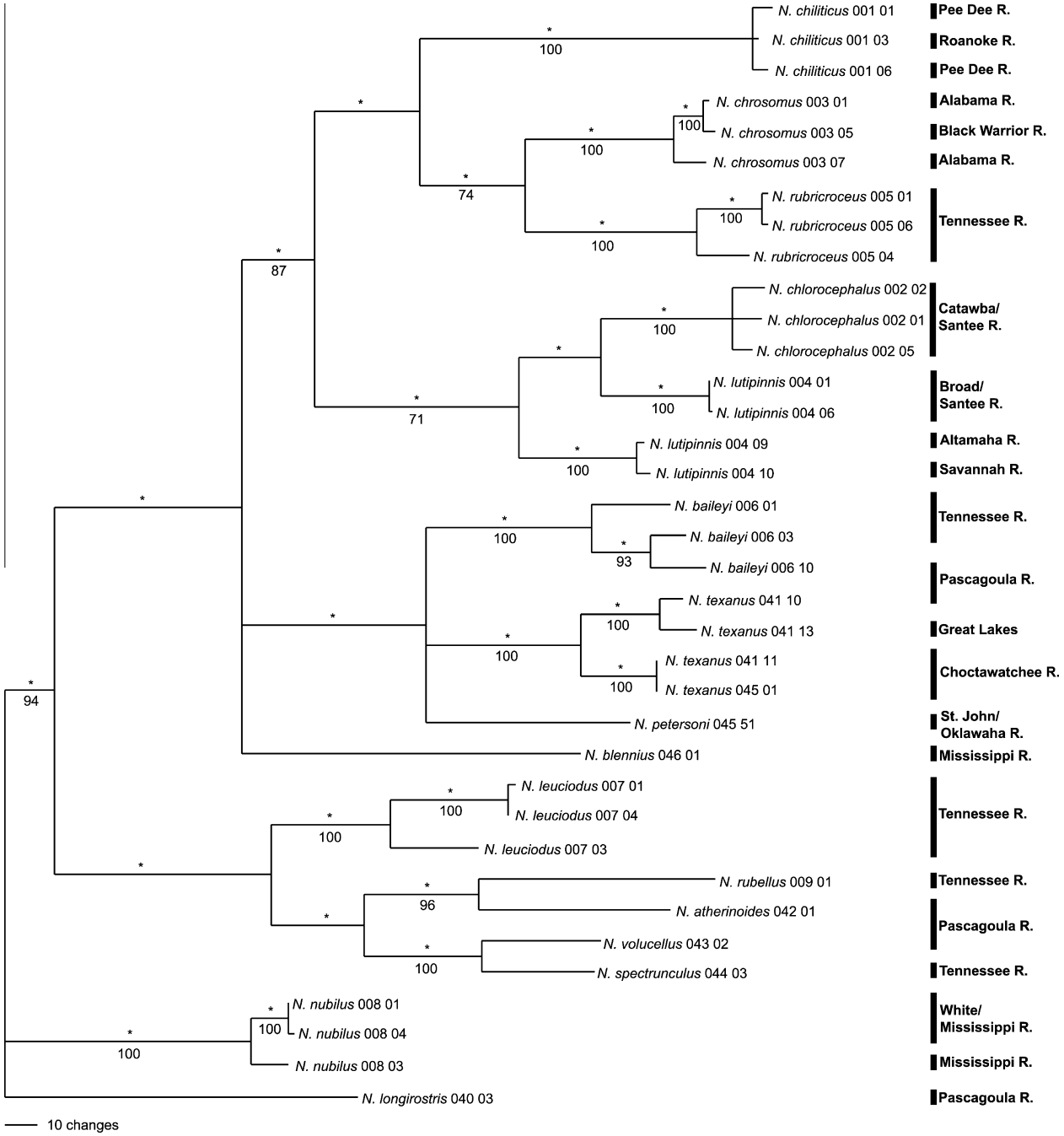

Fig. 3. 90% majority rule consensus tree from a partitioned mixed model Bayesian analysis of complete ND2 sequence data; Bayesian posterior probabilities ⩾95% are indicated with an asterisk above the node, numbers below the node are bootstrap pseudoreplicate values.

supports a sister relationship between core *Hydrophlox* and a *N. baileyi* + *texanus* group clade, with a basal placement of *N. blennius*. Additionally, *N. baileyi* was recovered as sister to *N. petersoni* and is part of the *texanus* species group.

Within core *Hydrophlox*, *N. chiliticus* and *N. rubricroceus* are sister taxa, as are *N. chlorocephalus* and *N. lutipinnis*. In the total evidence topology, *N. chrosomus* is in an unresolved polytomy within the core *Hydrophlox* clade, reflective of the different sister relationships in the ND2 and ITS1 topologies. *N. lutipinnis* is not resolved as monophyletic: a well-supported sister relationship occurs between *N. chlorocephalus* and *N. lutipinnis* from the Broad River system in North Carolina (genetic distances: 0.2% RH, 2% ITS1, and 7% ND2), with individuals from the Altamaha and Savannah rivers sister to that clade (genetic distances between nominal *N. lutipinnis* from the Broad River and the Savannah + Altimaha individuals: 0.4% RH, 3.4% ITS1, and 9% ND2). These genetic results are supported by the existence of chromatically distinct forms of *N. lutipinnis* between the Broad River and the Savannah + Altamaha Rivers (MFC, pers. obs.; Wood and Mayden, 1992).

4. Discussion

This is the first phylogenetic study to examine the relationships of the *Notropis* subgenus *Hydrophlox* inclusive of all putative members. Additionally, this is one of the few studies of *Notropis* to use

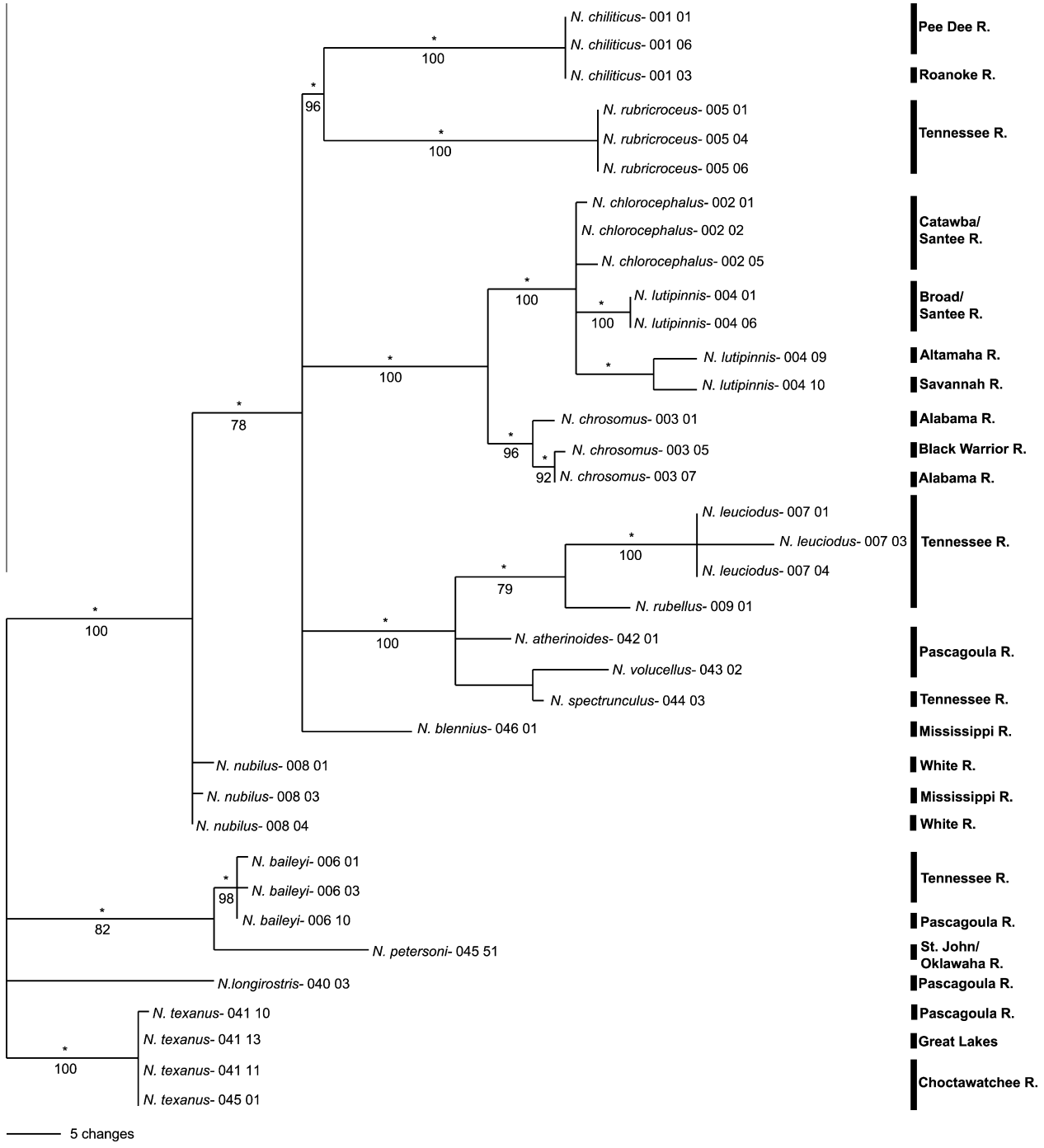

Fig. 4. 90% majority rule consensus tree from a Bayesian analysis of complete ITS1 sequence data using the GTR + G model; Bayesian posterior probabilities ⩾95% are indicated with an asterisk above the node, numbers below the node are bootstrap pseudoreplicate values.

both nuclear and mitochondrial DNA sequences (Schonhuth et al., 2008). Our results indicate that *Hydrophlox s.l.* is not monophyletic, but the monophyly of a core clade composed of five species (including the type species) is well supported. Thus, *Hydrophlox* should be restricted to five nominal species: *N. rubricroceus, N. chiliticus, N. lutipinnis, N. chlorocephalus,* and *N. chrosomus*. *Notropis baileyi*, *N. leuciodus,* and *N. nubilus* are not members of *Hydrophlox*: *N. baileyi* is more closely related to members of the *texanus* species group, *N. leuciodus* is a member of the subgenus *Notropis*, and *N. nubilus* is basal to all *Notropis* species included in this study.

Morphological and behavioral variation also supports the removal of *N. baileyi, N. leuciodus,* and *N. nubilus* from *Hydrophlox*. For many of the *Hydrophlox s.l.* species included in this study, body and fin coloration vary within the spawning season. In most months of spring and early summer, many individuals exhibit fin and body coloration with light washes of color; however when aggregated over a spawning site, the color intensifies to bright reds, yellows, and some orange. We consider this "peak nuptial coloration" and it lasts only as long as spawning aggregations are formed (1–5 h). When core *Hydrophlox* species are in peak nuptial spawning coloration, dorsal, pectoral, pelvic, and anal fins are nearly opaque, with colors ranging from white, yellow, red, to blue iridescence (in *N. chrosomus*). For these species, the fin coloration extends from the base to 2/3–3/4 of the fin, with a clear unpig-

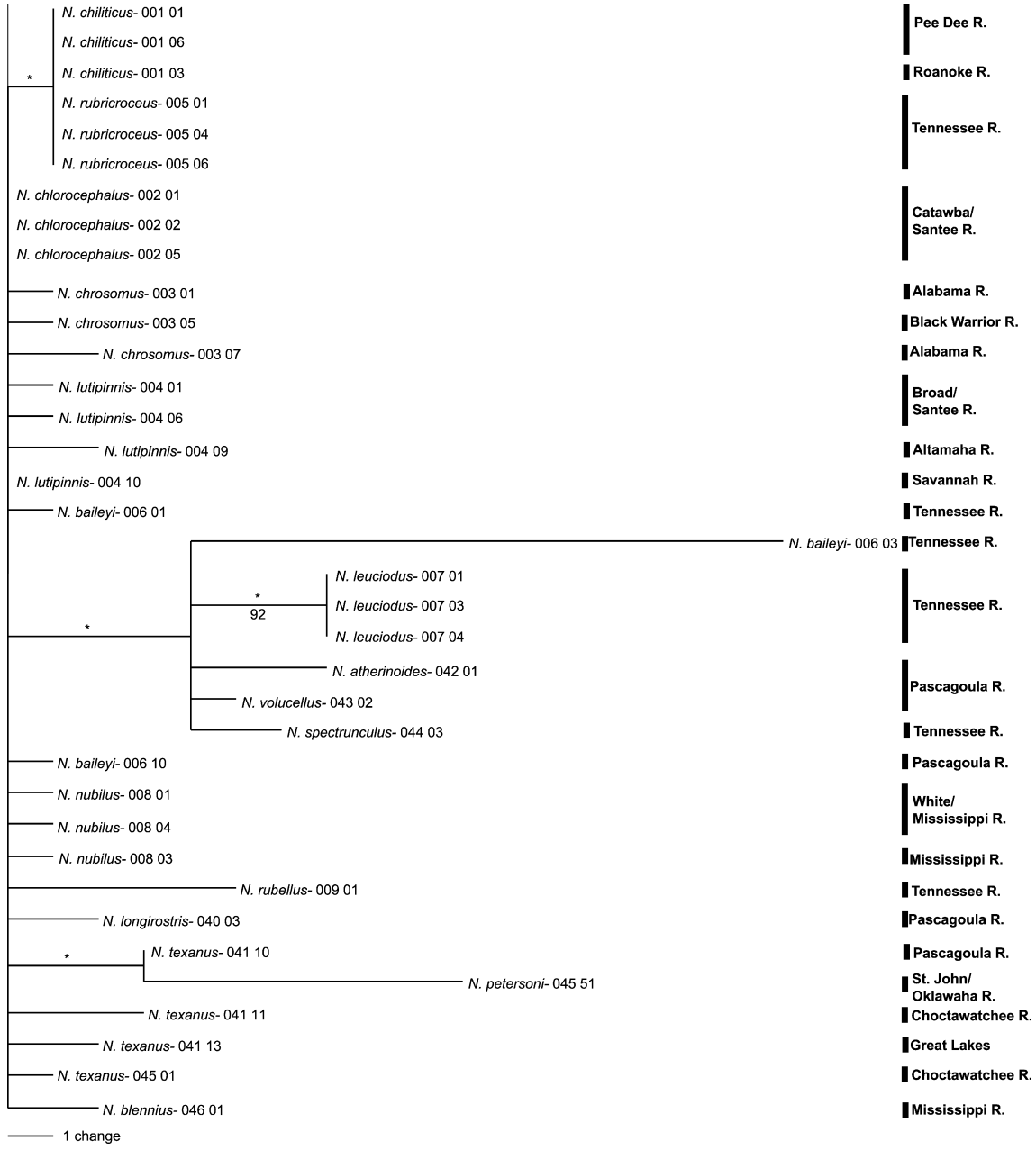

Fig. 5. 90% majority rule consensus tree from a partitioned mixed model Bayesian analysis of partial RH sequence data; Bayesian posterior probabilities ⩾95% are indicated with an asterisk above the node, numbers below the node are bootstrap pseudoreplicate values.

mented margin (MFC, pers. obs). In *N. baileyi, N. nubilus,* and *N. leuciodus* fins remain transparent with light washes of color usually not extending beyond the basal half (MFC, pers. obs). These observations were made by examining peak-condition males and females aggregated over spawning sites which were releasing ova and milt when lightly squeezed. Photos were taken and subsequently examined to determine fin coloration in life.

Swift (1970) also included iridescence in spawning coloration as a character uniting the *leuciodus* group (Table 1), but in our observations gold iridescent stripes above the lateral band are found in all core *Hydrophlox* as well as *N. baileyi*. The gold striping is absent in *N. leuciodus* and *N. nubilus*, despite some white to purple iridescence along the lateral band in both species. *Notropis chrosomus* is well known for brilliant nuptial coloration (hence the common name Rainbow Shiner), with much of the coloration manifested as blue iridescence in the paired, dorsal, and anal fins and in scattered scales along the dorsum. However, in peak nuptial coloration, some of that iridescence disappears and is masked by bright red to pink body pigment. Moreover, our data suggest that the iridescent gold striping along the dorsum and just above the lateral band of all core *Hydrophlox* is a plesiomorphy shared with *N. baileyi*. Therefore, iridescence is not an appropriate character for uniting members of *Hydrophlox*.

During active spawning, males of the five core *Hydrophlox* species hold poorly-defined territories, at most one body width apart (MFC, pers. obs.; Johnston, 1991; Johnston and Kleiner, 1994), whereas *N. baileyi* maintain large fairly static territories, in some cases up to 10 body lengths apart. Though there is often "jockeying" for position in core *Hydrophlox* aggregations among males, *N. baileyi* engages in highly aggressive displays and occasional fin biting (MFC, pers. obs.; Johnston and Kleiner, 1994). Additionally, male *N. baileyi* are particularly distinctive in their tuberculation, and the common name, Rough Shiner, is apt: breeding males have fine tubercles on margins of scales along the body (Suttkus and

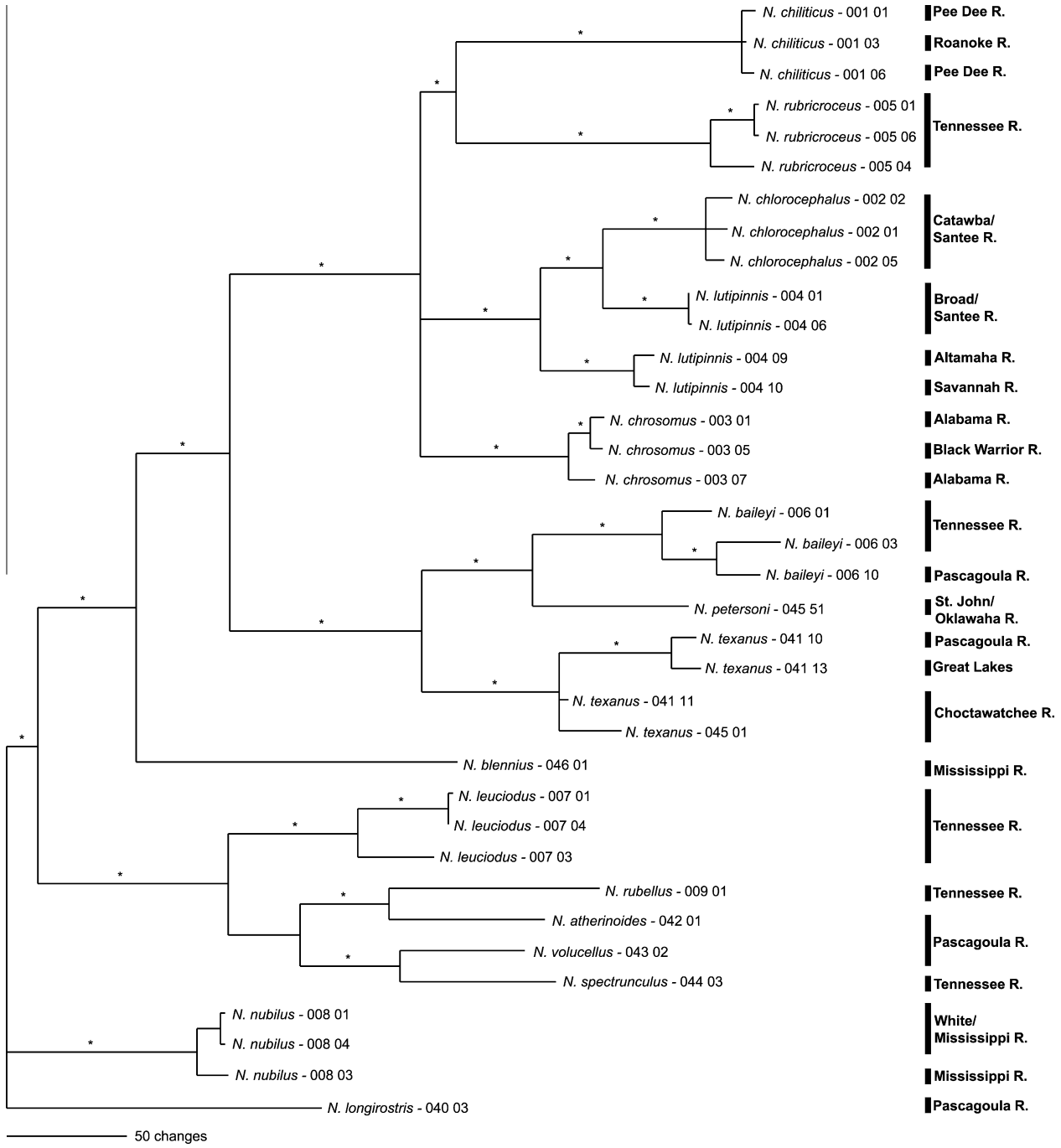

Fig. 6. 90% majority rule consensus tree from a partitioned mixed model Bayesian analysis of ITS1, RH, and ND2 sequence data; Bayesian posterior probabilities ⩾95% are indicated with an asterisk.

Raney, 1955; Swift, 1970) which can be easily felt when handling (female tuberculation is less pronounced and cannot be detected by touch). None of the core *Hydrophlox* have such prominent tuberculation, although all possess tubercles along scale margins (Swift 1970; Suttkus and Raney, 1955).

4.1. Genetic and morphological diversity within Hydrophlox

Within *Hydrophlox*, there is evidence that *N. lutipinnis* is composed of multiple taxa, from allozyme, pharyngeal tooth count, fin coloration (Wood and Mayden, 1992) and sequence data (presented here). During the course of this study, we observed notable color differences between populations of *N. lutipinnis* in the Broad River system versus those in the Altamaha and Savannah. Primary morphological differences include bright yellow head and fins in peak breeding condition fish in the Broad River system, versus mostly red bodies and heads and either orange or red fins in the other two river systems. Occasionally, some individuals in the Savannah exhibited a light yellow wash along the opercle and the gular region, but never intense yellow over the entire head as is seen in the Broad system. Additionally, *N. lutipinnis* in the Broad system do not have any obvious tuberculation on the head, whereas the Altamaha and Savannah forms both have a light shagreen of tubercles extending from the eyes to the snout on the dorsal portion of the head (Cashner, 2010). The Broad River system *N. lutipinnis* are more closely related to the *N. chlorocephalus* in the

Catawba River system (Wood and Mayden 1992; this study) than they are to the other *N. lutipinnis* populations. Recently, Rhode et al. (2009) published a distribution map of *N. chlorocephalus* which included the Piedmont of the Santee system (specifically, the Broad River and Saluda River systems) and the Lynches River system of the Pee Dee drainage in South Carolina. These populations were determined by Wood and Mayden (1992) to be sister to but distinct from *N. chlorocephalus*. We did not include samples from South Carolina in our study, however, our specimens 004.01 and 004.06 are from the Broad River system, and support the findings of Wood and Mayden (1992). Further study is warranted to investigate the number of species and the relationships among *N. lutipinnis* and *N. chlorocephalus*.

Chromatic differences occur among *N. chlorocephalus* found in the upper Catawba River system versus those in the lower Catawba River system. During peak spawning conditions (active aggregations over nest sites), those in the upper Catawba River system become completely red from snout to caudal peduncle with opaque white fins, while those in the lower part of the system have yellow heads and red bodies (Cashner, 2010). Some preliminary evidence from ND2, cyt*b*, and ITS1 suggest genetic differentiation as well (MFC, unpubl. data).

4.2. Relationships within Hydrophlox

Within the core *Hydrophlox* clade, there is a polytomy among the *N. rubricroceus* + *N. chiliticus* clade the *N. chlorocephalus* + *N. lutipinnis* clade and *N. chrosomus*. The ND2 (Fig. 3) and ITS1 (Fig. 4) topologies are incongruent with respect to the placement of *N. chrosomus*, either sister to the *N. lutipinnis* + *N. chlorocephalus* clade (ITS1) or sister to the *N. rubricroceus* + *N. chiliticus* clade (ND2). In the RH topology (Fig. 5), the only well-supported sister relationship within *Hydrophlox* is a *N. rubricroceus* + *N. chiliticus* clade, which may lend support to the ITS1 topology versus the ND2 topology. Incongruence among gene trees can be caused by multiple factors: recent or past hybridization, introgression, lineage sorting, and retention of ancestral polymorphisms. We can rule out recent hybridization because none of the species in question have ranges that currently overlap; however, historical hybridization and ancestral polymorphisms cannot be ruled out. Additional markers and larger taxon sampling may help to further elucidate the relationships within core *Hydrophlox*.

In conclusion, *Hydrophlox s.l.* is polyphyletic with respect to *N. baileyi*, *N. leuciodus*, and *N. nubilus*. We propose redefining the subgenus to include five nominal taxa: *N. rubricroceus, N. lutipinnis, N. chlorocephalus, N. chiliticus,* and *N. chrosomus*. Further studies are needed to clarify the taxonomic boundaries of the undescribed species within this monophyletic clade. The establishment of a solid phylogenetic hypothesis of relationships of *Hydrophlox* provides a framework from which to investigate the evolution of nest association in this group and in North American minnows in general.

Acknowledgments

This paper is based on the dissertation research of MFC. Funding was provided by grants from Sigma Xi (MFC), Graduate Women in Science Eloise Gerry Fellowship (MFC), American Museum of Natural History Theodore Roosevelt Memorial Fund (MFC), NSF Doctoral Dissertation Improvement Grant DEB 0710093 to MFC (HLB), NSF grants DEB 0237013 and EF 0431259 (HLB), and a Highlands Biological Station Grants in Aid (2005, MFC). We thank S. Woltmann, R.E. Blanton, J.W. Johansen, R.C. Cashner, and F.C. Cashner for help in specimen collection. Specimens were collected under scientific collecting permits on file with MFC and Tulane University IACUC protocol 0274R-UT-C.

References

Berendzen, P.B., Olson, W.M., Barron, S.M., 2009. The utility of molecular hypotheses for uncovering morphological diversity in the *Notropis rubellus* species comples (Cypriniformes: Cyprinidae). Copeia 2009, 661–673.

Berendzen, P.B., Simons, A.M., Wood, R.M., Dowling, T.E., Secor, C.L., 2008. Recovering cryptic diversity and ancient drainage patterns in eastern North America: historical biogeography of the *Notropis rubellus* species group (Teleostei: Cypriniformes). Mol. Phylogenet. Evol. 46, 721–737.

Berra, T.M., 2001. Freshwater fish distribution. Academic Press, San Diego, CA.

Bielawski, J.P., Gold, J.R., 2001. Phylogenetic relationships of cyprinid fishes in subgenus *Notropis* inferred from nucleotide sequences of the mitochondrially encoded Cytochrome *b* gene. Copeia 2001, 656–667.

Bielawski, J.P., Gold, J.R., 2002. Mutation patterns of mitochondrial H- and L-strand DNA in closely related cyprinid fishes. Genetics 161, 1589–1597.

Bortone, S.A., 1989. *Notropis melanostomus*, a new species of cyprinid fish from the blackwater-yellow River drainage of northwest Florida. Copeia 1989, 737–741.

Boschung, H.T., Mayden, R.L., 2004. Fishes of Alabama, Smithsonian Books, Washington, DC.

Breden, F., Ptacek, M.B., Rashed, M., Taphorn, D., Figueiredoss, C.A., 1999. Molecular phylogeny of the live-bearing fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae). Mol. Phylogenet. Evol. 12, 95–104.

Broughton, R.E., Gold, J.R., 2000. Phylogenetic relationships in the North American cyprinid genus *Cyprinella* (Actinopterygii: Cyprinidae) based on sequences of the mitochondrial ND2 and ND4L genes. Copeia 2000, 1–10.

Bufalino, A.P., Mayden, R.L., 2010. Molecular phylogenetics of North American phoxinins (Actinopterygii: Cypriniformes: Leuciscidae) based on RAG1 and S7 nuclear DNA sequence data. Mol. Phylogenet. Evol. 55, 274–283.

Cashner, M.F., 2010. Nest association in subgenus *Hydrophlox* (Cyprinidae: *Notropis*): a phylogenetic, behavioral and conservation approach. Ph.D. Dissertation, Tulane University.

Cavender, T.M., 1991. The fossil record of Cyprinidae. In: Winfield, I.J., Nelson, J.S. (Eds.), Cyprinid fishes: systematics biology and exploitation. Chapman and Hall, New York.

Chow, S., Hazama, K., 1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. Mol. Ecol. 7, 1255–1256.

Chow, S., Nakagawa, T., Suzuki, N., Takeyama, H., Matsunaga, T., 2006. Phylogenetic relationships among *Thunnus* species inferred from rDNA ITS1 sequence. J. Fish Biol. 68, 24–35.

Clayton, J.M., 2000. Life history aspects of three minnow species of the subgenus *Hydrophlox* (Pisces: Cyprinidae), *Notropis chiliticus, N. chlorocephalus,* and *N. lutipinnis*. PhD, George Mason University.

Cochran, P.A., 2000. William Bartram's early observation of a nest association between cyprinid fishes. Southeast. Fish. Counc. Proc. 40, 43–44.

Cunha, C., Mesquita, N., Dowling, T.E., Gilles, A., Coelho, M.M., 2002. Phylogenetic relationships of Eurasian and American cyprinids using cytochrome *b* sequences. J. Fish Biol. 61, 9290944.

Fletcher, D.E., 1993. Nest association of dusky shiners (*Notropis cummingsae*) and redbreast sunfish (*Lepomis auritus*), a potentially parasitic relationship. Copeia 1993, 159–167.

Fowler, J.F., James, P.W., Taber, C.A., 1984. Spawning activity and eggs of the Ozark minnow. Notropis nubilus. Copeia 1984, 994–996.

Gilbert, C.R., 1978. Type catalogue of the North American cyprinid genus Notropis. Bull. Fla. State Mus. Biol. Sci. 23, 1–104.

Glazier, J.R., Taber, C.A., 1980. Reproductive biology and age and growth of the Ozark minnow. Dionda nubila. Copeia 1980, 547–550.

Hillis, D.M., Davis, S.K., 1988. Ribosomal DNA: intraspecific polymorphism, concerted evolution, and phylogeny reconstruction. Syst. Zool. 37, 63–66.

Huelsenbeck, J.P., Bull, J.J., Cunningham, C.W., 1996. Combining data in phylogenetic analysis. Trends Ecol. Evol. 11, 152–158.

Humphries, J.M., Cashner, R.C., 1994. *Notropis suttkusi*, a new cyprinid from the Ouachita uplands of Oklahoma and Arkansas, with comments on the status of ozarkian populations of *Notropis rubellus*. Copeia 1994, 82–90.

Johnston, C.E., 1991. Spawning activities of *Notropis chlorocephalus, Notropis chiliticus,* and *Hybopsis hypsinotus*, nest associates of *Nocomis leptocephalus* in the southeastern United States, with coments on nest association (Cypriniformes: Cyprinidae). Brimleyana 17, 77–88.

Johnston, C.E., 1994a. The benefit to some minnows of spawning in the nests of other species. Environ. Biol. Fish. 40, 213–218.

Johnston, C.E., 1994b. Nest association in fishes: evidence for mutualism. Behav. Ecol. Sociobiol. 35, 379–383.

Johnston, C.E., Kleiner, K.J., 1994. Reproductive behavior of the rainbow shiner (*Notropis chrosomus*) and the rough shiner (*Notropis baileyi*), nest associates of the bluehead chub (*Nocomis leptocephalus*) (Pisces: Cyprinidae) in the Alabama River drainage. J. Ala. Acad. Sci. 65, 230–238.

Johnston, C.E., Page, L.M., 1992. The evolution of complex reproductive strategies in North American minnows. In: Mayden, R.L. (Ed.), Systematics Historical Ecology and North American Freshwater Fishes. Stanford University Press, Stanford, CA, pp. 600–621.

Jordan, D.S., Brayton, A.W., 1878. Contributions to North American Ichthyology, based primarily on the collections of the United States National Museum. III.–On the distribution of the Fishes of the Alleghany Region of South Carolina, Georgia, and Tennessee, with descriptions of new or little known species. Bull. US Natl. Music 12, 1–95.

Kocher, T.D., Conroy, J.A., McKaye, K.R., Stauffer, J., Jay, R., Lockwood, S.F., 1995. Evolution of NADH Dehydrogenase subunit 2 in east African cichlid fish. Mol. Phylogenet. Evol. 4, 420–432.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lavoue, S., Sullivan, J.P., Hopkins, C.D., 2003. Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. Biol. J. Linn. Soc. 78, 273–292.

Maddison, W.P., Maddison, D.R. 2009. Mesquite: a modular system for evolutionary analysis. V. 2.72. <http://mesquiteproject.org>.

Mayden, R.L., 1991. Cyprinids of the new world. In: Winfield, I.J., Nelson, J.S. (Eds.), Cyprinid fishes: systematics biology and exploitation. Van Nostrand Reinhold, New York, pp. 240–263.

Mayden, R.L., Chen, W.-J., Bart, J., Henry, L., Doosey, M.H., Simons, A.M., Tang, K.L., Wood, R.M., Agnew, M.K., Yang, L., Hirt, M.V., Clements, M.D., Saitoh, K., Sado, T., Miya, M., Nishida, M., 2009. Reconstructing the phylogenetic relationships of the earth's most diverse clade of freshwater fishes- order Cypriniformes (Actinopterygii: Ostariophysi): a case study using multiple nuclear loci and the mitochondrial genome. Mol. Phylogenet. Evol. 51, 500–514.

Mayden, R.L., Matson, R.H., 1988. Evolutionary relationships of eastern North American cyprinids: an allozyme perspective. ASIH Abstracts.

Menhinick, E.F., 1991. The Freshwater Fishes of North Carolina, North Carolina Wildlife Resources Commission, Raleigh, NC.

Moyer, G.R., Remington, R.K., Turner, T.F., 2009. Incongruent gene trees, complex evolutionary processes, and the phylogeny of a group of North American minnows (*Hybognathus* Agassiz 1855). Mol. Phylogenet. Evol. 50, 514–525.

Near, T.J., 2004. Investigating phylogenetic relationships of sunfishes and black basses (Actinopterygii: Centrarchidae) using DNA sequences from mitochondrial and nuclear genes. Mol. Phylogenet. Evol. 32, 344–357.

Near, T.J., Kassler, T.W., Koppelman, J.B., Dillman, C.B., Philipp, D.P., 2003. Speciation in North American black basses, *Micropterus* (Actinopterygii: Centrarchidae). Evolution 57, 1610–1621.

Nelson, J.S., 1994. Fishes of the World, third ed. John Wiley and Sons, Inc., Hoboken, NJ.

Outten, L.M., 1961. Observations on the spawning coloration and behavior of some cyprinid fishes. J. Elisha Mitchell Sci. Soc. 77, 118.

Piller, K.R., Bart, J., Henry, L., Hurley, D.L., 2008. Phylogeograpy of the greenside darter comples, *Etheostoma blennioides* (Teleostomi: Percidae): a wide-ranging polytypic taxon. Mol. Phylogenet. Evol. 46, 974–985.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Presa, P., Pardo, B.G., Martinez, P., Bernatchez, L., 2002. Phylogenetic congruence between mtDNA and rDNA ITS markers in Brown Trout. Mol. Biol. Evol. 19, 2161–2175.

Raley, M.E., Wood, R.M., 2001. Molecular systematics of members of the *Notropis dorsalis* species group (Actinopterygii: Cyprinidae). Copeia 2001, 638–645.

Raney, E.C., 1947. *Nocomis* nests used by other breeding cyprinid fishes in Virginia. Zoologica 32, 125–132.

Rhode, F.C., Arndt, R.G., Foltz, J.W., Quattro, J.M., 2009. Freshwater fishes of South Carolina. University of South Carolina Press, Columbia, SC.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Schonhuth, S., Doadrio, I., 2003. Phylogenetic relationships of Mexican minnows of the genus *Notropis* (Actinopterygii, Cyprinidae). Biol. J. Linn. Soc. 80, 323–337.

Schonhuth, S., Doadrio, I., Dominguez-Dominguez, O., Hillis, D.M., Mayden, R.L., 2008. Molecular evolution of southern North American cyprinidae (Actinopterygii with the description of the new genus *Tampichthys* from central Mexico. Mol. Phylogenet. Evol. 47, 729–756.

Schonhuth, S., Mayden, R.L., 2010. Phylogenetic relationships in the genus *Cyprinella* (Actinopterygii: Cyprinidae) based on mitochondrial and nuclear gene sequences. Mol. Phylogenet. Evol. 55, 77–98.

Sevilla, R.G., Diez, A., Noren, M., Mouchel, O., Jerome, M., Verrez-Bagnis, V., Van Pelt, H., Favre-Jrey, L., Krey, G., Consortium, T.F., Bautista, J.M., 2007. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome *b* and nuclear rhodopsin genes. Mol. Ecol. Notes. 7, 730–734.

Simons, A.M., Berendzen, P.B., Mayden, R.L., 2003. Molecular systematics of North American phoxinin genera (Actinopterygii: Cyprinidae) inferred from mitochondrial 12S and 16S ribosomal RNA sequences. Zool. J. Linn. Soc. 139, 63–80.

Simons, A.M., Mayden, R.L., 1998. Phylogenetic relationships of the western North American phoxinins (Actinopterygii: Cyprinidae) as inferred from mitochondrial 12S and 16S ribosomal RNA sequences. Mol. Phylogenet. Evol. 9, 308–329.

Suttkus, R.D., Raney, E.C., 1955. *Notropis baileyi*, a new cyprinid fish from the Pascagoula and Mobile Bay Drainages of Mississippi and Alabama. Tulane Studies in Zoology 2, 171–186.

Swift, C.C., 1970. A review of the eastern North American cyprinid fishes of the *Notropis texanus* species group (subgenus *Alburnops*), with a definition of the subgenus *Hydrophlox*, and materials for a revision of the subgenus *Alburnops*. Ph.D. dissertation, Florida State University.

Swofford, D.L., 1991. PAUP: Phylogenetic analysis using parsimony, Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Wallin, J.E., 1992. The symbiotic nest association of yellowfin shiners, *Notropis lutipinnis*, and bluehead chubs, *Nocomis leptocephalus*. Environ. Biol. Fish. 33, 287–292.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10, 506–513.

Warren, M.L., Burr, B.M., Grady, J.M., 1994. *Notropis albizonatus*, a new cyprinid fish endemic to the Tennessee and Cumberland River drainages, with a phylogeny of the *Notropis procne* species group. Copeia 1994, 868–886.

Wood, R.M., Mayden, R.L., 1992. Systematics, evolution, and biogeography of *Notropis chlorocephalus* and *N. Lutipinnis*. Copeia 1992, 68–81.

Wood, R.M., Mayden, R.L., Ronald, H.M., Kuhajda, B.R., Layman, S.R., 2002. Systematics and biogeography of the *Notropis rubellus* species group (Teleostei: Cyprinidae). Al. Mus. Nat. Hist. Bull. 22, 37–80.

Wyatt, P.M., Pitts, C.S., Butlin, R.K., 2006. A molecular approach to detect hybridization between bream *Abramis brama*, roach *Rutilus rutilus*, and rudd *Scardinius erythrophthalmus*. J. Fish. Biol. 69, 52–71.

Xia, X., Xie, Z., 2001. DAMBE: Software package for data analysis in molecular biology and evolution. J. Hered. 92, 371–373.