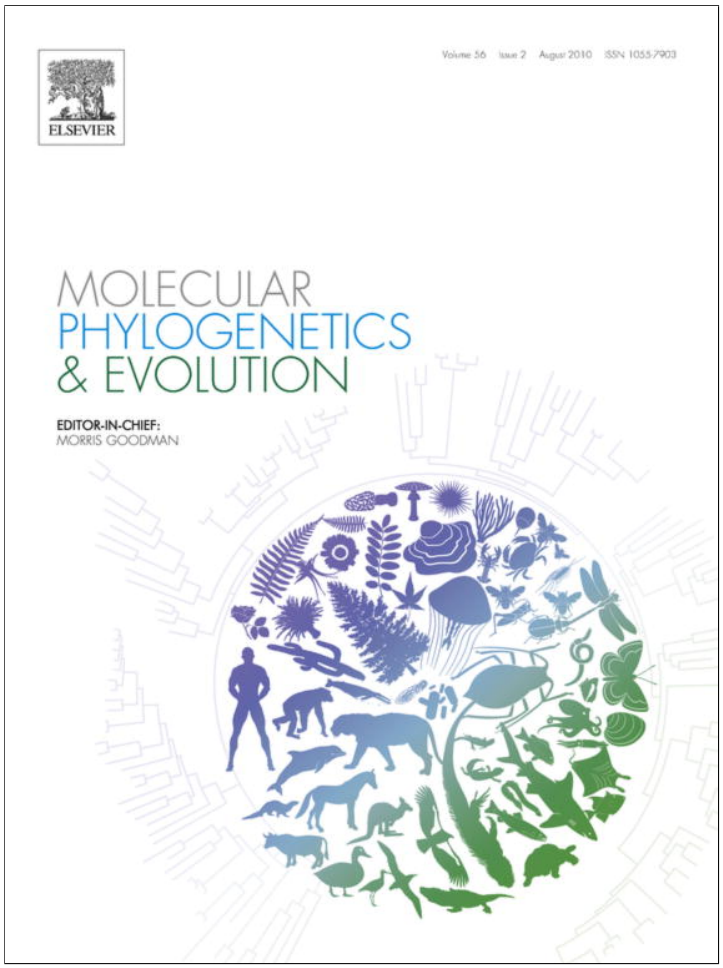

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Discordant molecular and morphological evolution in buffalofishes (Actinopterygii: Catostomidae)

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ABSTRACT

Buffalofishes (Genus *Ictiobus*) are large, robust-bodied suckers adapted to large rivers and lakes of North America. Currently recognized species are readily diagnosed by morphological characters, and the group is known from fossils dating back to the Miocene. However, sympatrically occurring species in the Mississippi River Basin are known to hybridize in nature and in the laboratory. Here we describe patterns of morphological (morphometric) and DNA sequence variation (mitochondrial and nuclear genes) across the geographic ranges of extant species of genus *Ictiobus*. We show that *Ictiobus* species form more of less discrete entities based on body morphometry, consistent with current taxonomy. However, except for *I. labiosus*, there is extensive sharing of alleles of nuclear and mitochondrial genes among species, and the species do not form reciprocally monophyletic groups in nuclear or mitochondrial gene trees. Moreover, the pattern is not confined to the broad area of sympatry in the Mississippi River Basin. We attribute this to a long history of introgressive hybridization and gene flow among species inhabiting the present-day Mississippi River Basin, and recent colonization of the Great Lakes, Hudson Bay drainage and gulf coastal rivers east and west of the Mississippi River by introgressed Mississippi River Basin stocks.

1. Introduction

Recent years have witnessed an upsurge of interest in the importance of hybridization and introgression as factors in animal evolution (Bullini, 1994; Dowling and Secor, 1997; Mallet, 2005). Well documented among plant species, instances of introgessive hybridization were thought to be rare and evolutionarily inconsequential among animals due to the common belief that hybridization favors rapid evolution of prezygotic, reproductive-isolating mechanisms (Mayr, 1963; Mallet, 2005). However, introgression has been documented in countless molecular studies involving all manner of animal species (Saetre et al., 2001; Salzburger et al., 2002; Linnen and Farrell, 2007).

When incipient species that have not evolved reproductive-isolating mechanisms establish secondary contact, the resulting hybridization leads either to introgression or evolution of barriers to gene flow. The mitochondrial genome introgresses much more rapidly than the nuclear genome, for reasons that are still a matter of debate (Funk and Omland, 2003; Ballard and Whitlock, 2004; Chan and Levin, 2005). Indeed, the most common examples of introgression among animal species involve studies of mitochondrial genes (Gerber et al., 2001; Bachtrog et al., 2006; Linnen and Farrell, 2007). Instances of introgression of nuclear genes are rare. However, recent studies have documented evidence of admixture and introgression involving small number of nuclear genes (Saetre et al., 2001) and broad spectrums of the nuclear genome, even among long divergent species (Kronforst et al., 2006; Kronforst, 2008). The latter studies demonstrated that butterfly species, differentiated at a small number of wing patterning loci, important for maintaining Mullerian mimicry, have experienced considerable gene flow at a number of other nuclear loci. These studies and other showed that species boundaries can remain porous to gene flow long after initial divergence (Chan and Levin, 2005; Kronforst, 2008).

Buffalofishes (Genus *Ictiobus*) are large, robust-bodied suckers (Family Catostomidae) adapted to large rivers and lakes of North America east of the continental divide, inclusive of Gulf drainages of Mexico. Four or five extant species of buffalofishes are recognized: the smallmouth buffalo, *Ictiobus bubalus* (Rafinesque); bigmouth buffalo, *I. cyprinellus* (Valenciennes); fleshylip matelote, *I. labiosus*

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(Meek); chopa, *I. meridionalis* (Gunther); and black buffalo, *I. niger* (Rafinesque). Extant species of *Ictiobus* are readily diagnosed by morphological characters such as body shape, size and position of the mouth, head size, gill raker number, and numerous osteological characters (reviewed in Smith 1992). Most recent workers treat *I. meridionalis* as synonymous with *I. bubalus* (Mayden et al., 1992; Smith, 1992; Miller et al., 2005; but see Bart et al., 2004).

Genus *Ictiobus* is naturally distributed throughout much of eastern North America (Fig 1). *Ictiobus labiosus* and *I. meridionalis* occur allopatrically in east-central and extreme southern Mexico, respectively. The native distributions of the other three species—*Ictiobus bubalus*, *I. cyprinellus* and *I. niger*—are centered mainly on the Mississippi River Basin and are broadly overlapping, although ranges of *I. bubalus* and *I. niger* extend considerable distances east and west of the Mississippi River along coastal drainages of the Gulf of Mexico. Populations of *Ictiobus bubalus*, *I. cyprinellus* and *I. niger*, most likely derived from captively-propagated stocks from the Mississippi River Basin, have been introduced elsewhere in North American and to other parts of the world (Welcomme, 1988).

Hybrids among sympatric species of *Ictiobus* have been widely reported in the Mississippi River Basin (Becker, 1983; Robison and Buchanan, 1988; Etnier and Starnes, 1993) and they are readily produced in the laboratory and in experimental ponds (Stevenson, 1964). Hybrid swarms have been reported in man-made reservoirs (Johnson and Minckley, 1969).

Ictiobus has an extensive fossil record. The earliest fossil evidence dates to the middle Miocene (~15 mya) of South Dakota (Cavender, 1986). Fossils identified as *I.* cf. *bubalus* are known from lower Pliocene deposits of Oklahoma (Smith, 1962), and fossil remains agreeing with *I. cyprinellus* were identified in pre-glacial, early Pleistocene deposits of Nebraska (1–1.5 mya, Smith and Lundberg, 1972). A remarkably well-preserved series of fossilized skeletal remains from upper Pliocene lacustrine deposits near Tula de Allende, Hidalgo, Mexico, was recently described *I. aguilerai* (Alvarado-Ortega et al., 2006).

A comprehensive phylogenetic analysis of Family Catostomidae, involving osteological, allozymic, developmental and external morphological characters (Smith, 1992) found *I. niger* to be the most basal of extant *Ictiobus* species. Among the remaining three species in the analysis, *I. bubalus* was sister to *I. cyprinellus* plus *I. labiosus*, suggesting an unusual pattern of vicariance involving a Mexican Plateau endemic and a species largely confined to the Mississippi River Basin.

In this study, we describe patterns of morphological (morphometric) and DNA sequence variation (mitochondrial and nuclear) across the geographic ranges of extant species of *Ictiobus*. The antiquity of the genus and tendency of species to form discrete, diagnosable units based on morphology suggest that species of *Ictiobus* species have been diverging for a long time (perhaps, several million years). Under this scenario, species should form reciprocally monophyletic clades, consistent with morphological variation, in both mitochondrial and nuclear genes trees. In contrast, however, we found extensive mitochondrial and nuclear DNA evidence of gene flow among four of the five extant species of *Ictiobus*. Moreover, the pattern is not confined to the Mississippi River Basin. We attribute this to a long history of introgressive

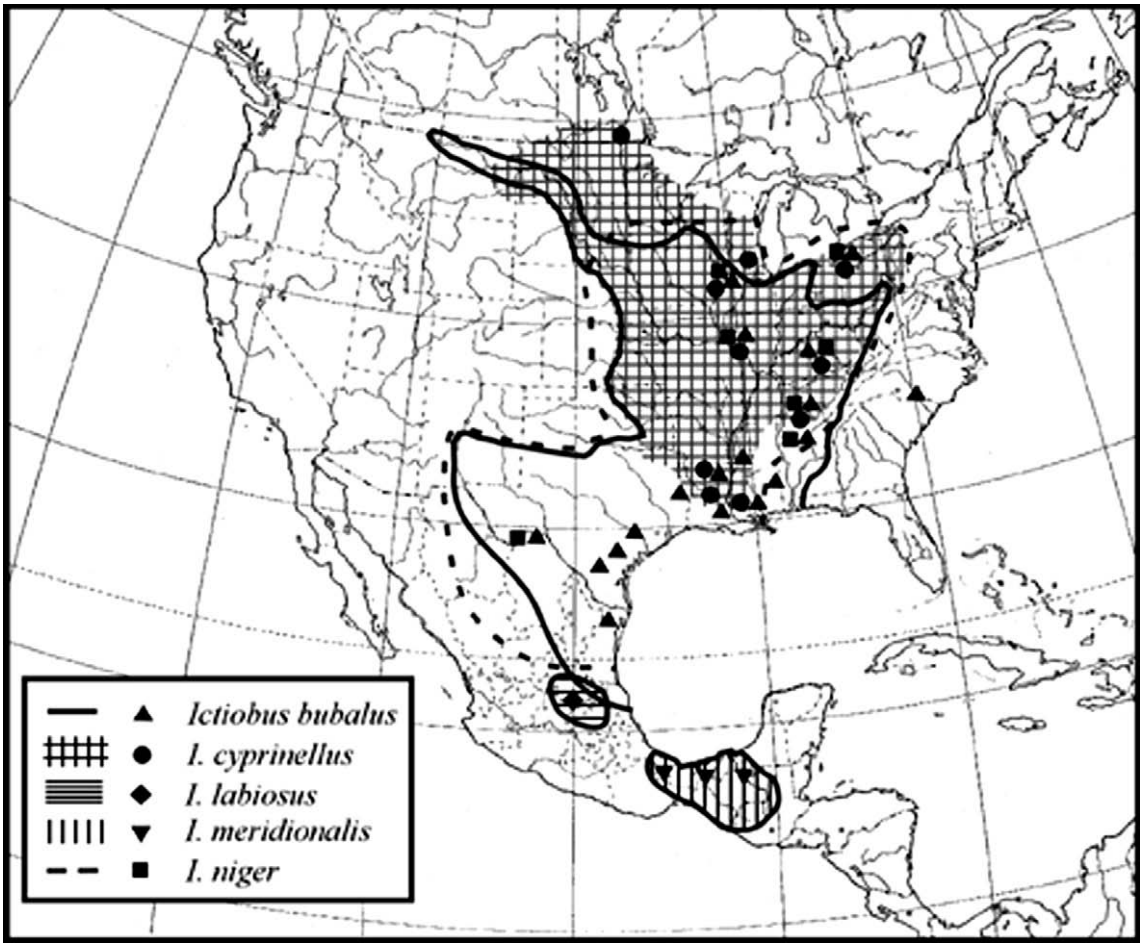

Fig. 1. Map of the distributions of extant *Ictiobus* species (outlines and shading), showing the locations of populations of five species sampled for this study.

hybridization among species inhabiting the present-day Mississippi River Basin, and possibly pre-Pleistocene rivers from which the basin formed. The pattern of genetic variation also suggests that gulf coastal rivers east and west of the Mississippi River were recently colonized by introgressed Mississippi River Basin stocks. The results of this study support the findings of other recent studies which suggest that interspecific hybridization and introgression are more important factors in animal evolution than previously thought.

2. Materials and methods

This study is based on shape analysis of 155 specimens, and DNA sequence variation from 179 specimens (the latter subsampled from 490 specimens), representing populations from across the geographic ranges of all extant species of *Ictiobus* (Table 1). In most instances, we were able to confidently assign specimens to species using established diagnostic characters we either applied in the field or applied to images of specimens produced by others. We avoided unusual specimens and specimens that we suspected might be hybrids. Only six specimens, one from the Red River of the North (Hudson Bay drainage) and five from the Grand River (Lake Erie System) could not be assigned to species because neither the specimens nor images were available for examination. We produced mitochondrial gene data for five of these specimens and nuclear gene data for two of them. Most tissues used for genetic analysis were taken from the same specimens used for morphometric analyses.

2.1. Shape variation

Geometric morphometric methods were used to characterize body shape differences among species of *Ictiobus* and to test whether known groups are morphometrically separable (Zelditch et al., 2004). The software program tpsDig ver 1.04 (© F.J. Rohlf, 2004) was used to digitize 15 homologous landmarks along the body outline of images of preserved or freshly collected specimens. The program tpsReg was used to perform procrustes superimposition analysis (Bookstein, 1991) to remove non-shape variation due to orientation, location, and scale. Size-free, shape variables (partial warps) were then derived by regressing landmark coordinates on centroid size (the square root of the sum of squared distances of a set of landmarks from their centroid). Partial warp scores were subjected to Canonical Discriminant Analysis (CDA) and MANOVA with species as the grouping variable, using the CANDISC and GLM (MANOVA option) procedures in SAS 9.1 for Windows (SAS Institute, Inc., Cary NC).

2.2. DNA sequencing and characterization

We obtained complete cytochrome *b* (hereafter, cytb) gene sequence data for 110 specimens of *Ictiobus* representing populations from throughout the geographic ranges of all extant species, including *I. labiosus* from Central Mexico, and *I. meridionalis* from southern Mexico (Table 1). A number of populations are represented by multiple individuals. The only form of *Ictiobus* not represented in our analysis is the population from the Nazas River of central Mexico, which has been variously referred to as *Ictiobus niger* and *I.* sp. cf. *niger* (Miller et al., 2005; Alvarado-Ortega et al., 2006).

Total genomic DNA was extracted from frozen or ethanol preserved tissues using the DNeasy Tissue Kit (Qiagen, Inc.) following the manufacturer's protocol, or by using a 5.0% Chelex solution with 3 μl of Protienase K and overnight digestion at 55 °C. Approximately 2 or 3 μl of DNA template and primers GLU (5′-TAACCGAG ACCAATGACTTG-3′) and THR (5′-ATCTTCGGATTACAAGACCG-3′) were used in polymerase chain reaction (PCR) amplification of the 1140 bp cytb gene. Reactions were cycled with the following temperature profile: initial denaturation at 94 °C for 2 min, followed by one cycle each of 94 °C for 30 s, annealing at 57–55 °C

Table 1
Species and populations of *Ictiobus* sampled for this study with groupings for geographic comparisons.

Species	Population	Grouping
Ictiobus bubalus	Pee Dee River, NC	No comparison
	Cahaba River, AL	Eastern Gulf
	Pasgagoula River, MS	Eastern Gulf
	Pearl River, LA	Eastern Gulf
	Amite River, LA	Eastern Gulf
	Red River, LA	Lower Mississippi River Basin
	Atchafalaya River, LA	Lower Mississippi River Basin
	Tennessee River, TN	Middle Mississippi River Basin
	Green River, KY	Middle Mississippi River Basin
	Kentucky River, KY	Middle Mississippi River Basin
	Mississippi River, IL	Upper Mississippi River Basin
	Wisconsin River, WI	Upper Mississippi River Basin
	Lake Erie, ON	Great Lakes/Hudson Bay
	Sabine River, TX	Western Gulf
	Neches River, TX	Western Gulf
	Colorado River, TX	Western Gulf
	Guadalupe River, TX	Western Gulf
	Nueces River, TX	Western Gulf
	Rio Grande, TX	Western Gulf
	Rio San Fernando, MX	Western Gulf
Ictiobus cyprinellus	Amite River, LA	Eastern Gulf
	Ouachita River, LA	Lower Mississippi River Basin
	Red River, LA	Lower Mississippi River Basin
	Atchafalaya River,LA	Lower Mississippi River Basin
	Green River,KY	Middle Mississippi River Basin
	Kentucky River, KY	Middle Mississippi River Basin
	Mississippi River, Illinois	Upper Mississippi River Basin
	Wisconsin River, WI	Upper Mississippi River Basin
	Lake Mendota, WI	Upper Mississippi River Basin
	Lake Huron, ON	Great Lakes/Hudson Bay
	Lake Erie, ON	Great Lakes/Hudson Bay
	Red River of the North, MB	Great Lakes/Hudson Bay
Ictiobus niger	Cahaba River, AL	Eastern Gulf
	Amite River, LA	Eastern Gulf
	Tennessee River, TN	Middle Mississippi River Basin
	Kentucky River, KY	Middle Mississippi River Basin
	Mississippi River, IL	Upper Mississippi River Basin
	Wisconsin River, WI	Upper Mississippi River Basin
	Lake Huron, ON	Great Lakes/Hudson Bay
	Lake St. Clair, ON	Great Lakes/Hudson Bay
	Rio Grande, TX	Western Gulf
Ictiobus labiosus	Rio Santa Maria, MX	Río Pánuco
Ictiobus meridionalis	Rio Papaloápan, MX	Southern Gulf
	Rio Coatzocoalcos, MX	Southern Gulf
	Rio Usumacinta, MX	Southern Gulf

for 30 s, extension at 72 °C for 75 s, followed by 32 cycles with annealing at 51 °C for 30 s, and a final extension of 72 °C for 10 min. Viable PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Inc.) or ExoSap-It (USB) and following manufacturer's protocols prior to sequencing.

Purified PCR products were cycle sequenced with ABI v. 3.1 (Applied Biosystems, Inc.). Four internal sequencing primers (designed with OLIGO, Molecular Biology Insights), three forward (1F, 5′-GAC TTG AAG AAC CAC CGT TG-3′; 254F, 5′-GGR GCA TCR TTY TTY TTY AT-3′; 500F, 5′-ATY TGA GGN GGR TTY TCR GT) and one reverse (4R, 5′-CCG ATG CTT TTA GGC TAA GC-3′), were used to obtain complete bi-directional sequences of the cytb gene. Prior to visualization, excess dye terminators, primers and nucleotides were removed from sequenced products using gel filtration with Performa DTR cartridges (Edge Biosystems). Purified products were visualized with either an ABI 373A or ABI 3100 automated sequencer performed at Tulane University or at the W.M. Keck Laboratory at the University of New Orleans, respectively, or sequenced by the Macrogen Corporation (Seoul, Korea). Resulting sequences were edited and aligned using Sequencher 4.1 (Gene Codes) and adjusted by eye when necessary. Amino acid sequences were determined from aligned sequences using MacVector 4.0 (Oxford Molecular).

We also sequenced the second intron of one of the two paralogous copies of the nuclear growth hormone gene (GH). Catostomids are tetraploids (Uyeno and Smith, 1972), with duplicate copies of most genes. In a previous study, we isolated and characterized cDNAs for the two paralogs of GH in *Ictiobus bubalus*, then used polymorphisms in the untranslated regions of the cDNAs to design locus-specific primers for amplifying and sequencing the genomic region (exons and introns) of both GH paralogs of *I. bubalus*, as described in Clements et al. (2004). To obtain GH intron data for this study, we designed primers specific to GH Copy 1 in exons flanking the second intron. Primers were designed so that sequence polymorphisms would be in the 3′ region of at least one primer (Oakley and Phillips, 1999). Amplifications of intron 2 used primers GH1-67F (5′-TCA GAN AAC CAG MGG CTC TTC-3′) and GH1-175R (5′-TTC TGG GTT TCA TGT TTG TCA-3′) and were subjected to the following PCR conditions: 94 °C for 2 min, then 40 cycles 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s.

We identified GH intron 2 alleles and genotyped individuals by Single Stranded Comformational Polymorphism (SSCP) analysis for 109 specimens of *Ictiobus* representing all species and virtually all of specimens sampled for cytb sequence data. Between 5 and 10 μl of the PCR was diluted with an equal volume of SSCP loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The mixture was heated at 95 °C for 3 min and immediately chilled on ice for 5 min. Samples were loaded onto a 19 cm (W) × 16 cm (L), 10% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) and electrophoresed in 0.5× TBE at 5 W for 15 h. The gel apparatus was kept cool during the run by placing it in a 4 °C refrigerator. At the end of the run, the gel was silver stained or fluorescently stained with EtBr or SYBR gold using a modified protocol described by Rodzen et al. (1998). Fluorescently stained gels were visualized with a UV transilluminator or scanned with a FUJIFILM (FLA-3000G) laser scanner. Silver stained gels were dried at room temperature overnight, then visualized with a light box and photographed with a digital camera.

Fig. 2 is a SSCP gel showing allele determinations for 13 *Ictiobus* specimens representing Mississippi River Basin populations of *I. bubalus*, *I. cyprinellus* and *I. niger*. Alleles were identified based on banding patterns on the top portion of the gel. Homozygotes had fewer bands than heterozygotes, and lacked heteroduplexes (circled in Fig. 2), which usually were visible on the lower portion of the gel.

Individuals determined by SSCP to be homozygous at the GH intron locus were directly sequenced. One or two homozygous individuals with matching SSCP profiles were sequenced per SSCP run. We confirmed the identity of sequenced products by comparing them to GH Copy 1 and 2, intron 2 sequences of *I. bubalus*. Genotypes of heterozygous individuals were determined by amplifying and running homozygous individuals of known genotype (and sequence) in adjacent lanes of the SSCP gel. For heterozygous individuals that contained rare alleles, and also to periodically confirm genotype sequences of heterozygous individuals, a small portion of each SSCP product, corresponding to a specific allele, was cut from the SSCP gel and diluted in 20 μl TE buffer or H2O. The dilution was kept at 4 °C overnight to elute the single stranded PCR product from the gel slice. Two μl of the eluted SSCP PCR product for the allele of interest was then used in a subsequent PCR reaction using the same PCR conditions and directly sequenced using methods described above.

2.3. Population genetic analysis

We performed AMOVA to test for significant genetic structure in haplotype and allele frequencies among sufficiently sampled

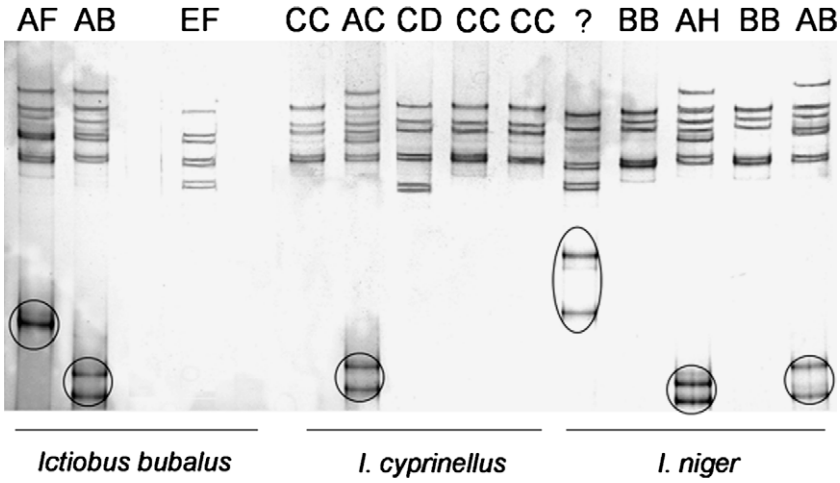

Fig. 2. SSCP gel showing allele determinations for 13 *Ictiobus* specimens representing Mississippi River Basin populations of *I. bubalus*, *I. cyprinellus* and *I. niger*. Alleles were identified based on banding patterns on the top portion of the gel. Homozygotes had fewer bands than heterozygotes, and lacked heteroduplexes (circled), which usually were visible on the lower portion of the gel (not visible on the portion of the gel photographed for two of the heterozygous specimens). Note the similarity in position of bands for like-lettered alleles.

species of *Ictiobus* (*I. bubalus*, *I. cyprinellus* and *I. niger*) and among geographic areas (Great Lakes/Hudson Bay, Mississippi River), using Arlequin 3.11 (Excoffier et al., 2005). AMOVAs that suggested significant genetic structure among species or geographic areas were followed up with Exact Tests of Differentiation (Raymond and Rousset, 1995) to explore pair-wise genetic differences between species. Contingency tables used in these tests were estimated using a Markov chain of 10,000 steps. Further tests of genetic structure among *Ictiobus* species were explored with a phylogenetic approach using the recently developed Genealogical Sorting Index (Cummings et al., 2008) available at http://www.genealogicalsorting.org/.

2.4. Phylogenetic Analysis

Phylogenetic trees were generated using Bayesian maximum likelihood optimality criteria (BML). Prior to the BML analysis, we determined the best-fit model of sequence evolution for each codon position of the cytb gene using 56 progressively complex models of sequence evolution and the Akaike Information Criterion (AIC) implemented in PAUP* 4.0b10 (Swofford, 2003) and Modeltest 3.7 (Posada and Crandall, 1998). Modeltest (3.7) selected different models of sequence evolution for each of the three codon positions: K80 for first positions, F81 for second positions, and TrN+I for third positions. A GTR + gamma model was selected for GH intron data. These models and their corresponding parameters were then used in partitioned mixed-model BML analyses in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). Model parameters were assigned to codon position using the LSET and APPLYTO commands. The appropriate model parameter values (e.g., transition/transversion ratios and empirical base frequencies) were estimated for each partition independently using the UNLINK command. Four Markov chains were used with a temperature profile of 0.2. Default substitution priors were used in all analyses and random trees were used to start each Markov chain. Runs consisted of 5 million generations of Markov chain Monte Carlo simulations (MCMC) with resampling of tree topologies every 100th generation. Four replicate runs were conducted to insure that the MCMC algorithm went through a sufficient number of iterations to provide convergence in the estimations of the tree topology with the best ML posterior probability. The burn-in of the MCMC analysis was determined by graphically examining the ML scores at each of the sampled generations to find where values converged. The posterior probability or frequency at which a particular clade occurred in the remaining trees was used as an indication of node support. Additional runs in which priors were modified were conducted, but these did not change the resulting topology.

To further examine haplotype and allele relationships, haplotype networks were generated for each data set (cytb and GH) using TCS (v.1.12, Clement et al., 2000), which implements a 95% statistical parsimony procedure and allows for reticulating patterns that may highlight instances of hybridization or gene flow among populations or species (Templeton et al., 1992, 1995; Crandall, 1996).

2.5. Test of species monophyly

The monophyly of each of the morphologically diagnosable *Ictiobus* species was tested using trees constrained to represent each species as a monophyletic clade. Constraint trees were generated using Mesquite v1.06 (Maddison and Maddison, 2009). Support for these alternative hypotheses of relationships (alternative gene tree topologies) was tested against recovered gene trees using a Bayesian approach, which examines the number of trees in the posterior distribution of post-burnin trees containing the alternative hypothesis (Farrell and Sequeira, 2004; Weisrock et al., 2006), and the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) using maximum likelihood constraint tree searches implemented in PAUP* 4.0b10. For the Bayesian test, if an alternative hypothesis (e.g., monphyletic *I. bubalus*), represented by one of the constrained trees was recovered in less than 5% of the post-burnin Bayesian trees, then the hypothesis was considered statistically rejected by the test (Weisrock et al., 2006).

3. Results

3.1. Shape variation

Species of *Ictiobus* are distinguishable based on overall body shape. The MANOVA of derived "size-free" shape variables detected significant differences among all five species of *Ictiobus* (Wilk's $\lambda = 0.0005$; $F = 22.88$; DF = 112; $p < 0.0001$). In the plot of PCA scores for the five species on PCA axes 1 and 2 (Fig 3A), *I. cyprinellus* and *I. labiosus* specimens form distinct clusters, widely separated from each other and from other *Ictiobus* specimens along PC1. The other three species form overlapping clusters, but show some separation along PC2. A separate MANOVA of derived shape variables for *Ictiobus bubalus*, *I. meridionalis* and *I. niger* (i.e., excluding *I. cyprinellus* and *I. labiosus*) revealed significant differences in body shape among these species (Wilk's $\lambda = 0.037$; $F = 9.91$; DF = 56; $p < 0.0001$). In the PCA plot based on these data (Fig 3B), separation among the species clusters is apparent, although there is still a fair amount of overlap, especially involving *I. niger*. Interestingly, the greatest separation in this plot is between *I. bubalus* and *I. meridionalis*, which some authors have argued are synonymous.

Thus, our geometric morphometric analysis results confirm that, with the exception of *I. niger*, traditionally recognized species of *Ictiobus* are morphometrically distinct. These results give us confidence about our assignment of most specimens to species in our genetic analyses.

3.2. Cytochrome b sequence variation

Complete cytb sequence data (1140 bp) were generated for 110 specimens of *Ictiobus* (GenBank Accession Nos. FJ226255–FJ226364) plus a specimen of *Carpiodes* sp. cf. *cyprininus* from the Apalachicola River, which served as the outgroup. Across all specimens of *Ictiobus*, the cytb gene varies at 100 sites, 85 of which are third codon position transitions. Most substitutions are synonymous, although three amino acid changes were detected, all at the 3′ end of the gene. The cytb sequence for *I. labiosus* and sequences from four specimens representing three other *Ictiobus* species (two *I. cyprinellus*, and one specimen each of *I. bubalus* and *I. niger*) code for Alanine at amino acid position 327, whereas all other specimens have Threonine at this position (a non-conservative change). The *I. labiosus* cytb sequence has Isoleucine in position 333, whereas all other *Ictiobus* species vary in having Isoleucine or Valine in this position. The *I. labiosus* sequence also codes for Isoleucine at position 364, whereas all other *Ictiobus* have Valine at this position (a conservative substitution).

We detected 42 cytb haploytpes among the 110 *Ictiobus* cytb sequences (Table 2, Fig. 4). Sequence divergence (corrected distance) among all *Ictiobus* specimens averaged 1.51% overall. Much of this is due to the relatively high degree of divergence of the *I. labiosus* cytb sequence from sequences of other specimens of *Ictiobus* (average of 6%). Excluding *I. labiosus*, pairwise distance among remaining species of *Ictiobus* (0.00–0.36%), is similar to cytb divergences observed within fish populations or species.

We detected a high degree of sharing of cytb haplotypes among *I. bubalus, I. cyprinellus, I. meridionalis* and *I. niger*. The 42 haplotypes cluster around two common haplotypes (H2 and H3), which are shared by all four of the above species. *Ictiobus bubalus* and *I.*

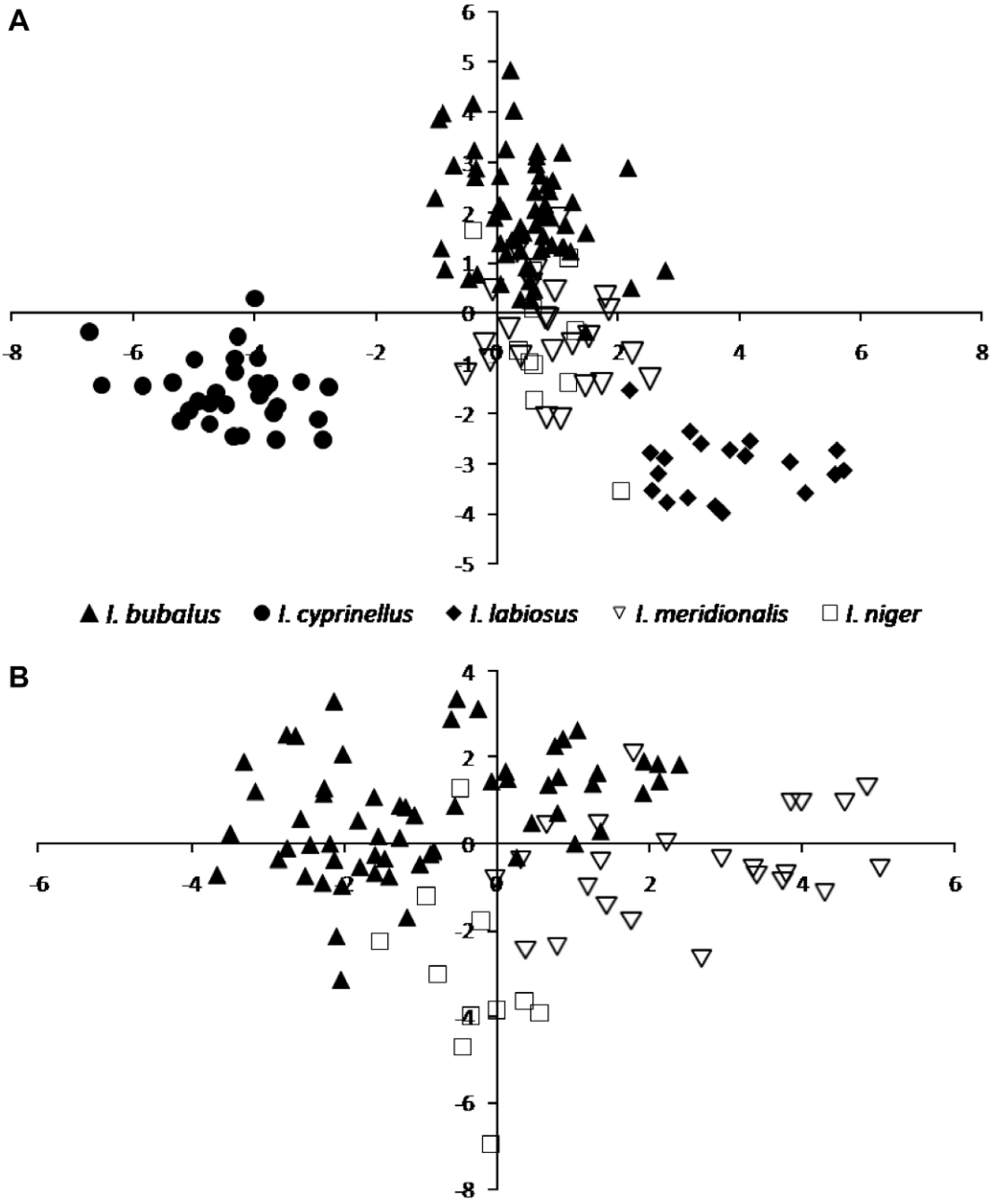

Fig. 3. Principal Components Analysis (PCA) results showing body shape differences among species of *Ictiobus* based derived shape variables. (A) Plot of the five extant species of *Ictiobus* on PCA axes 1 and 2. (B) Plot based on a separate PCA showing shape differences among *I. bubalus*, *I. meridionalis* and *I. niger*.

niger shared four other haplotypes not shared with *I. meridionalis* and *I. cyprinellus*. *Ictiobus cyprinellus* and *I. niger* shared two other haplotypes not shared with other species. Haplotype diversity is highest in *I. bubalus* (45 specimens with 20 unique haplotypes and six shared with other *Ictiobus* species). We detected 14 haplotypes among 26 *Ictiobus niger* specimens, eight shared, six unique, and nine among 32 specimens of *I. cyprinellus*, four shared, five unique.

Cytb haplotypes are shared across species separated by large geographic distances. The greatest distance involved Haplotypes 2 and 3, both of which were identified in specimens of *I. meridionalis* from extreme southern Mexico (Ríos Papaloapan, Coatzocoalcos, and Usumacinta), and specimens of *I. bubalus*, *I. cyprinellus* and *I. niger* from the Great Lakes and the Upper Mississippi River Basin. Frequencies of occurrence of cytb haplotypes across *Ictiobus* species and individuals suggest little structuring of cytb haplotype diversity (Table 2). Haplotype H2 is the dominant haplotype in *I. cyprinellus*, occurring in 34.4% of specimens, but it is also found in 31% of *I. niger* specimens and 43% of *I. meridionalis* specimens. Haplotype H3 is the dominant haplotype in *I. bubalus*, but was commonly encountered in specimens of *I. cyprinellus* (16% of specimens), *I. niger* (19% of specimens) and *I. meridionalis* (14% of specimens).

The AMOVA based on frequencies of cytb in *Ictiobus bubalus*, *I. cyprinellus* and *I. niger* detected evidence of significant genetic structuring at the species level ($F_{ST} = 0.07$, $p < 0.00001$). However, the F_{ST} value is quite low, indicating that a high portion of the variation is within groups rather than between groups. Exact Tests of Differentiation among these species detected significant differences in haplotype frequencies only between *I. cyprinellus*

Table 2
Cytochrome *b* haplotypes for the five *Ictiobus* morphotypes. Numbers are the number of individuals possessing a given haplotype, followed in parentheses by its frequency (%) of occurrence within and across *Ictiobus* species.

Haplotypes	Morphotypes				
	I. cyprinellus	*I. niger*	*I. bubalus*	*I. meridionalis*	*I. labiosus*
H18	–	–	1 (2.2; 100)	–	–
H19	1 (3.1; 100)	–	–	–	–
H20	2 (6.3; 100)	–	–	–	–
H21	3 (9.4; 100)	–	–	–	–
H22	–	–	1 (2.2; 100)	–	–
H23	–	–	1 (2.2; 100)	–	–
H24	–	–	1 (2.2; 100)	–	–
H25	–	–	–	1 (14.3; 100)	–
H26	–	–	–	2 (28.6; 100)	–
H27	–	1 (3.9; 50)	1 (2.2; 50)	–	–
H28	–	1 (3.9; 50)	1 (2.2; 50)	–	–
H29	–	1 (3.9; 50)	1 (2.2; 50)	–	–
H30	–	1 (3.9; 100)	–	–	–
H31	–	–	2 (4.4; 100)	–	–
H32	–	–	2 (4.4; 100)	–	–
H33	–	–	1 (2.2; 100)	–	–
H34	–	–	3 (6.7; 100)	–	–
H35	–	–	2 (4.4; 100)	–	–
H36	–	–	1 (2.2; 100)	–	–
H37	–	–	2 (4.4; 100)	–	–
H38	–	–	1 (2.2; 100)	–	–
H39	–	–	1 (2.2; 100)	–	–
H40	–	–	2 (4.4; 100)	–	–
H41	–	–	1 (2.2; 100)	–	–
H42	–	–	1 (2.2; 100)	–	–
Total # individuals	32	26	45	7	1
Total # haplotypes	9	14	26	4	1

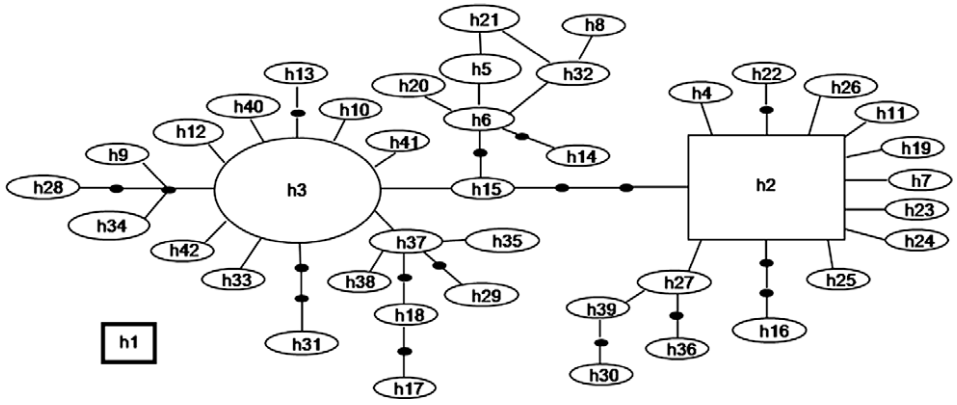

Fig. 4. 95% Statistical parsimony network for 42 cytochrome *b* haplotypes recovered from 110 *Ictiobus* specimens, representing all currently recognized species (haplotype designations are the same as in Table 2). Haplotype H1 (*I. labiosus*) differs from other *Ictiobus* cytb haplotypes by more than 14 substitutions.

and *I. bubalus* ($p < 0.001$). We detected significant Genealogical Sorting (i.e., phylogenetic structure) in cytb haplotype frequencies only for *I. cyprinellus* (GSI = 0.077, $p < 0.004$), suggesting that only individuals of this species tend cluster in phylogenetic trees.

3.3. GH intron

We identified 15 distinct GH Copy 1, intron 2 alleles among the 109 *Ictiobus* specimens studied (Fig. 5). We sequenced intron alleles for 11 specimens of *Ictiobus* representing all five species and all 15 intron alleles. The intron sequences are roughly 230 nt in length. We were able to identify all of the alleles by SSCP. The intron sequences have been assigned GenBank Accession Nos. FJ226079–FJ226254. We are confident that we amplified the second intron of only one paralog of GH. Between gene copies within a species (*I. bubalus*), the second GH intron differs at 66 positions (29% of sites), including 14 indels, which make sequences of the two intron copies difficult to align. Within a copy, the second intron differs by at most 34 positions (15% of sites) with a single large indel (*I. labiosus*).

The three specimens of *I. labiosus* for which the second GH intron was sequenced had three exclusive alleles that differed at 1–2 base positions. The *I. labiosus* GH intron alleles are the most divergent, differing from alleles in other *Ictiobus* specimens at as many as 34 positions (15% of sites), including a unique 19 bp indel. The 12 remaining GH intron alleles are shared across all *Ictiobus* species. Three of these alleles (denoted A, B and C) are common, being represented in a third or more of specimens depending on species. The most divergent of these alleles, allele A, differs from alleles B and C at 14–15 positions (7% of sites), including a five bp indel (Table 3). Alleles B and C are similar, differing at only 3 positions (1.3% of sites), including a 1 bp indel. The other 9 alleles were much less common and differed at only 1–2 positions.

Heterozygosity of GH intron alleles is high within (average of 61.8%) and among (68%) *Ictiobus* species (excluding *I. labiosus*). Specimens of *I. bubalus* and *I. niger* had a predominance of alleles A and B (equal frequencies for both, Table 4). However, individuals of these species also commonly possessed allele C, both as homozygotes and heterozygotes. Specimens of *I. cyprinellus* had a predominance of allele C (homozygotes and heterozygotes), but often had alleles A and B as heterozygotes. Specimens of *I. cyprinellus* were sometimes homozygous for allele B, but never allele A. Specimens of *I. meridionalis* most commonly had alleles B and G. Allele A was not found in any of the specimens of *I. meridionalis* studied.

Geographically, allele A was found primarily in the Mississippi River Basin, but was also detected in the Great Lakes and Hudson Bay drainage basins to the north, in the nearby Lake Pontchartrain Basin just east of the lower Mississippi River, and west of the Mississippi River as far as the Guadalupe River in Texas (Table 5). Alleles B and C, and variant alleles F and G, extend further east and west of the Mississippi River to the Mobile Basin in Alabama and the Usumacinta River Basin at the Mexican-Guatemalan border.

GH intron sequences were more variable than cytb gene sequences in *Ictiobus*, contrary to patterns of sequence variation typically seen when slower evolving nuclear gene regions are compared to mitochondrial genes. Mean GH intron sequence divergence among all *Ictiobus* specimens was 3%, compared to 1.5% mean divergence among all cytb sequences. Excluding divergent *I. labiosus* GH intron sequences (which were 5.6% divergent from GH intron sequences of other *Ictiobus*), mean divergence among remaining *Ictiobus* GH intron sequences was 1.9%, compared to 0.36% divergence among cytb sequences.

Similar to the cytb results, AMOVA revealed significant differences in GH intron allele frequencies among species of *Ictiobus* (F_{ST} = 0.04321, p = 0.00880 ± 0.00288). Once again, F_{ST} values are low, indicating high within group variation relative to between group variation. Exact Tests of Differentiation among *Ictiobus bubalus*, *I. cyprinellus* and *I. niger* indicate that GH intron allele frequencies of *I. cyprinellus* differ significantly from those of *I. bubalus* (p = 0.00000 ± 0.0000) and *I. niger* (p = 0.00000 ± 0.0000).

3.4. Phylogenetic analyses

Fig. 6 is a 50% Majority-rule consensus phylogram of 97,000 post-burnin trees resulting from partitioned, mixed-model Bayesian analysis of the cytb data (two separate runs stabilized at 50,000–60,000 generations with a burnin of 500–600 trees). *Ictiobus labiosus* is basal and highly divergent from all other species of *Ictiobus*. None of the other morphologically diagnosable species of *Ictiobus* is resolved as monophyletic in the cytb tree. Moreover, there is no statistical support for the monophly of any of these species based on results of the Shimodaira–Hasegawa tests and Bayesian Constraint Tree searches (Table 6). The high degree of sharing of cytb haplotypes across species and across broad geographic areas (described above) is evident in the tree.

The GH intron allele tree (Fig. 7) also shows the high degree of sharing of alleles among species. As in the cytb tree, the *Ictiobus labiosus* alleles form a group distinct from all other *Ictiobus* GH intron alleles.

4. Discussion

Species of *Ictiobus* are distinguishable morphologically. The species show significant differences in overall body shape and, with the exception of *I. niger*, form more or less distinct clusters in morphometric space. Yet, *Ictiobus* species do not form reciprocally

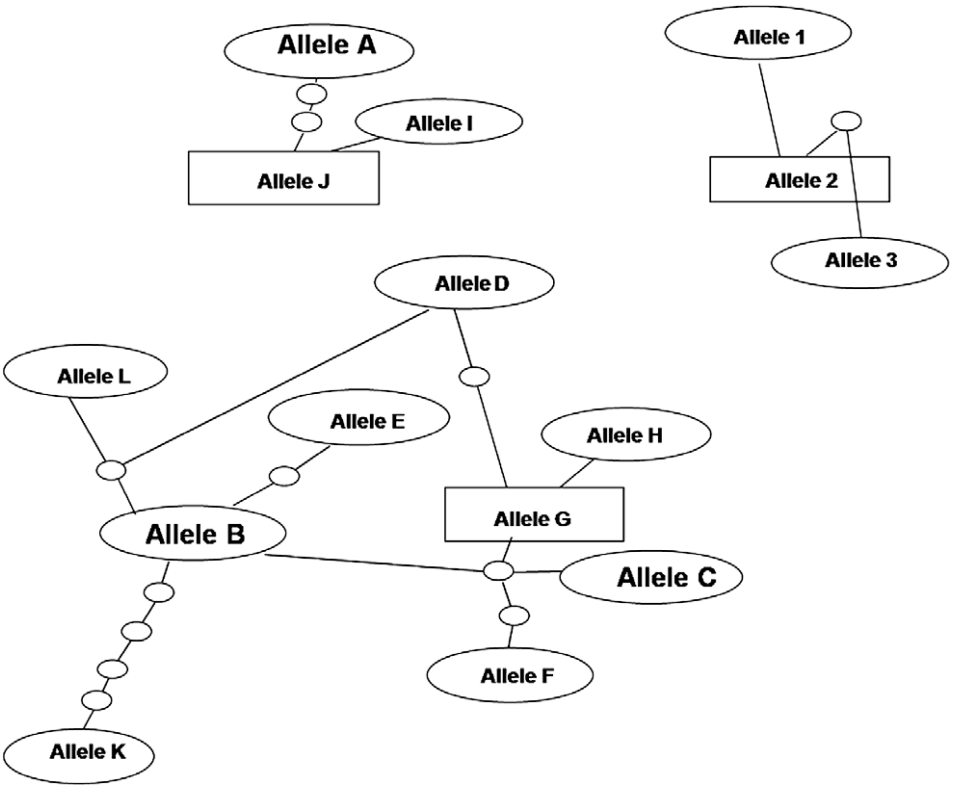

Fig. 5. 95% Statistical parsimony network for 15 GH intron alleles identified in 109 *Ictiobus* specimens representing all currently recognized species. Disconnected networks differ at more than 12 positions.

Table 3
Polymorphisms in intron alleles of *Ictiobus* species (excluding *Ictiobus labiosus*). Dashes denote gaps/indels inferred by the aligned seuqences.

Allele	Nucleotide position																				
	26	90	98	111	114	115	116	129	145	147	151	157	158	169	170	191	192	193	194	195	217	220
A	T	C	A	C	A	G	C	T	A	T	A	T	G	C	A	A	T	T	T	A	C	G
B	T	A	A	T	A	T	C	T	T	T	C	G	A	T	G	–	–	–	–	–	C	G
C	–	A	A	T	A	T	C	T	A	T	C	G	A	T	G	–	–	–	–	–	C	G
D	T	A	A	T	A	T	C	T	T	T	C	C	A	T	G	–	–	–	–	–	T	G
E	T	A	A	C	A	G	C	T	T	T	C	G	A	T	G	–	–	–	–	–	C	G
F	T	A	C	T	A	T	C	G	A	T	C	G	A	T	G	–	–	–	–	–	C	G
G	T	A	A	T	A	T	C	T	A	T	C	G	A	T	G	–	–	–	–	–	T	G
H	T	A	A	T	A	T	C	T	A	G	C	G	A	T	G	–	–	–	–	–	T	G
I	T	A	A	C	–	–	–	T	A	T	A	T	G	C	A	A	T	T	T	A	C	G
J	T	A	A	T	A	T	C	T	T	T	C	G	A	T	G	A	T	T	T	A	C	G
K	T	C	A	C	–	–	–	T	A	T	A	T	G	C	A	A	T	T	T	A	C	G
L	T	A	A	T	A	T	C	T	A	T	C	C	A	T	G	–	–	–	–	–	C	T

Table 4
Frequencies of 12 GH Copy 1, intron 2 alleles in four Ictiobus morphotypes.

	A	B	C	D	E	F	G	H	I	J	K	L
Ictiobus bubalus	0.318	0.318	0.121	0.015	0.015	0.061	0.076	0.030	0.015	0.030	–	–
I. niger	0.333	0.333	0.271	–	–	–	0.042	–	–	–	0.021	–
I. meridionalis	–	0.333	0.167	–	–	0.167	0.333	–	–	–	–	–
I. cyprinellus	0.194	0.081	0.581	0.097	–	0.016	–	–	0.016	–	–	0.016

Table 5
Frequencies of 12 GH Copy 1, intron 2 alleles in four *Ictiobus* morphotypes across geographic areas.

	A	B	C	D	E	F	G	H	I	J	K	L
Hudson Bay	0.200	0.300	0.500	–	–	–	–	–	–	–	–	–
Great Lakes	0.278	0.111	0.389	0.111	–	–	–	–	–	–	–	0.111
Upper Miss	0.238	0.226	0.345	0.048	–	0.024	0.036	0.024	0.024	0.024	0.012	–
Middle Miss	0.333	0.222	0.333	–	–	0.056	0.056	–	–	–	–	–
Lower Miss	0.300	0.200	0.400	–	0.100	–	–	–	–	–	–	–
Lake Pontchartrain	0.450	0.150	0.300	0.050	–	–	0.050	–	–	–	–	–
East Gulf	–	0.917	–	–	–	–	0.083	–	–	–	–	–
Western Gulf	0.375	0.375	0.125	–	–	0.125	–	–	–	–	–	–
Southern Gulf	–	0.333	0.167	–	–	0.167	0.333	–	–	–	–	–
N	48	49	60	7	1	5	7	2	2	2	1	2

monophyletic groups in either mitochondrial or nuclear gene trees. The pattern that emerges from both trees is one of high degrees of gene flow among species and across broad geographic areas (Great Lakes to extreme Southern Mexico). Only *I. labiosus*, which is confined to upland portions of the Río Pánuco on the eastern edge of the Mexican Plateau, is distinct both morphometrically and genetically. The divergent and phylogenetically basal position of this species suggests that it is the most ancestral of extant species of *Ictiobus*, not among the most derived, as hypothesized by Smith (1992). However, the high degree of genetic divergence of this taxon may simply reflect its limited opportunity for genetic exchange with other *Ictiobus* species. The species is known to overlap only slightly with the primarily lowland distributed Río Pánuco population of *Ictiobus bubalus* in the vicinity of Ciudad Valles, Mexico (Miller et al., 2005).

The relatively high degree of genetic divergence of *I. labiosus* from other *Ictiobus* species, combined with fossil evidence, provides a means of gauging DNA sequence divergence rates among *Ictiobus* species. Cavender (1986) assigned fossil catostomids from the middle Miocene of South Dakota to genus *Ictiobus*, suggesting that the genus diverged at least 15 mya. Using this value as the minimum age of the divergence of *Ictiobus* and *Carpiodes*, and the average cytb sequence divergence observed between these genera (11.6%, unpublished data) gives a rate of cytb sequence divergence of 0.78% per million years. This rate is comparable to rates calibrated based on geologic events (Zardoya and Doadrio,1999) and estimated in broad surveys of cytb variation for ectothermic vertebrates (Johns and Avise, 1998).

Applying this divergence rate to the divergence of *I. labiosus* from the common ancestor of other *Ictiobus* species places the minimum date of divergence at 4.5 mya (lower Pliocene), roughly the same age as fossils identified by Smith (1962) as *I.* cf. *bubalus*, and older than †*I. aguilerai*, recently described from lacustrine deposits near Tula de Allende, Hidalgo, Mexico (Alvarado-Ortega et al., 2006). The oldest fossils agreeing with *I. cyprinellus* were identified in pre-glacial, early Pleistocene deposits of Nebraska (1–1.5 mya; Smith and Lundberg, 1972). The earliest fossil evidence of *I. niger* is from late Pleistocene deposits (<0.2 mya; Smith, 1981). Excluding *I. labiosus*, mean cytb sequence divergence among *Ictiobus* species is 0.36% (range 0–0.62%), which is lower than expected based on fossil ages and morphological divergence.

A more plausible explanation for the low levels of interspecific cytb sequence divergence is that it is the result of introgressive hybridization. Seven of the 42 recovered cytb haplotypes are shared across 61 specimens (55% of all specimens sequenced for cytb) representing four species of *Ictiobus* (20 *I. cyprinellus*, 19 *I. niger*, 16 *I. bubalus*, 4 *I. meridionalis*) distributed from the Great Lakes to the Mexican/Guatemalan border. The broad geographic extent of this introgression suggests that species of *Ictiobus* have been hybridizing for long periods of time.

Mitochondrial introgression is commonly encountered where closely related fish species occur in sympatry (Dowling and DeM-

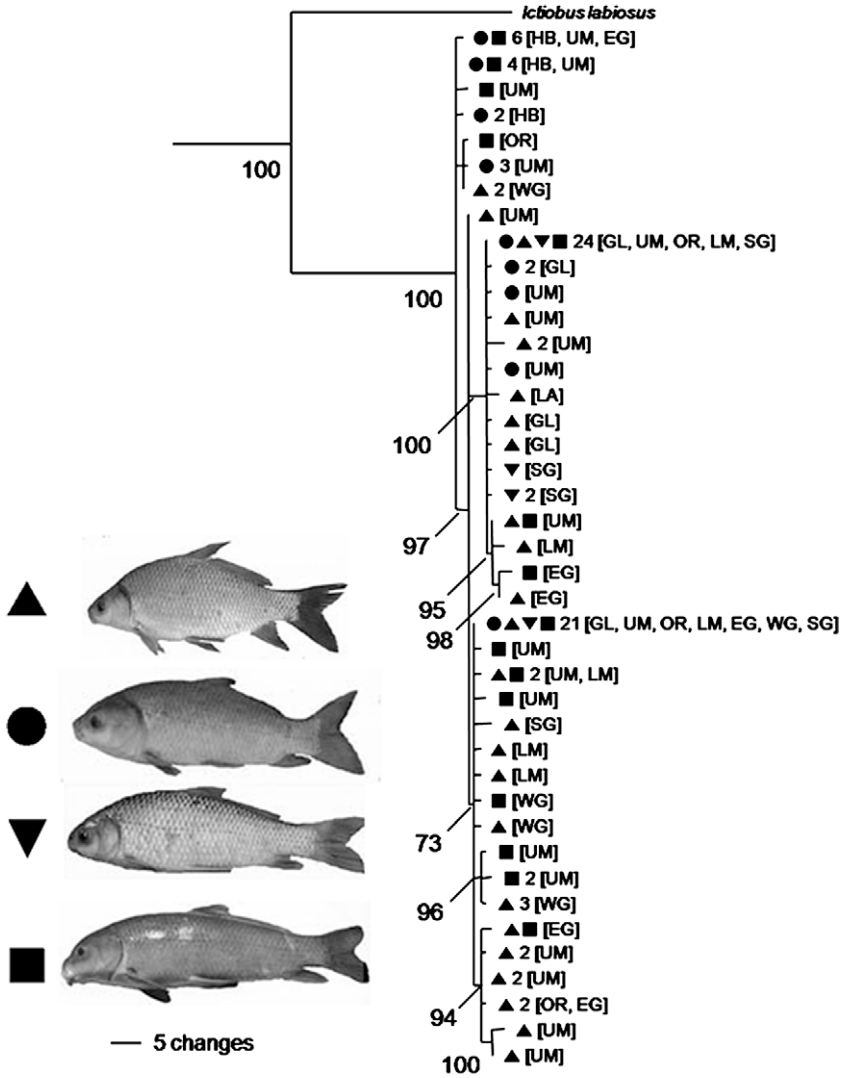

Fig. 6. Phylogenetic relationships of 110 *Ictiobus* specimens representing all species and multiple populations based on mixed-model Bayesian maximum likelihood analysis of complete cytochrome *b* sequence data. Numbers on branches are posterior probabilities and provide an indication of node support. Numbers of individuals at each branch tip and geographic areas represented (in brackets) follow species symbols. EG = Eastern gulf; GL = Great Lakes; HB = Hudson Bay; OR = Ohio River; LM = Lower Mississippi River; WG = Western Gulf; SG = Southern Gulf.

Table 6
Results of Shimodaira–Hasegawa topology tests and Bayes Constraint Tree searches of monophyly of *Ictiobus* species.

Tree	Shimodaira–Hasegawa test			Bayes Constraint Tree searches			Tree
	−ln L	Diff −ln L	P	Total trees	Trees matching constraint	Percentage	
Bayes Consensus Tree	3094.23935	Best		NA	NA	NA	1
I. meridionalis monophyletic	3785.08526	690.84591	0.000*	48,500	2408	4.96%	2
All species monophyletic	3946.32746	852.08812	0.000*	48,500	0	0.00%**	3
I. cyprinellus monophyletic	3777.46467	683.22532	0.000*	48,500	0	0.00%**	4
I. bubalus monophyletic	3776.45349	682.21414	0.000*	48,500	0	0.00%**	5
I. bubalus + *I. meridionalis* monophyletic	3784.09680	689.85745	0.000*	48,500	0	0.00%**	6
I. niger monophyletic	3787.87333	693.63399	0.000*	48,500	0	0.00%	7

* $p < 0.05$.
** Anything with 5% or less rejected at 95% limit.

arais, 1993; Sullivan et al., 2004; Wilson and Bernatchez, 1998), and discordant patterns of morphological and mtDNA sequence variation have been observed in many of these instances (Dowling and DeMarais, 1993; Gerber et al., 2001; Wilson and Bernatchez, 1998). Nuclear genes, which encode morphological, ecological and behavioral traits, tend to vary more consistently with these traits (Piller et al., 2008; Ray et al., 2007; Sullivan et al., 2004). Species of *Ictiobus* exhibit patterns of variation in both nuclear and mitochondrial genes that disagree with morphology.

Species of *Ictiobus* show less overall allelic diversity and surprisingly higher levels of sequence divergence in GH introns than in cytb gene sequences. However, the predominant pattern of varia-

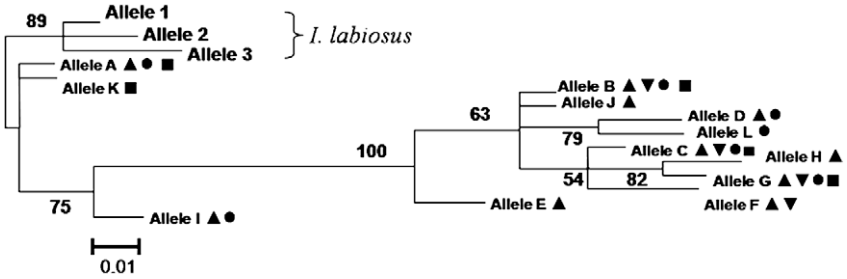

Fig. 7. Phylogenetic relationships among 15 GH intron alleles recovered from 109 *Ictiobus* specimens representing all species (symbol notation same as in other figures) based on Bayesian maximum likelihood analysis. Numbers beside branches are posterior probabilities and provide an indication of node support.

tion is the same: extensive sharing of alleles across *Ictiobus* species, excluding *I. labiosus*. The three GH intron alleles seen most commonly in *I. bubalus*, *I. cyprinellus*, *I. meridionalis* and *I. niger* (alleles A, B and C) show different degrees of divergence suggestive of different timings of origin, and confounding patterns of geographic distribution relative to species distribution, but no clear pattern of association with any of these species.

Distributions and speciation patterns of many groups of fishes and other organisms in eastern North America were strongly influenced by Pleistocene Glaciation (Mayden, 1988; Kozak et al., 2006; Berendzen et al., 2008). Advancing ice sheets caused formerly separate, north flowing, Pliocene-aged drainage systems to flow southward, eventually merging to form the present day Ohio and Mississippi Rivers. Fossil evidence suggests that ancestors of some of the currently recognized species of *Ictiobus* were diverging as early as Pliocene or early Pleistocene times in different areas of what is now the Mississippi River Basin. It is possible that the merger of these formerly separate river basins during the Pleistocene caused recently evolved modern species of *Ictiobus* to establish secondary contact. The genetic results of this study suggest that alleles of both nuclear and mitochondrial genes have flowed freely among species of *Ictiobus* in the Mississippi River Basin, and that introgressed *Ictiobus* stocks recently colonized the Great Lakes, Husdon Bay and Gulf coastal drainages north, east, west and far south of the Mississippi River. Gene flow to the north can be explained by repeated connections between the Mississippi River, the Great Lakes and Hudson Bay drainages during and since the end of Pleistocene Glaciation (Murdoch and Hebert, 1997; Widmer and Lexer, 2001; Burdick and White, 2007; Kawamura et al., 2009).

It is also possible that occurrences of species of *Ictiobus* in the Great Lakes, and perhaps also the Hudson Bay basins, are based on artificial introductions, mostly likely from stocks in the Mississippi River Basin. An early report of occurrence of *I. cyprinellus* in Lake Erie (ca. 1854) is now thought to be based on a record from the Green River, Ohio (Bailey et al., 2004). The species was successfully introduced into Lake Erie in Ohio in 1920 and soon spread to neighboring states bordering the lake (Trautman, 1981). Specimens of *I. niger* reported in the southern part of Lake Michigan are thought to have entered via the Chicago River drainage canal, which is connected to the Illinois River (Mississippi River Basin, Bailey et al., 2004).

The invasion of the Gulf Coast likely was facilitated by sea level changes, which alternately connected (low sea stands) and isolated (high sea stands) coastal rivers, and by movements of buffalofishes through low-salinity, coastal estuaries. Buffalofishes are tolerant of low-salinities and are even known to spawn in brackish water (Perry, 1976). The *I. bubalus* specimen from the Río San Fernando in Mexico, for which the cytb gene was sequenced, was taken in water with a salinity of 0.4%. *Ictiobus bubalus* is frequently taken in brackish coastal lagoons in the US and Mexico (Miller et al., 2005; numerous museum records). The species may actively disperse through coastal waterways and low-salinity estuaries, especially at times when estuaries are freshened by high river flows.

The break between the distributions of *I. meridionalis* and its presumed closest relative, *I. bubalus*, occurs at an interesting and little studied ichthyogeographic barrier in the vicinity of Punta del Moro, Veracruz, Mexico, where the distributional limits of nine other putative fish sister species or subspecies occur (Obregon-Barboza et al., 1994; Contreras-Balderas et al., 1996; Bart et al., 2004; Hulsey et al., 2004). No large rivers occur in the region and lowland coastal habitat is greatly restricted by an area of steep topographic relief very near the coast. The nature of this distributional pattern, its relationship to the coast, and the close relatedness of the taxa involved, suggests that the vicariance resulted from recent sea level rise.

Another possible explanation that cannot be completely discounted for the extensive allele sharing in four of the species of *Ictiobus* is that it is the result of allele retention from a recent genetically polymorphic ancestor. The large body sizes and relatively long life spans of buffalofishes would tend to slow the rate of lineage sorting. Among the four species that share alleles, only *I. cyprinellus* is known from older fossils (early Pleistoncene). This species also exhibits the highest degree of lineage sorting. The much older fossil identified as *I.* cf. *bubalus* (Smith, 1962) could be a distinct ancestral species. The fact that it is difficult to fix the ages of *I. bubalus*, *I. meridionalis* and *I. niger* suggests that these species are very young and may be retaining alleles from a polymorphic ancestor.

We suggest that all species of *Ictiobus* should continue to be recognized taxonomically, despite the high levels of allele sharing among species, and the lack of phylogenetic resolution of four of the five species in gene trees. We base this conclusion on the fact that all *Ictiobus* species (except, perhaps, *I. niger*) form discreet, diagnosable units based on morphology, and our observation of significant genetic structure and genealogical sorting in at least one of the species. The morphological characteristics that distinguish *Ictiobus* species (head size, body depth and shape, mouth position, gill raker number) are likely are under strong selection pressure because of their importance to feeding ecology. Natural selection could be maintaining the distinctiveness of regions of the genome programming these traits, while other parts of the genome are being homogenized by gene flow, as has been postulated in a number of different insect species (Emelianov et al., 2004; Turner et al., 2005; Kronforst et al., 2006).

For most species of *Ictiobus* (excluding *I. labiosus*) morphological evidence is in conflict with levels of differentiation in genetic markers and hypotheses of relationships based on these markers. The pattern suggests either that genes are flowing among species and have been since shortly after the species were formed, or that the species are much younger than previously thought and have retained alleles from a polymorphic ancestor. We feel that evidence presented in this study more strongly supports the former explanation than the latter.

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