Received: 18 January 2018 | Accepted: 29 March 2018

DOI: 10.1111/zsc.12288

WILEY Zoologica Scripta

ORIGINAL ARTICLE

Patterns of diversification in a North American endemic fish, the Blackbanded Darter (Perciformes, Percidae)

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Funding information
Southeastern Louisiana University's STAR program

The coastal plain of the south-eastern United States shows multiple biogeographic patterns of plant and animal dispersal; however, few freshwater fish taxa span these biogeographic barriers. *Percina nigrofasciata*, the Blackbanded Darter (Teleostomi: Percidae), is a small, benthic, freshwater fish species with an extensive range in the south-eastern United States. Recently, two species have been elevated from within *P. nigrofasciata*: *P. crypta* and *P. westfalli*, but their ranges have not been established. We broadly sampled across the south-eastern United States, encompassing the range of *P. nigrofasciata* sensu lato. We reconstruct the phylogeny of *Percina* using both mitochondrial and nuclear markers. Eighty-four specimens of *Percina nigrofasciata* were sampled for the mitochondrial gene cytochrome b (1,119 bp) to form a base phylogeny. The nuclear marker S7-I1 was subsampled across populations to detect instances of hybridization. Phylogenetic relationships with other members of the genus *Percina* were assessed through Bayesian inference. Our results suggest that *Percina nigrofasciata* sensu stricto occurs from the Lake Pontchartrain Basin in Louisiana to the rivers of the Mobile Basin with little genetic structuring throughout its range. *Percina westfalli* occurs from the Apalachicola River drainages to the Atlantic Slope from the Savannah River to the St. Johns River. We find that *P. crypta* is not genetically distinct from *P. westfalli* in the Chattahoochee and Flint Rivers. Possible ancestral hybridization occurred between the *P. nigrofasciata* and *P. westfalli* in the panhandle of Florida between Mobile Bay and the Apalachicola River.

1 | INTRODUCTION

The south-eastern United States, especially the North American Coastal Plain, is a hotbed of biodiversity with over 1,500 endemic plant and animal species (Noss et al., 2015; Soltis, Morris, McLachlan, Manos, & Soltis, 2006). The Coastal Plain functioned as a refuge for species during the last glacial maxima (Bagley, Sandel, Travis, Lozano-Vilano, & Johnson, 2013; Krysko, Nuñez, Lippi, Smith, & Granatosky, 2016; Soltis et al., 2006), and the adjacent uplands or piedmont regions acted as refugia during periods of sea-level rise. Due to the dynamic nature of the region's climate, endemism is especially high for freshwater fishes (Avise, 2000; Boschung & Mayden, 2004; Noss et al., 2015; Page & Burr, 2011; Soltis et al., 2006; Swift, Gilbert, Bortone, Burgess, & Yerger, 1986). South-eastern freshwater fishes often exhibit genetic structuring associated with the boundaries of the major river systems including the Mississippi River, Mobile Bay and Apalachicola Rivers, although slightly different patterns exist across different groups of fishes (Bermingham & Avise, 1986; Lydeard, Wooten, & Meyer, 2014; Soltis et al., 2006; Strange & Burr, 1997; Wiley & Mayden, 1985).

Darters, members of subfamily *Etheostomatinae* (Teleostei: Percidae), are endemic to North America and include five genera: *Ammocrypta*, *Crystallaria*, *Etheostoma*, *Nothonotus* and *Percina* (Near et al., 2011). All darters are benthic or semibenthic and many are geographically restricted to particular river basins; however, some species, such as the Blackbanded Darter, *Percina nigrofasciata*, have very broad distributional ranges (Page & Burr, 2011). *Percina nigrofasciata* was

described from near Mobile, Alabama, by Agassiz (1854) and occupies both coastal and piedmont river systems from as far west as the Lake Pontchartrain Basin in Louisiana, eastward to the Edisto River in South Carolina (Crawford, 1956; Lee, Gilbert, Hocutt, & Jenkins, 1980; Page & Burr, 2011). Its range does not appear to be affected by well-known biogeographic boundaries such as the Fall Line or major river systems. The species occurs both above and below the Fall Line, the physiographic border between the coastal plain and piedmont region (Boschung & Mayden, 2004; Lee et al., 1980; Page & Burr, 2011), and spans the Apalachicola River Basin, Appalachian Mountains and Tombigbee River discontinuities, three repeated phylogeographic breakpoints identified for terrestrial and freshwater taxa (Bermingham & Avise, 1986; Soltis et al., 2006).

In addition to its widespread distribution, *P. nigrofasciata* is extraordinarily variable in its pigmentation (Crawford, 1956). This has led researchers to question whether *P. nigrofasciata* is truly a single species or a complex of multiple species. The subspecies *Percina nigrofasciata westfalli* was described from the St Johns River in Florida (Fowler, 1942) nearly a century after the original description of *P. nigrofasciata* (Agassiz, 1854). Its description was based on a single specimen, and the extent of its geographic distribution was not identified. Shortly thereafter, in a comprehensive review of morphological variation, Crawford (1956) synonymized *P. n. westfalli* with *P. n. nigrofasciata* and described a new subspecies, *P. nigrofasciata raneyi,* from the Savannah River. Crawford (1956) also recognized eight distinct races within *P. n. nigrofasciata*, which could be assigned to their specific drainages based on meristic and mensural characters; however, there was substantial overlap in many of these characters. The subspecies recognized by Crawford (1956) have not been used in subsequent literature (Boschung & Mayden, 2004; Freeman, Freeman, Burkhead, & Straight, 2008; Lee et al., 1980; Page & Burr, 2011); however, *Percina nigrofasciata raneyi* was mentioned, but not discussed, in the description of the Halloween Darter, *P. crypta,* from the Apalachicola River drainage (Freeman et al., 2008), a former conspecific of *P. nigrofasciata*. *Percina crypta* and *P. nigrofasciata* are sympatric in the Chattahoochee and Flint River systems, but can be differentiated from one another by coloration, behaviour and meristics (Freeman et al., 2008). Most recently, specimens of *P. nigrofasciata*, *P. westfalli* (elevated from subspecies status by Near et al., 2011) and *P. crypta* were included in a multigene phylogeny of Etheostomatinae (Near et al., 2011). In that study, *Percina crypta* and *P. westfalli* were recovered as sister taxa, but not sister to *P. nigrofasciata* (Near et al., 2011).

Percina nigrofasciata has typically been considered a widespread species throughout the south-east; however, its distribution and distinctiveness from *P. crypta* and *P. westfalli* require clarification. *Percina crypta* occurs sympatrically with *P. nigrofasciata* throughout the Chattahoochee River (type locality of Chattahoochee River at Nora Mill*,* Freeman et al., 2008), and the range of *P. westfalli* is unclear. Near et al. (2011) recognized *P. westfalli* as a species based on a single genetic specimen from a tributary to the Savannah River; however, its range is not discussed, nor is the possibility of sympatry with *P. nigrofasciata* (Near et al., 2011). We hypothesize that the distribution of *P. westfalli* will correspond to the Atlantic drainages of the south-east, known as the Appalachian Mountain discontinuity (Soltis et al., 2006), and both *P. nigrofasciata* and *P. crypta* will be found in the Apalachicola drainage, with *P. nigrofasciata* extending westward to Louisiana.

Percina nigrofasciata is a widespread, morphologically variable taxon that is comprised of multiple distinctive populations and subspecies. Previous morphological diagnoses and studies of morphological variation and taxonomic distinctiveness of some populations remain unclear, geographic boundaries of these morphologically variable populations are uncertain, and to date, no study has specifically focused on this group in an evolutionary context. Therefore, we broadly sampled across the south-eastern United States to obtain tissue samples from populations of *P. nigrofasciata* sensu lato, including individuals currently recognized as *P. nigrofasciata*, *P. crypta* and *P. westfalli* (Figure 1). Although no samples were taken near Mobile, AL, the type locality of *P. nigrofasciata*, we did sample near type localities for *Percina crypta* (type locality, Nora's Mill, Helen, GA; sampled, tributary to Chattahoochee River, north of Helen)*, P. westfalli* (type locality, Wekiwa River, tributary of St Johns River, FL; sampled Etonia Creek, a tributary to St Johns River) and *P. raneyi* (type locality, tributary to the Savannah River, 7.6 miles east of Calhoun Falls, SC; sampled Little River, north of Abbeville). All of these taxa were previously thought to be closely related or conspecific (Agassiz, 1854; Crawford, 1956; Fowler, 1942; Freeman et al., 2008; Near et al., 2011). DNA sequence data from mitochondrial and nuclear markers were gathered to address two objectives. First, we reconstructed a phylogeny to determine the relationships of the populations within the group to identify the lineages and better understand the distribution of genetic variation across the range. Second, we used the results from the molecular phylogenetic analyses to clarify the species diversity and geographic distribution of the lineages with *P. nigrofasciata s. l.* and to make comments on the taxonomic status of the species within the complex.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

Specimens of *Percina nigrofasciata s. l.* were collected from 33 sites (Figure 1, Table S1) across the south-eastern

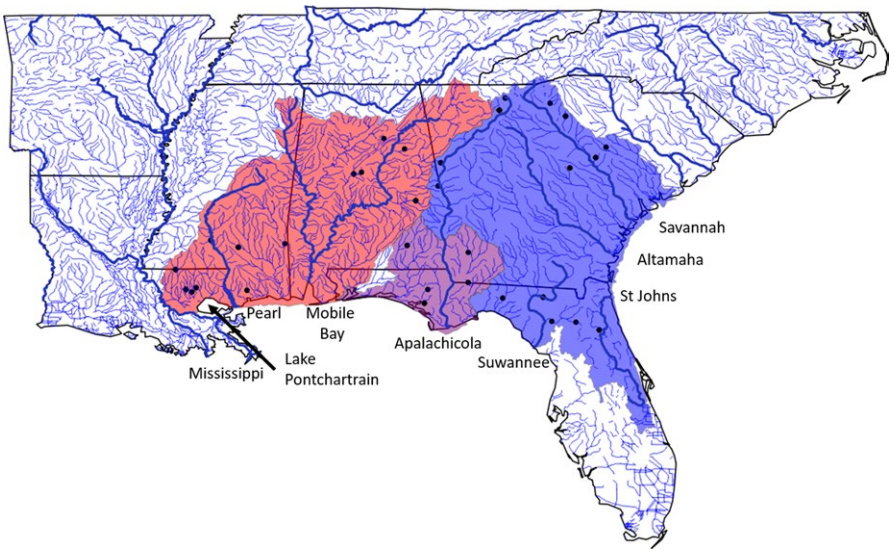

FIGURE 1 Map of the southeastern United States. Black dots represent collection sites in this study. Distribution of species is indicated by shaded HUC-8 watersheds. Black lines represent state borders. The distribution of *Percina nigrofasciata* is coloured red, and the distribution of *P. westfalli* is coloured blue. Watershed-coloured purple indicates areas where taxonomic uncertainty remains (see text). River names are shown at the mouth of each river

United States using seines, dip nets and/or a backpack electrofisher. Individual fin clips or whole specimens were preserved in 95% ethanol, and voucher specimens were preserved in 10% formalin. Tissue samples were deposited into the Southeastern Louisiana University Tissue Collection (SLU-TC), and voucher specimens were deposited into the Southeastern Vertebrate Museum (SLU). Other tissue samples were provided by colleagues or natural history museums. Museum abbreviations follow Sabaj (2016). Specimen localities are summarized in Table S1. Eighty-four individuals of *P. nigrofasciata s. l.* were used in a phylogenetic analysis of cytochrome b. Using the mitochondrial phylogeny as a guide, a subset of individuals ($n = 58$) was sampled for subsequent S7 intron 1 phylogenetic analysis. Other members of *Percina,* as well as other outgroup taxa, were used in the analysis, and sequences for these individuals were gathered from GenBank from previously published phylogenies (Bossu & Near, 2009; Keck & Near, 2008, 2009; Near, 2002; Near et al., 2011).

2.2 | Molecular methods

Whole genomic DNA was extracted from fin clips preserved in 95% ethanol using a Chelex extraction protocol (Walsh, Metzger, & Higuchi, 1991) or the DNeasy Tissue Kit (Qiagen, Inc.). Two genes, cytochrome b (cytb) and S7 intron 1 (S7-I1), were amplified through polymerase chain reactions. The cytb gene was amplified using primers L14724 and H15915 (Schmidt & Gold, 1993) in 25 μl polymerase chain reactions. Cytochrome b reactions adhered to the following protocol: initial denaturation step of 120 s at 94°C then 25 to 30 cycles of denaturation (60 s at 94°C), annealing (60 s at temperatures between 47°C and 48°C), and extension (120 s at 72°C) followed by a final extension of 120 s at 72°C. Primers used for PCR amplification were also used for DNA sequencing. The S7 intron 1 (S7-I1) gene was amplified using the primers S7RPEX1F and S7RPEX2R and prescribed conditions from Chow and Hazama (1998) with annealing temperature varying between 58°C and 64°C. Primers used for PCR amplification were also used for DNA sequencing.

PCR amplicons were visualized on a 0.8% agarose gel with a 1Kb ladder to assure appropriate product size and intensity. PCR products were purified using ExoSAP-IT (USB Corp.). When sequencing was outsourced to the Genomics Core Facility at Pennington Biomedical Laboratory, sequencing reactions were conducted using the same amplification primers for sequencing and BigDye terminator sequencing kits (Applied Biosystems) according to manufacturer's recommendations. The samples were then run on an Applied Biosystems 3130XL automated DNA sequencer. Other samples were sent to the DNA Analysis Facility on Science Hill at Yale University, where BigDye reactions were run and purified by laboratory technicians at Yale University. Samples were run on an Applied Biosystems 3730x automated DNA sequencer.

Sequences were aligned using Geneious v.6.0.3 (Kearse et al., 2012). Heterozygous genotypes for S7-I1 were coded using standard IUPAC degeneracy codes. Additional sequences from members of Percidae were downloaded from GenBank. All GenBank sequences for cytb were 1,140 base pairs in size; however, due to inconsistencies with the beginning sequencing reads for some specimens of *P. nigrofasciata* generated in this study, the final cytb data set was truncated to 1,119 base pairs. This truncation was done with respect to the first, second and third codon positions, so the reading frame was not misaligned. The total length of S7-I1 individual sequences ranged from 383 to 445 base pairs with an aligned total of 452 base pairs across all taxa, inclusive of insertions and deletions. Simple uncorrected mean pairwise genetic distances for cytb and for S7-I1 (with standard

errors) were computed using MEGAv.6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

2.3 | Phylogenetic analyses

Bayesian inference was used to infer independent phylogenetic hypotheses of cytb and S7I1 in *Percina nigrofasciata*. For the Bayesian analysis, cytb was partitioned into three codon positions and tested in jModelTest (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003) for the best-fit model of evolution using the Akaike Information Criterion (AIC) (Posada & Buckley, 2004). The S7-I1 data set was not partitioned because it is a non-coding intron. A Metropolis-coupled Markov chain Monte Carlo (MCMC) was used to estimate posterior probabilities in MrBayes (Ronquist et al., 2012) through CIPRES (Miller, Pfeiffer, & Schwartz, 2010). The Bayesian analyses were carried out using MrBayes v3.2.6 generating 10,000,000 generations and sampling every 1,000 generations. Two separate runs were performed, and burn-in was determined using the sump command in MrBayes. A 50% majority rule consensus tree with posterior probabilities was obtained from the postburn-in tree files from the runs.

3 | RESULTS

3.1 | Phylogenetic analyses, cytochrome b

Phylogenetic analyses on the cytochrome b (cytb) gene were performed on a total of 171 samples of the family Percidae. Of the 171 sequences analysed, 90 were generated in this study, with a total fragment length of 1,119 bp (GenBank Accession Nos: MH203798–MH203807). Results of the Bayesian inference resulted in a monophyletic *Percina* and a non-monophyletic clustering of *P. nigrofasciata s. l.* (Figure 2a).

The first clade to diverge from other *Percina* species is the eastern clade, a highly structured assemblage of samples

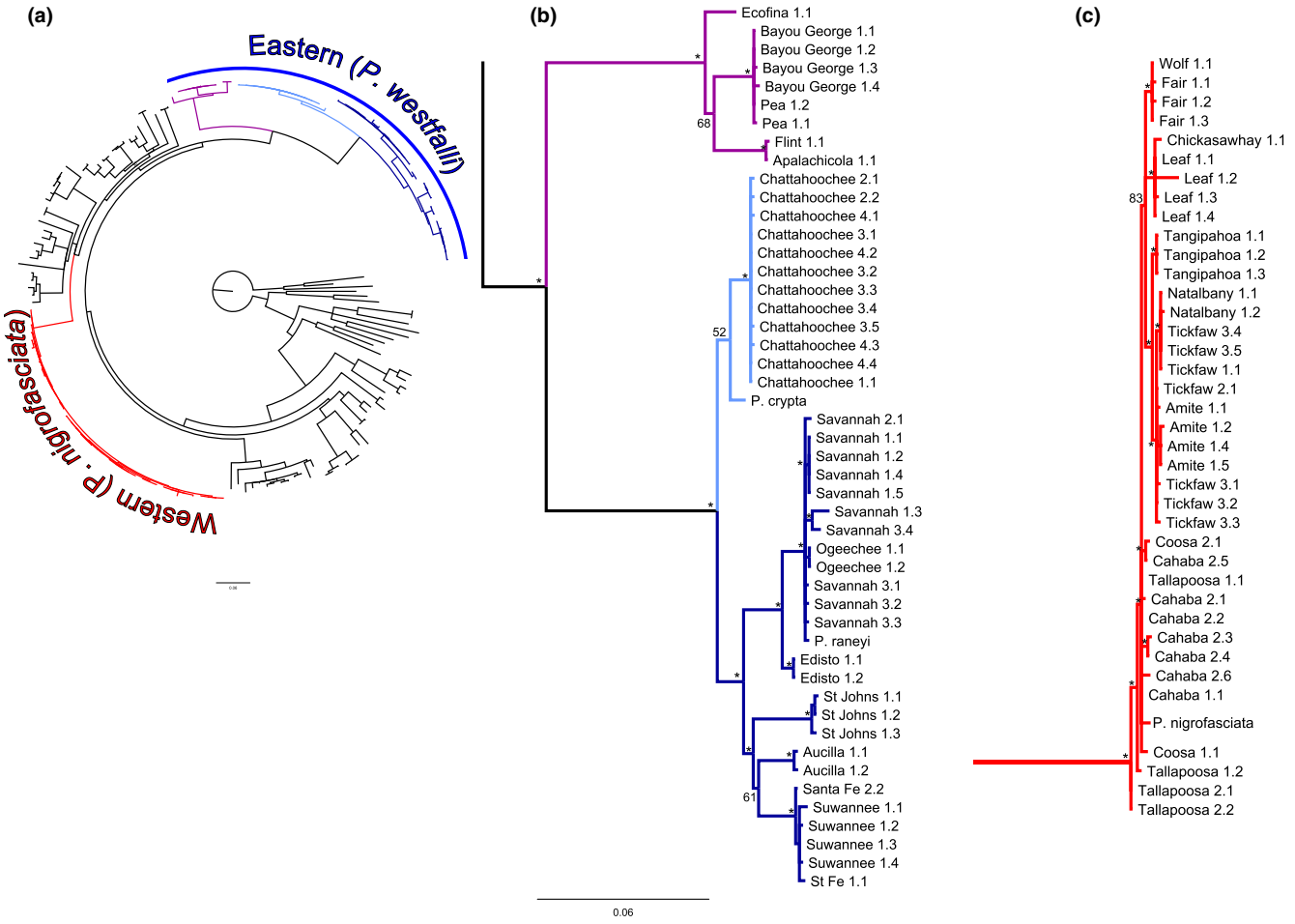

FIGURE 2 (a–c). Fifty per cent majority rule consensus tree from Bayesian inference of cytochrome b sequences. Nodes labelled with an asterisk (*) indicate posterior probabilities >90%. Values less than 90% are written on the trees. (a) Cytochrome b phylogeny of all *Percina* with two clades of *P. nigrofasciata* sensu lato coloured and labelled. (b) Detailed view of the eastern clade. Tips are labelled with names corresponding to Table S1. (c) Detailed view of the western clade. Tips are labelled with names corresponding to Table S1

consisting of individuals of *P. nigrofasciata s. l.*, *P. crypta* and *P. westfalli* from the basins in the south-east from the Choctawhatchee River, Florida, eastward to the Atlantic slope basins (Figure 2b). Within the eastern clade, there are three distinct subclades. The first consists of specimens from the Choctawhatchee River, which form a clade with other lowland systems surrounding the Apalachicola River, such as Econfina Creek, Pea River and the Ochlocknee River. The second subclade consists of samples exclusively from tributaries of the Chattahoochee River and a specimen of *P. crypta* from the type locality (voucher YPM 23800). The sequence from *P. crypta* was generated in a previous analysis (Near et al., 2011) and included in this study. The third subclade consists of specimens from the Aucilla, Chattahoochee, Edisto, Sampson, Savannah, St Johns and Suwannee Rivers. This clade encompasses the type localities of *P. westfalli* and *P. raneyi* (Crawford, 1956*).* These rivers flow to the eastern Atlantic coast with the exception of the Aucilla and Suwannee Rivers, which flow into the Gulf of Mexico.

The second clade*,* the western clade, includes specimens of *P. nigrofasciata* sampled from the northernmost tributaries of the Coosa River (Mobile Drainage), westward to the Natalbany River (Lake Pontchartrain Basin) (Figure 2c). This large clade is in a polytomy with two other clades composed of 13 other species of *Percina:* One of *P. aurolineata, P. sciera, P. sipsi* and another consisting of *P. cymatotaenia, P. gymnocephala, P. maculata, P. nasuta, P. notogramma, P. oxyrhynchus, P. pantherina, P. phoxocephala, P. squamata* and *P. stictogaster.* This relationship is not consistent with the taxonomy of the subclades designated by Near et al. (2011). Unlike the eastern clade, the western clade has short branch lengths and multiple polytomies. There appears to be no clear resolution among major river drainages and relationship within the western clade aside from the monophyletic localities draining to the Lake Pontchartrain Basin in Louisiana (Tangipahoa, Natalbany, Tickfaw and Amite Rivers).

3.2 | Phylogenetic analysis: S7-I1

Phylogenetic analysis was performed on a total of 165 sequences representing members of the family Percidae and especially focused on the genus *Percina.* Of the 165 sequences, 61 sequences were generated in this context of this study. Total fragment length of the S7-I1, including insertions and deletions, is 452 bp (GenBank Accession Nos: MH203096–MH203119). Results of the Bayesian inference resulted in a non-monophyletic clustering of *P. nigrofasciata* (Figure 3).

A monophyletic *Percina* is recovered in the Bayesian inference of S7-I1 data. Unlike cytochrome b, the evolutionary rate of S7-I1 marker is reduced due to its evolutionary constraint in association with the ribosomal protein (Lavoué, Sullivan, & Hopkins, 2003). Due to this lower rate of variation in the marker, the resolution within the phylogeny is reduced, especially at the tips. Although the genus is recovered as monophyletic (posterior probability = 100%), the intraspecific relationships are poorly resolved, often resulting in a polytomy or weakly supported clades. Despite the lack of overall resolution in the S7-I1 tree, two clades are recovered.

With the S7-I1 marker, *Percina apristis* renders the two clades of *P. nigrofasciata* paraphyletic; a relationship not found in the cytochrome b phylogeny; however, this relationship is poorly supported (71% posterior probability). The two clades in the S7-I1 analysis are similar to those found in the cytb analysis: a phylogenetically structured eastern clade and an unstructured western clade. Despite the low levels of phylogenetic resolution across the S7-I1 tree, both clades have a >90% posterior probability.

The eastern clade shows structure similar to the cytb phylogeny. The specimen from Econfina Creek is basal to other members of the eastern clade, a pattern echoing the cytb analysis. A Chattahoochee River clade is next recovered sister to a clade composed of rivers found in the Florida peninsula as well as the Savannah and Edisto Rivers. Both *Percina crypta* and *P. westfalli* are found within the eastern clade and nested within the polytomy of the Atlantic drainage and Florida peninsular clades.

The western clade in the S7-I1 analysis has less genetic structuring than the clade recovered in the cytochrome b analysis, with the entire subsampled group resulting in a polytomy and a single specimen from the Flint River basal to the clade. Branch lengths within this clade are low suggesting little genetic variation across the sampled sites.

Although the overall patterns are similar between the cytochrome b and S7-I1 analysis, one clade in the cytochrome b analysis is found dispersed between the two clades in the S7-I1 analysis. The Choctawhatchee clade with specimens from the Bayou George, Econfina Creek, lower Flint River and Apalachicola River is not recovered as a monophyletic in the S7-I1 analysis. Instead, the Econfina Creek and Apalachicola River specimens are found in the eastern clade, but the Bayou George and lower Flint River specimens are found in the western clade. The single specimen from the lower Flint River is basal to the polytomy encompassing the rest of the western clade samples.

4 | DISCUSSION

4.1 | Taxonomy and distribution of the Blackbanded Darter

The results from this study indicate that the Blackbanded Darter, *Percina nigrofasciata,* is not a single taxon with a

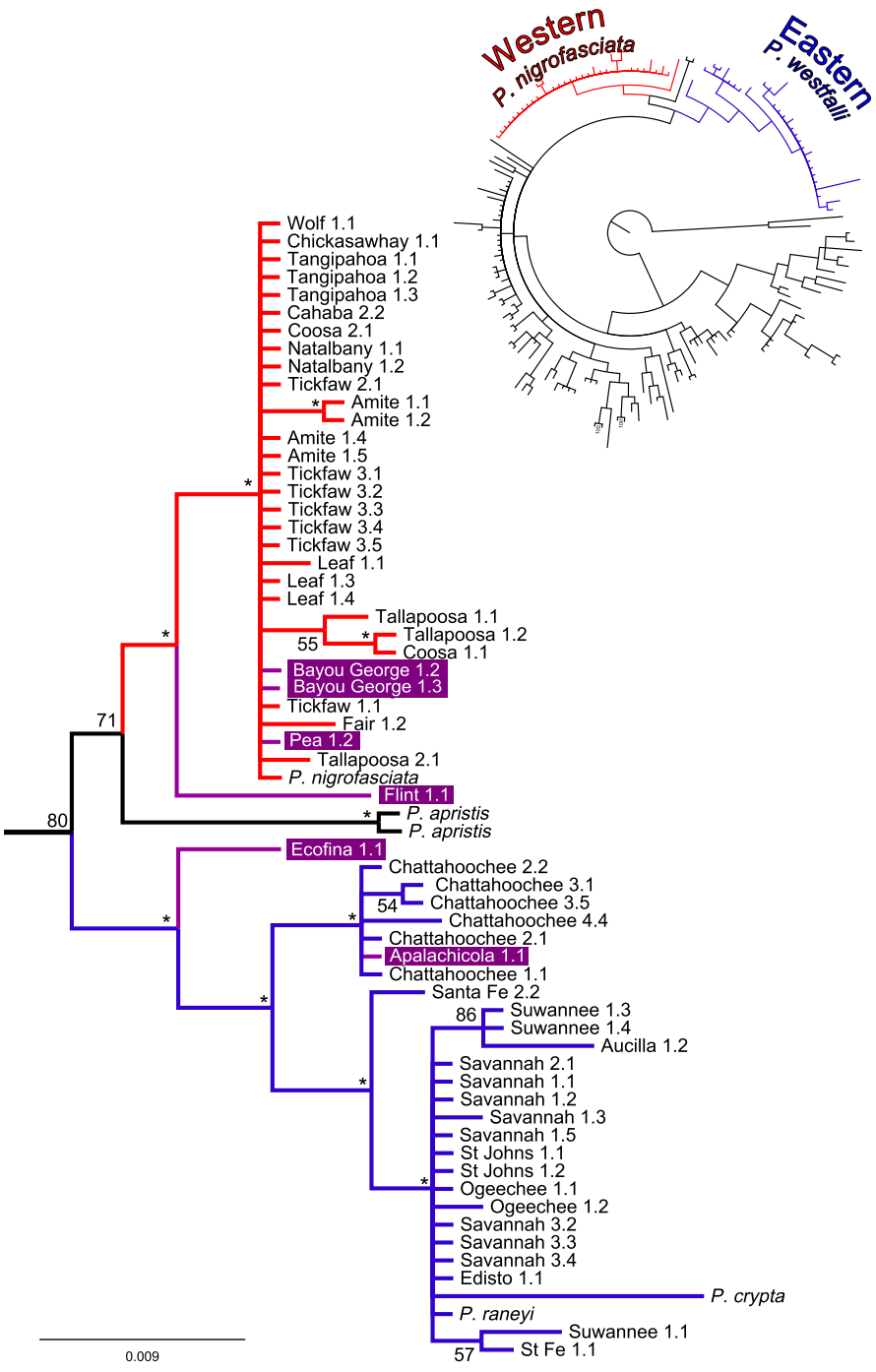

FIGURE 3 Fifty per cent majority rule consensus tree from Bayesian inference of S7 intron 1 sequences. Detailed view of the two clades of *Percina nigrofasciata* sensu stricto. Tips are labelled with names corresponding to Table S1. Nodes labelled with an asterisk (*) indicate posterior probabilities >90%. Values less than 90% are written on the trees. Circle phylogeny is all *Percina* with two clades of *P. nigrofasciata* sensu lato coloured and labelled. Branches are coloured with respect to the cytochrome b phylogeny. Names with a purple background are samples that moved clades between the two analyses

broad distribution across the south-eastern United States. The western clade recovered in our analysis represents the distribution of *Percina nigrofasciata* sensu stricto. The taxon occurs throughout the Mobile drainage as far north and east as northern Georgia and Alabama and westward through Mississippi to Louisiana. A specimen of *P. nigrofasciata* was archived (CU16300) from Alexander Creek, a tributary to Thompson Creek (West Feliciana Parish, LA) in the Lower Mississippi River Basin (Crawford, 1956); however, no other specimens of *P. nigrofasciata* have been collected from any Mississippi River tributaries, and it likely that this specimen record is an error. *Percina nigrofasciata* should be confined to the Lake Pontchartrain Basin eastward including the Pearl River, Pascagoula River, Wolf River and Mobile Bay drainages.

Near et al. (2011) recognized *P. westfalli* from a specimen from Hard Labor Creek, a tributary to Steven Creek (McCormick Co., SC) in the Savannah River Basin (Near, M. M. Hayes & K. R. Piller, personal communication); however, its range and consideration of *P. raneyi,* the subspecies described from the Savannah River by Crawford (1956), were excluded from discussion. Although the

Savannah River is the locality for *P. raneyi* (Crawford, 1956), we find no major genetic distinction between the Savannah River populations and other members of the eastern clade. Because the eastern clade includes the St Johns River, Florida, the type locality of *P. westfalli* (Fowler, 1942), we assign the clade the name with seniority. This greatly expands the range of *P. westfalli* from its original description. Thus, Westfall's Darter, *Percina westfalli*, has a range from the Apalachicola River Basin eastward as far north as the Edisto River in South Carolina and as far south as the St Johns River in Florida.

Percina westfalli shows population-level genetic structuring associated with the rivers in which it occurs. This echoes the morphological races described by Crawford (1956) and includes the Chattahoochee River, the Savannah and Ogeechee Rivers, the St Johns River, and the Suwannee River (including Santa Fe). The presence of distinct genetic clades and discrete morphology across these populations suggests that further study in this area may reveal additional unrecognized diversity within *P. westfalli*.

Additional diversity and unique genetic exchange likely occurred in the lower Apalachicola Basin, the lower Flint River and the surrounding coastal drainages. These rivers represent an area with a complex genetic and biogeographic history which remains to be explored and understood. Recently, studies on the alligator snapping turtle have shown this region to contain multiple unique genetic lineages (Echelle et al., 2010; Folt & Guyer, 2015). Additionally, the Alabama Map Turtle has been split into multiple species based on drainage (Ennen, Lovich, Kreiser, Selman, & Qualls, 2010). Our cytb analysis recovers a clade including samples from the lower Apalachicola and coastal drainages sister to other *P. westfalli*; however, in the S7-I1 analysis, the same individuals were assigned to either *P. nigrofasciata* or *P. westfalli*, suggesting the occurrence of ancestral hybridization in this region. The populations in this region also vary based on their reproductive life history, which may indicate further genetic isolation (Hughey, Heins, Jelks, Ory, & Jordan, 2012). Due to the genetic uncertainty in this region and our small sample size, we are unable to assign these populations to either *P. nigrofasciata* or *P. westfalli* with any confidence. We suggest that the complex genetic history of this area should be further examined with more samples and loci.

Further north, the Chattahoochee and Flint Rivers contain two sympatric and closely related species: *P. crypta* and *P. westfalli*. *Percina crypta* has a disjunct distribution throughout the Chattahoochee and Flint Rivers, and co-occurs with *P. westfalli* (Freeman et al., 2008). *Percina crypta* can be differentiated from *P. westfalli* primarily based on breeding coloration (Freeman et al., 2008). We find the species is genetically similar to *P. westfalli*, with cytb sequence divergence 1.5% (compared to 12.6% divergence between *P. nigrofasciata* and *P. westfalli*). It is important to note that the two species are syntopic, and the possibility of hybridization may decrease genetic differentiation; however, it is also notable that *P. westfalli* is highly variable in its coloration throughout its range. Based on the lack of genetic differentiation and the existence of a highly variable coloration pattern, we suggest that the validity of *P. crypta* requires further investigation.

ACKNOWLEDGEMENTS

We would like to thank Southeastern Louisiana University's STAR program for funding fieldwork for this project. Thanks to Aaron Geheber, Caleb McMahan, Carys Kenway-Lynch, Mollie Cashner, Rebecca Johansen, Jim Williams and C. Keith Ray for fieldwork, specimens and insight into this project.

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SUPPORTING INFORMATION

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How to cite this article: Hayes MM, Piller KR. Patterns of diversification in a North American endemic fish, the Blackbanded Darter (Perciformes, Percidae). *Zool Scr*. 2018;00:1–9. https://doi.org/10.1111/zsc.12288