The genus *Pteronotropis* is widely distributed along the gulf slope of eastern North America from Louisiana to Florida and rivers in South Carolina along the Atlantic slope. *Pteronotropis* have very distinctive, flamboyant coloration. The habitats most frequently associated with these species include heavily vegetated backwater bayous to small sluggish or flowing tannin-stained streams. Although *Pteronotropis* is recognized as a valid genus, no phylogenetic analysis of all the species has corroborated its monophyly. In recent years, four additional species have been either described or elevated from synonymy: *P. merlini*, *P. grandipinnis*, *P. stonei*, and *P. metallicus*, with the wide-ranging *P. hypselopterus* complex. To examine relationships within this genus and test its monophyly, phylogenetic analyses were conducted using two nuclear genes, recombination activating gene 1, RAG1, and the first intron of *S7* ribosomal protein gene in both maximum parsimony and Bayesian analyses. In no analysis was *Pteronotropis*, a currently recognized, recovered as monophyletic without the inclusion of the currently recognized *Notropis harperi*, herein referred to as *Pteronotropis*. Two major clades are supported: one inclusive of *P. hubbsi*, *P. welaka*, and *P. harperi* and the second inclusive of *P. signipinnis*, *P. grandipinnis*, *P. hypselopterus* plus *P. merlini* sister to *P. euryzonus*, and *P. metallicus* plus *P. stonei*.

### 1. Introduction

The subfamily (or family) Leuciscinae includes all cyprinid species in North America, except *Notemigonus*, and species across Eurasia. Many of the species of this North American fauna have been examined in different phylogenetic studies at varying degrees of universality using both morphological and molecular data. Initial morphological studies by Mayden [1] and Coburn and Cavender [2] revealed exciting new relationships and a reclassification of the North American fauna. These studies were followed with several molecular analyses of different major lineages, genera, and species groups that supported many, but not all, of the monophyletic groups previously identified in one or both of the above studies [3–7]. However, not all proposed genera have been examined for species relationships using molecular markers.

One such genus in North America with an increasing and intriguing diversity, biology, and geographic distribution, as well as complex taxonomic history, is *Pteronotropis*. This genus contains one of North America’s most colorful shiners. *Pteronotropis hubbsi* and *P. welaka* are relatively slender-body species that, in breeding males, possess enlarged dorsal fins, whereas the remaining species, *P. euryzonus*, *P. hypselopterus*, *P. merlini*, *P. grandipinnis*, *P. stonei*, *P. metallicus*, and *P. signipinnis*, are more deep-bodied and lack enlarged dorsal fins in breeding males. The most frequently associated habitat of these species across their ranges includes deep, backwater bayous, small sluggish tannin-stained streams, and flowing tannin-stained streams, all with ample aquatic vegetation. However, despite several studies on shiners and relatives to date, *Pteronotropis* has received essentially no recent attention as to their relationships and has been proposed to be an unnatural grouping. Herein, we provide the first examination of phylogenetic relationships of all species in the genus (formerly subgenus of *Notropis* [1]) and a test of the monophyly of this purported lineage. Two nuclear genes are used in this analysis because of their previously demonstrated genetic distances and resulting ability to resolve nodes deeper than at
the crown of trees. These genes have been used successfully for resolution of more basal lineages of North American cyprinids by several recent papers [3–10]. Resulting phylogenetic inferences of species of this group and their eventual placement relative to other North American cyprinids are critical as they largely facilitate more process-level questions as to the evolution of the biology of the species and other lineages to better understand the processes of anagenesis and speciation. While multiple papers listed above have made groundbreaking strides in providing a phylogenetic framework where one previously did not exist for North American cyprinids, Hollingsworth et al. [10] provide an excellent evaluation of a subset of the fauna and a novel hypothesis as to habitat shifts for clades with differing rates of speciation. Given that no study has examined all of the species of Pteronotropis, we provide a review of the history of the genus and molecular phylogenetic analyses of the species using two nuclear genes that result in identical species using two nuclear loci (RAG1, S7), found both parsimony and likelihood analyses, Pteronotropis [18] used mitochondrial cytochrome b gene and failed to corroborate Pteronotropis as a monophyletic group. With both parsimony and likelihood analyses, P. euryzonus was sister to P. hypselopterus and an unrelated clade included P. signipinnis sister to P. hubbsi plus P. welaka. Later, in a subsample of Pteronotropis species, Simons et al. [4], using two mitochondrial genes (12S, 16S), and Bufalino and Mayden [5, 6], using two nuclear loci (RAG1, S7), found Pteronotropis as monophyletic but, again, only with the inclusion of “Notropis” harperi; however, neither of these analyses included all species of the genus. Other early molecular data and analyses also failed to resolve the phylogenetic relationships of the above species that were generally phenetically similar. Most recently a study by Hollingsworth et al. [10], using one mtDNA gene and nDNA genes, corroborated the monophyly of a subsample of species of Pteronotropis that also included N. harperi.

While there have been several efforts testing the monophyly of Pteronotropis, its composition, and at resolving the phylogenetic relationships of species since its elevation to genus, no single study has included all of the species in the genus and appropriate outgroups based on earlier studies and some did not include the morphologically similar Notropis harperi. With the elevation of species from synonymy with P. hypselopterus and the description of a new species [12], the complexity involved in testing the monophyly of the genus and species relationships have become even more biologically interesting. While Suttkus and Mettee [12] did provide dialogue invoking phylogenetic terminology as to species relationships, their study contained no phylogenetic analyses, no discussions of character homology, or any morphological or molecular synapomorphies. To date, no investigation has been completed for this group inclusive of all of the purported species of Pteronotropis. Thus, the objectives of the current study are twofold: (1) testing the monophyly of the genus and (2) examining relationships of all of the purported species of the genus using two nuclear genes.

2. Materials and Methods

2.1. Specimens and DNA Extraction/Amplification and Alignment. Museum catalogue numbers for vouchers in this study include UAIC (University of Alabama Ichthyological Collection) and SLUM (Saint Louis University Museum). Specimens examined in this study were either frozen at Saint Louis University, preserved in 95% ethanol, or captured alive and transported to Saint Louis University (Table 1). Outgroup taxa included species from the genera Cyprinella, Lythrurus, and Notropis. Species of Cyprinella were included
### Table 1: Species, localities, and GenBank numbers of specimens used for sequencing and analyses of S7 and RAG1.

(a)

| Species and drainage | Stream, county, state     | Catalogue number | S7         | RAG1         | Extraction |
|----------------------|---------------------------|------------------|------------|--------------|------------|
| **Pteronotropis euryzonus** |                           |                  |            |              |            |
| Chattahoochee R.      | Maringo Cr., Russell, AL  | UAIC 12229       | KM048270   | KJ634252     | 22         |
| Chattahoochee R.      | Snake Cr., Russell, AL    | UAIC 10493       | KM048276   | KJ634258     | 51         |
| Chattahoochee R.      | Snake Cr., Russell, AL    | UAIC 10493       | KM048277   | KJ634259     | 52         |
| **Pteronotropis grandipinnis** |                     |                  |            |              |            |
| Apalachicola R.        | Irwin Mill Cr., Houston, AL | No voucher      | KM048265   | KJ634247     | 12         |
| Apalachicola R.        | Irwin Mill Cr., Houston, AL | No voucher      | KM048266   | KJ634248     | 13         |
| **Pteronotropis hypseloceptorus** |                        |                  |            |              |            |
| Mobile R.              | Cedar Cr., Mobile, AL     | UAIC 12730       | KM048256   | KJ634238     | 01         |
| Mobile R.              | Cedar Cr., Mobile, AL     | UAIC 12730       | KM048257   | KJ634239     | 02         |
| Mobile R.              | Cedar Cr., Mobile, AL     | UAIC 12730       | KM048258   | KJ634240     | 03         |
| Alabama R.             | Little Reedy Cr., AL     | UAIC 14326       | KM048269   | KJ634251     | 18         |
| **Pteronotropis hubbsi** |                          |                  |            |              |            |
| Ouachita R.           | Backwater pond, Ouachita, LA | UAIC 11928   | KM048261   | KJ634243     | 06         |
| Ouachita R.           | Backwater pond, Ouachita, LA | UAIC 11928   | KM048262   | KJ634244     | 07         |
| Little R.             | Little R., McCurtain, OK  | UAIC 12053       | KM048273   | KJ634255     | 41         |
| **Pteronotropis merlini** |                     |                  |            |              |            |
| Pea R.                | Clearwater Cr., Coffee, AL | No voucher      | KM048267   | KJ634249     | 16         |
| Pea R.                | Clearwater Cr., Coffee, AL | No voucher      | KM048268   | KJ634250     | 17         |
| **Pteronotropis metallicus** |                     |                  |            |              |            |
| Suwannee R.           | Sampson R., Bradford, FL  | UF 158855        | KM048278   | KJ634260     | 96         |
| Suwannee R.           | Sampson R., Bradford, FL  | UF 158855        | KM048279   | KJ634261     | 97         |
| **Pteronotropis signipinnis** |                     |                  |            |              |            |
| Pascagoula R.         | Beaverdam Cr., Forest, MS | UAIC 13416       | KM048259   | KJ634241     | 04         |
| Pascagoula R.         | Beaverdam Cr., Forest, MS | UAIC 13416       | KM048260   | KJ634242     | 05         |
| Mobile R.             | Cedar Cr., Mobile, AL     | UAIC 12730       | KM048271   | KJ634253     | 23         |
| Mobile R.             | Cedar Cr., Mobile, AL     | UAIC 12730       | KM048272   | KJ634254     | 24         |
| **Pteronotropis stonei** |                          |                  |            |              |            |
| N. Fork Edisto R.     | Murphy Mill Cr., Calhoun, SC | SLUM 1121   | KM048281   | KJ634263     | 101        |
| N. Fork Edisto R.     | Murphy Mill Cr., Calhoun, SC | SLUM 1121   | KM048280   | KJ634262     | 100        |
| Combahee R.           | Savannah Cr., Colleton, SC | SLUM 1122   | KM048282   | KJ634264     | 102        |
| **Pteronotropis welaka** |                          |                  |            |              |            |
| Cahaba R.             | Lightsey pond, Bibb, AL   | UAIC 10391       | KM048263   | KJ634245     | 10         |
| Cahaba R.             | Lightsey pond, Bibb, AL   | UAIC 10391       | KM048264   | KJ634246     | 11         |
| Pearl R.              | Lees Cr., Washington, LA  | UAIC 12205       | KM048274   | KJ634256     | 48         |
| Mobile Bay            | Lees Cr., Washington, LA  | UAIC 12205       | KM048275   | KJ634257     | 49         |
| **Pteronotropis harperi** |                      |                  |            |              |            |
|                      |                           | GU134235         | GU136332   |              |            |

(b)

Outgroup taxa (note that *Pteronotropis harperi* was also originally an outgroup species)

| Species                      | S7       | RAG1   |
|------------------------------|----------|--------|
| *Cyprinella formosa*         | GU134192 | GU136293 |
| *Lythrurus fumeus*           | GU134222 | GU136231 |
| *Lythrurus umbratilis*       | GU134223 | GU136322 |
| *Nocomis leptoccephalus*     | GU134236 | GU136333 |
| *Notropis asperifrons*       | GU134231 | GU136330 |
consisted of 35 cycles of an initial denaturation of 95 °C.

Amplifications were performed using a BigDye labeled dideoxy sequencing kit (QIA-GEN, Valencia, CA). Sequencing was performed using a BigDye labeled dideoxy sequencing kit (BigDye) and visualized on an ABI 377 automated sequencer performed using a BigDye labeled dideoxy sequencing kit (QIA-GEN, Valencia, CA). The two nuclear genes included recombination activating gene 1, RAG1, and the first intron of S7 ribosomal protein gene. Both genes were amplified, via PCR, and internal primers amplification and sequencing were developed for S7. These include the forward primers 5'-GCCACTGCAGGCAGCTAAT-3' and 5'-GCCCCA-GCTTTCCACCATTAC-3' and reverse primers 5'-CCC-GAGGGCTGTGAGGAGTAA-3' and 5'-CCCCCTCAG-CGGCCGACTA-3'. Universal primers for RAG1 and S7 were developed in López et al. [19] and Chow and Hazama [20], respectively. In addition, both forward and reverse internal primers were developed for S7. For RAG1, each 25 μL PCR reaction consisted of 2 μL of DNTPs, 2.5 μL of 10X Taq buffer, 3 μL of both forward and reverse primers, 10.375 μL of dH2O, 1 μL of Taq polymerase, or 0.125 μL of HotStart Taq Polymerase (QIA-GEN, Valencia, CA). Amplifications consisted of 35 cycles of an initial denaturation of 95°C for 15 minutes with an additional denaturation of 94°C for 40 seconds. This was followed by an annealing temperature of 55°C for 1 minute, an initial extension of 72°C for 90 seconds, and a final extension of 72°C for 5 minutes. Conditions for S7 were identical except the annealing temperature was set at 59°C. For the S7 intron, products that failed to amplify using the universal primers were reamplified using nested primers. Taxa failing to amplify with internal primers were cloned using the pGEM-T Easy Vector System kit (PROMEGA, Madison, WI) as outlined in Lang and Mayden [9]. PCR products were purified using QIA-GEN gel extraction kits (QIA-GEN, Valencia, CA). Sequencing was performed using a BigDye labeled dideoxy sequencing kit (BigDye) and visualized on an ABI 377 automated sequencer (Auburn University Molecular Genetics Instrumentation Facility, Auburn, AL) or an ABI 3700 (Macrogen Sequencing Facility, Seoul, South Korea). Both the heavy and light strands were sequenced for all samples and the sequences were aligned with Clustal X [21] with reference to the accompanying electropherograms. Some individuals contained heterozygote peaks in the RAG1 data and these heterozygote base pair positions were coded using standard degeneracy codes.

### Results and Discussion

As the ILD test was nonsignificant for heterogeneity between RAG1 and S7, the gene sequences were analyzed both individually and as a concatenated data set. MP analysis of the aligned 1001 bp of S7 (aligned sequence lengths ranged from 839 to 919 bp) yielded 245 bp parsimony informative sites (12.9%). Analyses of these data resulted in 90 equally parsimonious trees (Figure 1: length = 697, CI = 0.803, and RI = 0.875). The more conservative RAG1 sequences included 1521 bp with 151 bp sites (9.9%) being parsimony-
informative. MP analyses of RAG1 resulted in 46,668 equally parsimonious trees (Figure 1; length = 371 steps, CI = 0.658, and RI = 0.866). Individual BA analyses for each gene resulted in some variations in sister-group relationships but all were consistent and supported the monophyly of *Pteronotropis* (Figure 2). Both MPA and BA of the combined S7 + RAG1 data recovered identical topologies (Figure 3).

As in previous studies involving species of *Pteronotropis*, nuclear sequence variation, neither individual nor combined [5, 6], resolved *Pteronotropis* as a monophyletic group if *Notropis harperi* is excluded from the genus. Constraining *Pteronotropis* to be monophyletic in the S7 + RAG1 data set without *N. harperi* resulted in a significantly worse tree (1246 steps). In both BA and MPA, *Notropis harperi* is resolved as sister to *P. welaka* within the ingroup, a sister-group relationship with strong PP and BS support (Figures 1 and 2). *Pteronotropis hubbsi* is resolved as sister to this clade, also with strong PP and BS support. All three of these taxa (*P. hubbsi* (*P. welaka + *N. harperi*)) are resolved as monophyletic and sister to the remaining species traditionally referred to as *Pteronotropis* (PP 95, bootstrap 75; Figure 2). The strong support for the monophyly of the (*P. hubbsi* (*P. welaka + *N. harperi*)) clade (Figures 1 and 2) is logical as the three species are phenetically and ecologically similar. They possess aspects of similar body coloration in life when not in breeding condition and have similar habitat associations [5, 26, 27]. They are found in deep pools with ample aquatic vegetation and in areas where *P. welaka* and *N. harperi* are sympatric they are often taken syntopically in a sample (pers. obs.). The authors are unaware of any studies corroborating nest association in *N. harperi*, as observed in *P. welaka* and *P. hubbsi* [28–30]. In light of the relationships presented here and in Bufalino and Mayden [4, 5] and Hollingsworth et al. [10], studies of *N. harperi* may reveal ecological and behavioral synapomorphies.

In all analyses, *P. signipinnis* is resolved as sister to a clade of remaining species of *Pteronotropis* (Figures 1 and 2). In analyses of S7 and S7 + Ragi data sets, the latter clade formed two clades: one inclusive of *P. hypselopterus*, *P. grandipinnis*, and *P. merlini* and the other inclusive of *P. euryzonus*, *P. stonei*, and *P. metallicus*. Resolution of the former clade was not complete in either Ragi or S7 analyses, but both are fully consistent with the phylogeny recovered with the Ragi + S7 data set. These relationships are in contrast to those hypothesized by Simons et al. [4] based on 12S and 16S ribosomal RNA sequences wherein *P. signipinnis* was resolved as sister to
These relationships are also consistent with those presented by Bailey and Suttkus [13] using mitochondrial gene ND2. In recent years, the general trend in phylogenetics has been to place greater emphasis on the use of nuclear genes, largely because of issues associated with hybridization, intergradation, lineage sorting, and disagreement between gene and species trees [13]. While these nuclear genes have shown a greater ability to resolve relationships at supraspecific levels for this group with greater consistency and stronger branch support, the results presented herein illustrate the benefit in using nuclear genes. However, it is also true that mitochondrial genes have been extremely useful in phylogenetic resolutions [26, 27], and like nuclear genes they also vary in their degree of anagenesis and abilities to resolve trees at different levels of universality. While these and other nuclear genes used in the above-cited papers for Cypriniformes clearly display a reduced phylogenetic signal and are more limited in phylogenetic resolution for relationships of populations and species, they are essential for resolution of deeper nodes. This is to be expected as rates of mutation of many nuclear genes (especially protein coding) are generally not as high as that typically found in most mitochondrial genes.

4. Conclusions

Given the consistent sister-group relationship between formerly recognized Notropis harperi and Pteronotropis welaka, the former species is herein referred to as Pteronotropis. Nuclear genes RAG1 and S7 support the long-standing question/hypothesis regarding the monophyly of Pteronotropis and provide new insight into the phylogenetic placement of Pteronotropis harperi and the basal-most relationships between the species groups (P. hubbsi, P. welaka, and P. harperi) relative to the remaining species of Pteronotropis. These relationships are also consistent with those presented by Bailey and Suttkus [13] using mitochondrial gene ND2. In recent years, the general trend in phylogenetics has been to place greater emphasis on the use of nuclear genes, largely because of issues associated with hybridization, intergradation, lineage sorting, and disagreement between gene and species trees [13]. While these nuclear genes have shown a greater ability to resolve relationships at supraspecific levels for this group with greater consistency and stronger branch support, the results presented herein illustrate the benefit in using nuclear genes. However, it is also true that mitochondrial genes have been extremely useful in phylogenetic resolutions [26, 27], and like nuclear genes they also vary in their degree of anagenesis and abilities to resolve trees at different levels of universality. While these and other nuclear genes used in the above-cited papers for Cypriniformes clearly display a reduced phylogenetic signal and are more limited in phylogenetic resolution for relationships of populations and species, they are essential for resolution of deeper nodes. This is to be expected as rates of mutation of many nuclear genes (especially protein coding) are generally not as high as that typically found in most mitochondrial genes.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.
Figure 3: Inferred species relationships of species of *Pteronotropis* based on maximum parsimony and Bayesian analyses of combined Rag1 + S7 (a) and Rag1 + S7 (b), respectively. Nodal values indicate posterior probabilities.

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