Journal of the Arkansas Academy of Science

Volume 71 Article 25

2017

Phylogeography and Vicariant Separation of Two River Darters, Percina uranidea and Percina vigil, from rivers that drain the North American Interior Highlands

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Running Title: Ouachita and White River Darter Phylogeography

Abstract

The phylogeography and separation of two river darters, *Percina uranidea* and *P. vigil* were investigated through sequencing of the mitochondrial Cytochrome B and Cytochrome Oxidase genes. These molecular markers revealed the darters exhibit moderate genetic divergence between two large river drainage systems within the Mississippi River basin associated with the Interior Highlands of midwestern North America. An additional haplotype network analysis also supported these trends. Phylogenetic divergence dating indicated that population separation between the river systems occurred after recent Pleistocene glacial events rather than an early Pleistocene separation.

Introduction

The aquatic fauna of the southern United States exhibit a diverse evolutionary and ecological structure due to processes such as regional geology, anthropogenic impacts, climate change and subsequent habitat alterations. In the eastern Gulf Coastal Plain, fish in river systems draining the Appalachian and associated highlands were affected by upland stream changes but also through specific processes more common in lowlands, e.g., river meanders, stream capture, sea level changes, as well as streamflow and sediment load alteration from Pleistocene glacial cycles (Galloway *et al*. 2011; Shen *et al*. 2012 Egge and Hagbo 2015). These and other historical effects created the unique distributions of Gulf Coastal Plain aquatic taxa. In the western Gulf Coastal Plain, consisting primarily of the Mississippi Embayment, river systems evolved through similar processes, but created their own unique faunal distributions (Egge and Hagbo 2015). One of the unique geological features that affected the current streamflow patterns in western Mississippi Embayment was the Interior Highlands.

The Interior Highlands of Arkansas, Missouri, and Oklahoma, USA represent a unique and distinct biogeographic region in North America. The aquatic fauna in the Interior Highlands are often associated with Appalachian and eastern North American connections as many species are derived from eastern species, and the Interior Highlands are considered the western disjunct region of the eastern North American Central Highlands (Mayden 1985; Strange and Burr 1997; Bossu *et al*. 2013). Many of these eastern species exhibit their western North American boundary in the Interior Highlands or near the western periphery of the Highlands (Robison and Buchanan 1988; Trauth *et al*. 2004). The Interior Highlands are separated into the Ouachita and Ozark mountain regions and the Arkansas River Valley, each with a unique geologic structure (Robison and Buchanan 1988; Guccione 1993; The Nature Conservancy 2003; Zollner 2003). The Interior Highlands are considered glacial refugia for many taxa and possess a complex mixture of aquatic fauna. Stream changes precipitated by Pleistocene glaciation cycles resulted in altered species distributions, endemism, relict populations, range expansion, and speciation that have led to the contemporary aquatic fauna (Mayden 1985; Near *et al*. 2001; Near and Keck 2005; Berendzen *et al*. 2010).

Not only have these Pleistocene events caused aquatic species separation between the eastern Highlands and the Interior Highlands, they have affected species distributions within and surrounding the Interior Highlands, i.e., the Ouachita and White Rivers systems. Rivers within the unglaciated Interior Highlands were altered when glacial cycles changed river volumes, lowered sea levels, and allowed stream capture (Mayden 1985, 1988; Elfrink *et al*. 2008; Blanton *et al*. 2013). Several hypotheses highlight events that affected aquatic fauna within and surrounding the Interior Highlands. The Pre-Pleistocene Ouachita River in southern Arkansas may have originated further west and encompassed portions of the present Red River instead of its current origins in the Ouachita Highlands and caused vicariant

population separation in the smaller streams within the pre-Pleistocene Ouachita River (Mayden 1985; Ross 2013). Mayden (1985) proposed that stream drainage alterations throughout the Pleistocene, such as those between the Ouachita and Red River systems, may have caused peripheral isolation and microvicariance in rivers within the Interior Highlands. One large river drainage change includes the extension of the pre-Pleistocene Arkansas River to its larger, current drainage and stream flow pattern, which separated Ozark from Ouachita populations. Another major change occurred when the Pleistocene Mississippi River altered its course multiple times from the eastern edge of the Interior Highlands across the Mississippi Embayment (Mayden 1988; Saucier 1994; Blum *et al*. 2000). These cyclic expansions and reductions in stream volume, flow, and drainage patterns created a unique and complex pattern seen in many aquatic Ozark fauna (Mayden 1988; Hardy *et al*. 2002; Ray *et al*. 2006; Sabatino and Routman 2008; Blanton *et al*. 2013).

Pleistocene glaciation not only affected aquatic populations in Interior Highlands higher gradient, clear streams, they have impacted the fauna in larger streams of the Mississippi Embayment that drain the Interior Highlands. The alteration of drainage patterns in these larger streams such as the formation of the contemporary Mississippi and Arkansas Rivers also changed stream habitat, current flow, and separated populations (Mayden 1985; Ray *et al*. 2006; Lang and Echelle 2011). Understanding the geographic distribution patterns in lowland fish may be further confounded as these populations may have experienced greater connectivity among populations for longer time periods due to the reduced gradient and higher water volumes in these rivers with increased duration of high water events (Lang and Echelle 2011; Egge and Hagbo 2015). Additionally, these populations may have historically experienced greater streamflow stability as larger streams are more likely to persist during drought conditions. These factors may have resulted in larger, more stable fish populations with sufficient genetic variation to slow genetic differentiation among populations. Even with larger population density, these lowland populations were affected by large perturbations such as drainage alterations that occurred during the Pleistocene and isolated populations. To determine Pleistocene effects upon fish species distributions in larger streams and discriminate how vicariance affected fish inhabiting larger river systems draining the Interior Highlands, a phylogeographic analysis was conducted with *Percina uranidea* (Jordan and Gilbert 1887) and *P. vigil* (Hay 1882), two darter species with limited geographic distributions in the larger rivers that drain the Interior Highlands.

Although both species inhabit medium-sized streams that drain the Interior Highlands and inhabit the western Mississippi embayment, *P. uranidea* exhibits a limited distribution when compared to *P. vigil* with the current distribution of *P. uranidea* confined to Arkansas and Missouri. The bulk of *P. uranidea* distribution occurs in Arkansas (Page 1983) with disjunct populations occurring in the White River and Ouachita River drainages. Kuehne and Barbour (1983) reported that *P. uranidea* also occurs in the St. Francis River although the species has not been captured from that river in many years (Robison and Buchanan 1988). Historical records show that the species occurred in, but has since been extirpated from, the lower Wabash River of Indiana and Illinois (Page 1983) and the Ouachita River of Louisiana (Chris Davidson, USFWS, *pers. comm*.). *Percina uranidea* is currently listed as a species of lower risk near threatened (Gimenez 2008), or vulnerable (Arkansas Natural Heritage Commission 2007), and a species of greatest conservation need (Anderson 2006).

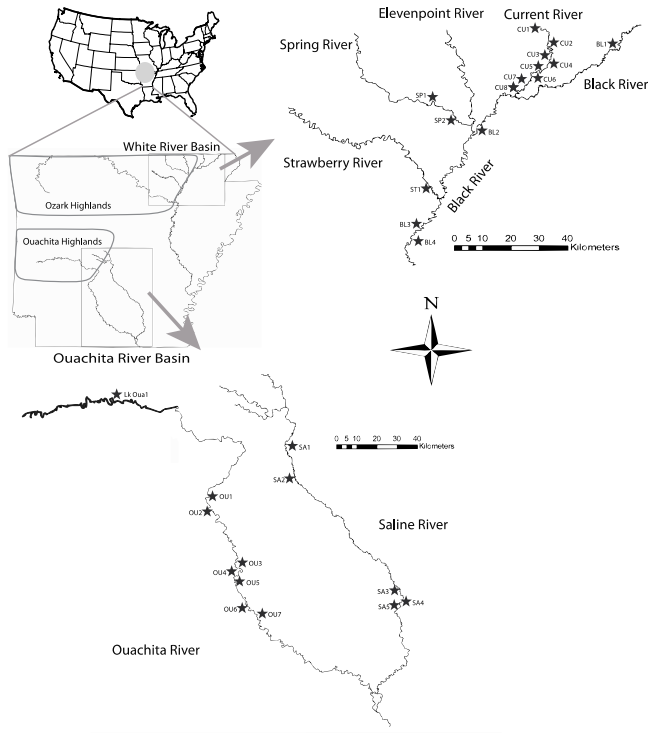

Figure 1. Location map for collected *P. uranidea* and *P. vigil* samples in the White River and Ouachita River basins. See Table S1 for locality information and GenBank Accession data.

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DOI: https://doi.org/10.54119/jaas.2017.7125

Percina vigil, with its greater distribution than *P. uranidea*, ranges from northwest Indiana to southeast Missouri, south to east central Louisiana to northwest Florida. Page (1983) reported that its distribution is sporadic, with locally abundant populations; however, within the state of Arkansas, its distribution mirrors *P. uranidea*, with disjunct populations occurring in the White River and Ouachita River drainages. *Percina vigil* and *P. uranidea* are reportedly found in different habitats within moderate-sized rivers, with *P. vigil* found in shallow habitats with fine gravel or sand bottoms and *P. uranidea* found along gravel bottoms in deeper water, although they are usually syntopic within the state of Arkansas (Robison and Buchanan 1988).

The unique distribution of *P. vigil* and *P. uranidea* in Arkansas can also illuminate if vicariance separation occurred earlier versus later in the Pleistocene. If these fish were affected through the alteration of connections disrupting dispersal during the Pleistocene, the resultant phylogenetic tree would show shorter branches and more haplotype sharing among populations indicating a recent disruption of gene flow among streams. Consequently, molecular clock dating analysis should indicate a more recent divergence between White River and Ouachita River populations. Alternatively, if these populations were separated via early vicariance events, the phylogenetic tree should show a deep separation between the two river drainages with reduced haplotype sharing within river drainages, i.e., a hierarchical haplotype distribution showing reduced haplotype similarity among populations within a river and unique haplotypes among different rivers in a river drainage. The molecular clock dating analysis should also show deep divergence times between river drainages. Furthermore, if early Pleistocene peripheral isolation affected these populations, the phylogenetic tree should exhibit deep branches at the tips with shorter divergence among river basins.

Materials and Methods

Sample collection and preparation

Sixty three *P. uranidea* and 40 *P. vigil* specimens were collected from the Arkansas portions of seven rivers in these darter's historical distribution (28 collection sites): the Black River, Current River, Spring River, Strawberry River, and Eleven Point River of the White River drainage and the Ouachita River and Saline River of the Ouachita River drainage (Figure 1 and S1). Collection sites were based on access ease and favourable sampling conditions, and included an upper, middle, and lower segment of each river within the Arkansas border. The length of the 24 sampled segments measured approximately 123 kilometers.

Fish were sampled with a Missouri trawl (a modified balloon trawl) towed behind a boat at an average depth of 1.76 m (range: 0.46-3.74 m). The net is composed of larger mesh netting (38 mm) encased by smaller mesh netting (6 mm). Compared to other sample methods, the Missouri trawl has been shown to more effectively capture small-bodied, benthic fishes, such as *P. uranidea* and *P. vigil*, in moderate to large river systems (Herzog *et al*. 2005). When sampling conditions were not conducive to using the Missouri trawl (patchy environments, untrawlable stretches due to too much debris, and too shallow water), kick-electrofishing with a backpack shocker and a downstream blocknet was a secondary method to capture darters. Upon capture, the left pectoral and caudal fins were removed and preserved in 100% v/v ethanol in a -20°C freezer. Voucher specimens were deposited in the Arkansas Tech University Fish Collection.

DNA sequencing

Total genomic DNA from fish fin clips was extracted with the FastID genomic DNA extraction kit (GeneticIDNA Inc., USA). Extracted genomic DNA was stored in molecular biology grade water (Sigma Chemical Co., USA) at -20°C until molecular analyses. The entire mitochondrial Cytochrome *b* gene (Cyt *b*) was amplified with primers described in Near *et al.* (2000) and Brogdon *et al*. (2003). In addition, new primers were developed for the mitochondrial Cytochrome Oxidase I (*COX I*) gene through alignment of the gene in three species (*E. radiosum*, GenBank Accession: AY 34348; *P. macrolepida*, DQ 536430; and *I. Furcatus*, AF484165.2): *COX* 1F (Forward primer) 5'- GTG-GCC-ACC-ACA-CGT-TGA-TTC-TTC-TCG -3' and *COX I*-1500R (Reverse Primer) 5'- GCR-GGC-TCT-TCA-AAT-RTR-TGG-TAG-GG -3'. These mitochondrial genes appear to be well suited for delineating intraspecific relationships and may be better suited for this purpose than nuclear genes such as RAG1 and S7 intron (Near *et al*. 2011, Blanton *et al*. 2013). However, mitochondrial introgression is reported in some darters, but does not appear to be as significant in *Percina* species (Near *et al*. 2011).

Each PCR reaction for Cyt *b* and *COX I* was performed in 25-µL aliquots with the following ingredients: 10-µL total genomic DNA (10 – 50 ng),

1X Taq Buffer (150 mM Tris-HCl pH 8.5, 40mM $(NH_4)_2SO_4$, 3.0mM $MgCl_2$. 0.2% v/v Tween 20), 1 mM for each dNTP, 0.5 µM of each primer, 6.25 units REDTaq DNA polymerase (Sigma Chemical Co., USA), 1.6% v/v Dimethyl sulfoxide, 0.6% w/v BSA, and 1.6% v/v Formamide. The cycling conditions consisted of an initial denaturation period of five minutes at 94 °C followed with 30 one-minute cycles of 94 °C, 50 °C annealing, 72 °C extension, and a final seven-minute extension at 72 °C. After PCR products were verified with agarose electrophoresis in a 0.9% w/v agarose concentration, they were GeneCleaned to remove PCR impurities (Bio 101 Inc., USA). Forward and reverse DNA sequencing was performed with PCR primers for both sequences at the UAMS DNA Core Sequencing Facility on an Applied Biosystems 3100 Genetic Analyzer, Big Dye Terminator Chemistry, Kit version 1.1 (Foster City, CA, USA). For *COX I*, two additional internal primers along with the previous PCR primers were employed to provide additional sequencing products for a more complete sequence contig: *COX I*-961F 5'- TTT-AGC-TGA-CTC-GCA-ACY-CTT-C -3' and *COX I*-1185R 5'- GCC-CGA-GAA-TAG-MGG-GAA-TCA-GTG -3'.

After sequencing, all trace files were reviewed by eye and all ambiguous bases removed from further analysis. Alignment of the sequence data was conducted with Clustal X and Geneious Pro 3.7 (Thompson *et al*. 1997; Drummond *et al*. 2009). After the initial alignment and contig creation, all sequences were converted into their amino acid sequences to verify if any internal stop codons existed. All sequences were deposited in GenBank with the following accession numbers for Cyt *b*: KC211117-KC211117. GenBank accession numbers for *COX I* sequences are KC211058-KC211116.

Data Analysis and phylogenetic tree production

Several outgroups from GenBank records were included for the analyses. The Cyt *b* and *COX I* sequences were not concatenated as outgroup sequences retrieved from GenBank were varied in sequence size among individuals and *COX I* sequences were typically smaller than our sequences (650bp vs >1kb). For the Cyt *b* Bayesian analysis, 18 sequences were retrieved from GenBank that included the following outgroups: *P. caprodes, P. macrolepida, P. lenticula, P. antesella, P. copelandi, P. aurora, P. brevicauda, P. tanasi,* and *P. shumardi* (Table S1). The *COX I* outgroups included these species: *P. caprodes, P. maculata, P. lenticula, P. antesella, P. copelandi, P. aurora, P. brevicauda, P. tanasi,* and *P. shumardi* (Table S1). These outgroups were identified through examination of the closest species to *P. uranidea* and *P. vigil* (Near *et al*. 2011). A total of 65 additional *P. uranidea* and *P. vigil* sequences were added for *COX I* from GenBank to include samples from outside Arkansas (Table S1).

All aligned DNA sequences were entered into MODELTEST version 3.7 in HyPhy, and the model of nucleotide sequence evolution (Cyt *b*: GTR+I+G, -lnL = 4342.0; *COX I*: GTR+I+G, -lnL = 4650.9) was chosen with the Akaike (AIC) criteria (Posada and Crandall 1988; Posada 2009; Kosakovsky *et al.* 2006). These sequences were analysed with Bayesian methods with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with these parameters: four separate Metropolis-coupled Monte Carlo Markov chains, random starting trees with 20 X 10^6 generations with samples taken every 100 generations, and 25% of the resultant trees removed as burn-in. A 50% majority-rule consensus tree was produced with nodal posterior probability support from the four runs post burn-in. The average standard of split frequencies was examined to determine if they dropped to a low, convergent value below 0.005. The outputs from the Bayesian analyses with TRACER v1.6 (Drummond *et al*. 2012) were reviewed to evaluate the robustness of the Bayesian analyses with respect to burn-in, effective sample size, stationary distribution, and posterior.

Population Statistics

As population divergence was considered to be potentially minor, additional analyses were conducted to better understand population structure and evolution. Analyses that consider population level processes such as a multitude of haplotypes in populations and recombination encompass parameters that may not be considered in strict phylogenetic analyses (Clement *et al.* 2000; Althoff and Pellmyr 2002; Hey and Machado 2003). Haplotype network analysis was conducted on Cyt *b* sequences in TCS with 95% connection limits (Clement *et al*. 2000). Any network loops that caused ambiguities were resolved according to Pfenninger and Posada (2002).

To further explore patterns in our data, several population genetics statistics were conducted. These statistics were summarized with Arlequin 3.01 (Excoffier *et al*. 2005). Populations were grouped into two regional groups corresponding to their current disjunct distributions in the Ouachita and White river basins. These statistics were conducted with both *P. uranidea* and *P. vigil* to determine if any evidence of recent expansion and non-neutrality of DNA sequences

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DOI: https://doi.org/10.54119/jaas.2017.7125

existed in these regional groups. To test this hypothesis, Fu's F_s and Tajima's D were calculated in Arlequin 3.01 (Tajima 1989; Fu 1997; Excoffier *et al*. 2005). Significant negative values of these statistics indicate non- neutrality and population expansion: Fu's F_s below a p-value of 0.02 indicate population expansion (Fu 1997; Excoffier *et al*. 2005).

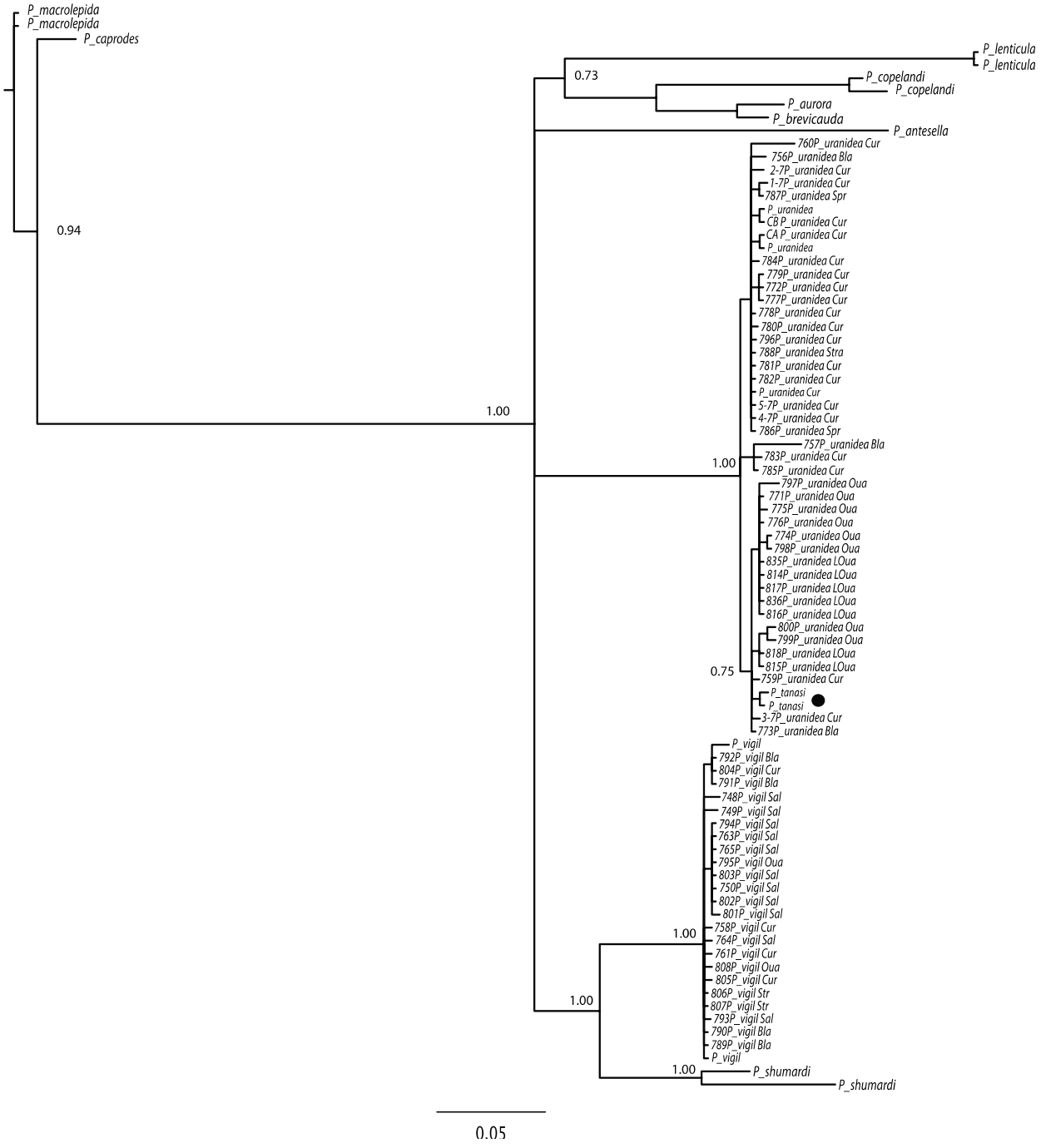

Figure 2. A 50% majority rule consensus phylogram created with Cyt b sequences in Mr Bayes. Clade posterior probabilities are shown at the major nodes. *P. tanasi* sequences are identified with a •. River designations are as follows: Bla = Black river, Cur = Current River, Spr = Spring River, Stra = Strawberry River, Oua = Ouachita River, LOua = Lake Ouachita, and Sal = Saline River

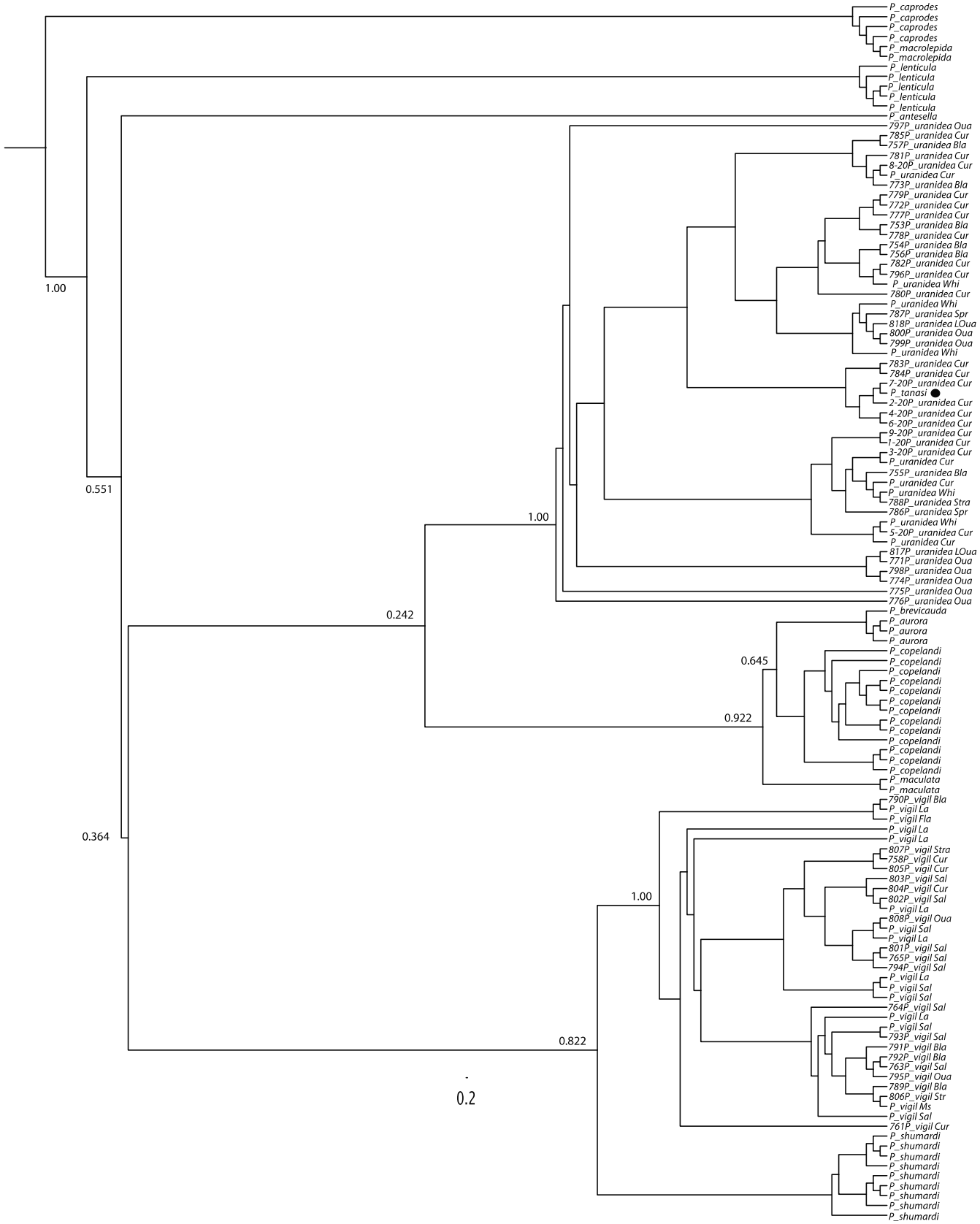

Figure 3. A 50% majority rule consensus phylogram created with COX I sequences in Mr Bayes. Clade posterior probabilities are shown at the major nodes. *P. tanasi* sequences are identified with a •. River designations are as in Figure 2.

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Divergence Dating

To further investigate migration and date population separation, Cyt *b* coalescent analyses were conducted with *BEAST v.2.1.2. This analysis estimates several parameters (phylogeny & divergence dates) using a relaxed clock model (Kumar *et al*. 2009; Drummond *et al*. 2012; Bouckaert *et al*. 2014). Dating analysis was not conducted with *COX I* as outgroup sequences length were significantly shorter than those produced in this study. The divergence date estimates with the BEAST software were produced with similar parameters to Bayesian analysis done in MrBayes but increased generation time ($100X10^6$ generations & 20% burn-in). The clock model was calibrated with the proposed Arkansas River expansion in the Sangamon ~ 0.105 mybp (Mayden 1985; Elfrink 2007; Tripsanas *et al.* 2007). The Cyt *b* estimated pairwise rate of nucleotide substitution was set to 1.80% per myr as reported in Near *et al*. (2011). In these analyses, *P. uranidea* was constrained from *P. vigil*, then a further constraint was done within these species to reflect separation into Ouachita River and White River drainages. These constraints were conducted with a normal distribution in the nodes with a calibrated Yule model prior. All Bayesian outputs produced through BEAST were also reviewed in TRACER for robustness in a similar manner to the Mr Bayes simulations. The resultant trees were summarized in TreeAnnotator v1.6.1 to create a 50% majority-rule consensus maximum clade credibility tree.

Results

Data Analysis and phylogenic tree production

For Cyt *b*, 65 samples of 1190bp were sequenced and an additional 18 sequences were added from GenBank. The mean base composition of the sequenced samples was A = 0.22, C = 0.32, G = 0.17, and T = 0.28 with 309 variable sites. For Cyt *b*, 36 haplotypes were recovered for *P. uranidea* and 19 for *P. vigil*. The 58 individuals sequenced for *COX I* produced contigs of 1495bp with 261 variable sites and a mean base composition of A = 0.24, C = 0.29, G = 0.19, and T = 0.29, with an additional 65 sequences of 670bp included from GenBank to improve the phylogenetic analysis with this locus. For COX1, 39 haplotypes were recovered for *P. uranidea* and 26 for *P. vigil*.

Both Bayesian trees mirrored the trees for these species reported in Near *et al*. (2011) with reciprocal monophyly in both *P. uranidea* and *P. vigil* and high posterior probabilities (Figures 2 and 3). The Cyt *b* tree was characterized with a harmonic mean –ln*L* value of 4676.33 and the *COX I* tree exhibited a harmonic mean –ln*L* value of 5129.55. The Cyt *b* tree did not resolve relationships among *Percina* taxa as well as the *COX I* tree, but exhibited higher posterior probabilities for all nodes when compared to the *COX I* tree. Furthermore, both the *Cyt b* and *COX I* trees suggest *P. uranidea* is paraphyletic with *P. tanasi* nestled within this clade, and neither tree reveals a distinct structuring of White River and Ouachita River drainages as haplotypes were not exclusive to a drainage nor river, i.e., each tree showed a mixture of Ouachita and White River individuals without clearly separating the two drainages.

Population and gene diversity statistics, with divergence dating

The Cyt *b* haplotype network analysis created three unconnected networks at the 95% connection limit. These networks consisted of two *P. uranidea* and one *P. vigil* (Figure 4). All networks mimic the phylogenetic analyses with only one network showing a clear separation between White River and Ouachita River drainages. The genetic diversity statistics summarized in Arlequin suggests population expansion took place for both species in the two river drainages for all values of Fu's Fs except for *P. uranidea* in the Ouachita River drainage (Table 1).

The divergence dating analysis under a coalescent expansion growth prior created a tree with a likelihood of -4185.49 (Figure 5). The dates calculated for the separation of White River drainage populations from Ouachita River drainage populations correlates with a Sangamon divergence date for both species (*P.uranidea*, 0.0997 mybp and *P. vigil*, 0.1326 mybp).

Discussion

The limited divergence between the White River and Ouachita River drainage populations suggests these populations were connected until recently or populations retained substantial genetic variation due to slow allele loss after a separation event (Figure 4 and 5). Based upon the ecological characteristics of both species, it is likely that the contemporary streamflow patterns of the large rivers, such as the Arkansas and Mississippi, altered habitat requirements with gradual local extinctions to create the distributions observed in *P. uranidea* and *P. vigil*. As both species exist in deeper waters of medium-sized rivers, populations likely contained densities that prevented

bottlenecks and random effects due to genetic drift. As the data also suggest population expansion (Table 1), some populations acted as source populations from which recolonization could occur to nearby habitats, newly created as these complex and dynamic Gulf Coastal Plain river systems evolved to their modern distributions and stream characteristics.

The *COX I* tree and the haplotype network (Figure 2 and 4) shows *P. uranidea* with greater haplotype segregation in the White River as compared to *P. vigil*. These results suggest specific habitat requirement differences between the species may not only affect the geographic distribution of the species, but may also affect gene flow among populations. *Percina uranidea's* preference for deeper waters and gravel bottoms may promote more isolation among populations and limit gene flow.

Limited genetic divergence is also present in the Ouachita River drainage as *P. uranidea* populations in the mid Ouachita River (below Lake Catherine) possess different haplotypes from those in the upper Ouachita River (Lake Ouachita population - *LOua*), yet exhibit low divergence from mid Ouachita River and do not form a separate clade in either the Cyt *b* or *COX I* trees (Figure 2 and 3). However, the 11step separation in haplotype network B (Figure 4) are the *LOua* population samples and shows gene flow disruption via river impoundment affects population genetic structure. The upper Ouachita population is approximately 150 km upstream from the mid Ouachita population and also separated by three large reservoirs. *Percina vigil* also reflects the limited *COX I* divergence in the Ouachita River drainage as sequences from Louisiana, Mississippi, and Florida are mixed with those in the Saline and even those in the White River drainage (Figure 3).

Table 1. River drainage diversity indices for *P. uranidea* and *P. vigil* in *Cyt b* and *COX 1* sequences.

River Drainages	Sample & Haplotype #'s ( )	Gene Diversity ± SE	Nucleotide Diversity ± SE	Fu's Fs	Tajima's D
P. uranidea					
Cyt b White River	30 (16)	0.862 ±0.0579	0.0033 ±0.0019	-6.345 p=0.002	-2.00 p=0.006
Ouachita River	15 (7)	0.781 ±0.1020	0.0019 ±0.0012	-1.685 p=0.134	-1.17 p=0.132
COX 1 White River	36 (30)	0.984 ±0.0125	0.0047 ±0.0028	-26.28 p=0.000	-2.02 p=0.006
Ouachita River	9 (9)	1.000 ±0.0520	0.0036 ±0.0022	-4.843 p=0.003	-0.09 p=0.519
P. vigil					
Cyt b White River	13 (10)	0.949 ±0.0500	0.0016 ±0.0011	-7.687 p=0.000	-1.71 p=0.035
Ouachita River	12 (9)	0.939 ±0.0580	0.0011 ±0.0008	-7.817 p=0.000	-1.28 p=0.115
COX 1 White River	10 (10)	1.000 ±0.0450	0.0029 ±0.0018	-6.650 p=0.000	-0.67 p=0.272
Ouachita River	24 (16)	0.917 ±0.0482	0.0028 ±0.0019	-15.533 p=0.000	-2.085 p=0.003

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DOI: https://doi.org/10.54119/jaas.2017.7125

With regards to the dispersal or vicariance models, a recent vicariance event is supported rather than a pre-glaciation vicariance event as neither the phylogenetic analyses nor the haplotype network analysis show a haplotype distribution consistent with dispersal from a refugia; i.e., basal haplotype populations with derived haplotype populations that reflect expansion (Berendzen *et al*. 2008; Blanton *et al*. 2012). In addition, the divergence dating analysis suggests a Pleistocene division between the two river basins (Figure 5). However, the *COX I* tree shows that the most basal *P. uranidea* haplotypes are those from the Ouachita River drainage, which may suggest a longer divergence period in this region.

The phylogenetic data also suggests a paraphyletic relationship in *P. uranidea* as *P. tanasi* is nested within *P. uranidea* haplotypes in both Cyt *b* and *COX I* trees. This paraphyletic relationship in these darters is not reported in other *Percina* phylogenetic studies (Near *et al*. 2011) and may represent incomplete lineage sorting in specific populations of these darters. As *Percina* darters inhabit deeper waters of streams and rivers, which may house larger populations, population divergence, speciation, and lineage sorting may require longer divergence periods. Conversely, this relationship could be an artifact of mitochondrial introgression into *P. tanasi*. If mitochondrial introgression has occurred, this relationship would provide evidence of a second example in the *Percina* genus (Near *et al*. 2011). As nuclear genes were not sampled, a definitive conclusion regarding introgression is premature.

In conclusion, our results provide support that the Pleistocene Arkansas River expansion created a substantial barrier, reducing gene flow between the Ouachita and White River systems. In addition, our results suggest that further examples of incomplete lineage sorting may exist in other darter species and may lay hidden within unsampled haplotypes further complicating the phylogenetic resolution of species within this genus. Due to the complexity of darter phylogenetics, it appears to be fruitful to conduct further extensive population level sampling within species in the genus *Percina* to better illustrate the extent and complexity of speciation within this genus.

Acknowledgements

We thank Brian Wagner, the Arkansas Game and Fish Commission, and the United States Fish and Wildlife Service for project funding. We thank Arkansas Tech University for laboratory, logistical, and financial support. The following students were instrumental in fish collections or laboratory analyses: A Orr, C Ford, C Steelman, B Peoples, N Carmean, C Vrba, K McArthur, and K Rudolf. We thank the comments from two anonymous manuscript reviewers.

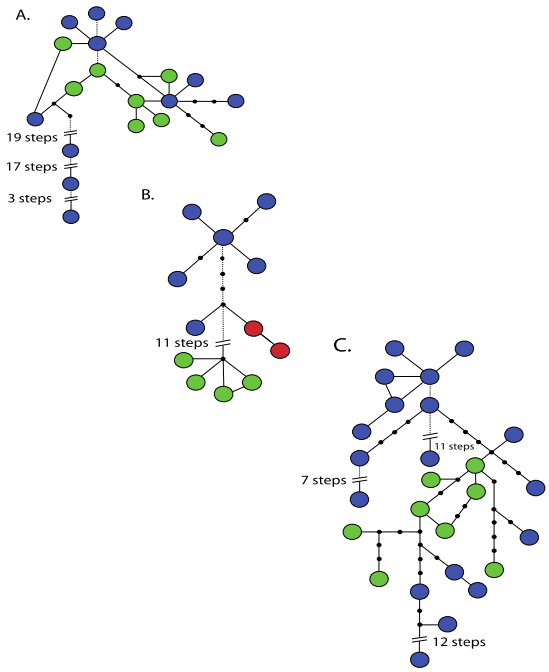

Figure 4. Haplotype networks for Cyt b created in TCS. Blue circles represent White River basin haplotypes, green circles represent Ouachita River haplotypes, and red circles represent *P. tanasi*. *P. uranidea* haplotypes are shown in network A & B: *P. vigil* in network C.

Supporting Information

Table S1. Individual sample information with GenBank Acession numbers. Taxa accessed through GenBank follow DNA sequences created in this study.

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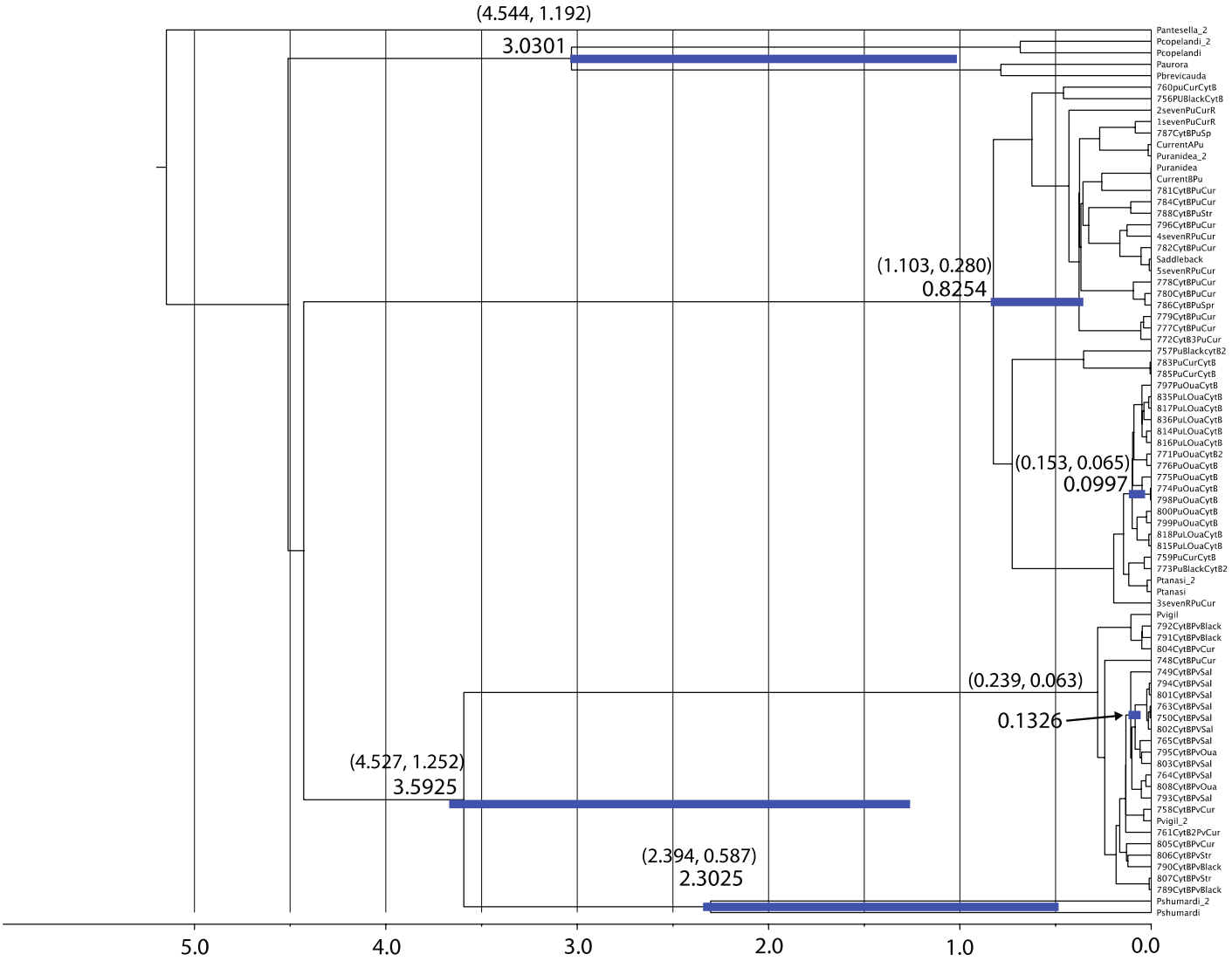

Figure 5. A 50% majority-rule consensus maximum clade credibility tree showing estimated divergence estimates created through a relaxed clock model in BEAST* with Cyt b sequences. *P. uranidea* and *P. vigil* were constrained into Ouachita River and White River populations. Mean clade ages are shown at the nodes with 95% uncertainty lower bound ranges shown in blue and 95% uncertainty ranges shown in parentheses. The scale represents age estimates in mybp.

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DOI: https://doi.org/10.54119/jaas.2017.7125