Aquat. Living Resour. 23, 325–333 (2010)

DOI: 10.1051/alr/2010029
www.alr-journal.org

Aquatic Living Resources

Mitochondrial DNA sequence revealed contrasting demographic history between the black bullhead (*Ameiurus melas*) and its cryptic lineage in North America

Abinash Padhi[a,b]

Department of Biological Science, University of Tulsa, 800 S. Tucker Drive, Oklahoma-74104, USA

Received 21 July 2010; Accepted 17 November 2010

Abstract – The black bullhead, *Ameiurus melas* of the family Ictaluridae, is a freshwater fish native to North America that was introduced throughout Europe in the late 1800s. Using mitochondrial DNA (mtDNA) as a genetic marker, the present study investigates the genetic structure and historical demography of *A. melas* in North America. MtDNA-based phylogenetic analyses revealed the existence of two distinct lineages (A and B) of *A. melas*. While lineage A clustered with the previously reported sequences of *A. melas*, lineage B emerged as a unique clade like other species of the genus *Ameiurus*. Individuals belonging to lineage B were mostly from the south central region of the United States, the region that never glaciated during the last Ice Age. Results of a fossil-based molecular clock analysis suggest that lineage A and the paraphyletic lineage B of *A. melas* diverged from their respective common ancestors approximately 3 (±0.2) and 15.9 (±1.3) million years ago. Lineage B could possibly be a hybrid species, possessing the mtDNA haplotype of its maternal parent, an *Ameiurus* species that has gone extinct. While lineage A showed evidence of population expansion, lineage B did not show any evidence of population expansion, but rather is comprised of geographically structured populations.

Key words: Siluriformes / *Ameiurus* / mtDNA / Phylogeny / Cryptic species / Molecular Clock / Population dynamics

1 Introduction

Black bullhead, *Ameiurus melas* of the family Ictaluridae, is native to North America and has a distribution ranging from the Great Lakes in southern Canada to northern Mexico (Page and Burr 1991). Since its first introduction in France in 1871, this species has spread widely all over Europe (Wheeler 1978; Novomeska and Kovac 2009). This omnivorous fish inhabits low-gradient streams with turbid waters and silt bottoms and can survive in heavily polluted water with high temperatures (Simon and Wallus 2004).

Based on the fossil evidence (Lundberg 1975), morphological characters (Lundberg 1992), and molecular phylogenetic analyses (Hardman and Page 2003) *A. melas* was previously reported to be more closely related to the brown bullhead, *A. nebulosus* than any other species of the genus *Ameiurus.* Both species have overlapping distributions across their distributional ranges (Page and Burr 1991), and therefore it may not be unusual to find hybrid populations of *A. nebulosus*-*A. melas* (Hunnicutt et al. 2005). Fossil evidence of *A. melas* from Kansas (Smith 1962), Texas (Swift 1968) and Oklahoma (Smith 1954) and *A. nebulosus* from Florida (Weigel 1963), Kansas (Smith 1962), Pennsylvania (Leidy 1889) and Indiana (Teller and Bardack 1975) suggest that while *A. nebulosus* had a dynamic distribution across the northern and southern range, *A. melas* was restricted to the southern range during the Pleistocene epoch. The recovery of distinct matrilineal lineages of *A. nebulosus* in the Great Lakes region corroborated the fossil evidence of its dynamic distribution during the last Ice Age and further indicates that this species might have emerged from multiple glacial refugia (Murdoch and Hebert 1997). However, the origin and divergence of *A. melas*, more specifically the importance of Pleistocene glacial cycles on the distribution of genetic variation across its native range, is unclear. Like many other North American freshwater fishes (e.g., Avise 2000), Pleistocene glacial cycles might have had profound influences on the diversity and distribution of *A. melas*. These Pleistocene glacial cycles played a dominant role in intraspecific divergence of many freshwater fishes of temperate zones (Bernatchez and Wilson 1998; Avise 2000; Culling et al. 2006). During glacial periods, the majority of the species in temperate zones were believed to have survived in southern refugia and expanded towards the north after the glacial retreat (Hewitt 1999; Avise 2000). Although fossil records would

[a] Corresponding author: aup17@psu.edu
[b] *Present address:* Department of Biology, 208 Mueller Laboratory, The Pennsylvania State University, University Park, PA-16802, USA

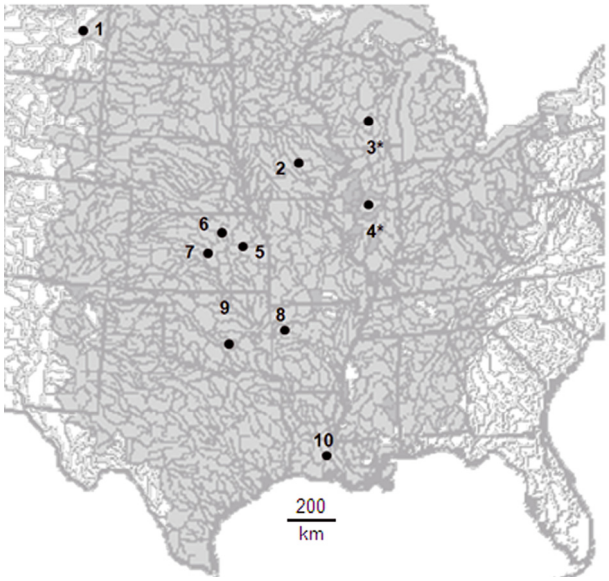

Fig. 1. Current distribution (grey area) range of *Ameiurus melas*. Approximate sampling locations of *A. melas* are shown. Distribution map is adapted from NatureServe, 2006. See Table 1 for drainage code. The drainage names for 3 and 4 are not known.

provide the minimum age and the distributional range of the species, it would be difficult to infer the postglacial dispersal of species. The maternally inherited, rapidly evolving and recombination free nature of mitochondrial DNA (mtDNA) makes it a suitable genetic marker to track the past population dynamics of vertebrates and invertebrates (Avise 2000). Utilizing mtDNA sequence data, the present study used both population genetic and phylogenetic methods to explore the genetic structure and historical demography of this extreme environment-tolerant species *A. melas* with the aim of investigating its origin and divergence, and its connectivity to different geographically separated populations in North America.

2 Materials and methods

2.1 Sample collection, DNA extraction, PCR, and Sequencing

A total of 95 *A. melas* representing its distributional range (Fig. 1) were collected by the fisheries biologists of the respective states. Ten mitochondrial control region (CR) sequences representing drainages of Illinois and Wisconsin were included from Hunnicutt et al. (2005). Fin clip samples were preserved in 75% ethanol and shipped to the University of Tulsa for genetic analyses. Total DNA was extracted from preserved tissue and a portion of the CR gene was amplified using the previously described method in Murphy and Collier (1996). The primers CR-F (5′-AACTCTCACCCCTAGCTCCCAAAG-3′) and CR-R (5′-CCTGAAGTAGGAACCAGATG-3′) (Kocher et al. 1989; Meyer et al. 1990) were used for amplification and sequencing of 402-base pairs (bp) of the mtCR gene. For phylogenetic analyses, representatives of each *Ameiurus* species from different drainages were sequenced for the 12S, 16S, and cytochrome b (cyt b) region using previously designed universal primers (Kocher et al. 1989; Meyer et al. 1990; Palumbi et al. 1991). A 436 bp sequence of 12S, 505 bp of 16S and a 422 bp of cyt b, respectively, were used in phylogenetic analyses. Following the amplification, excess dNTPs and primers were separated from PCR products using 10^6 MW low-binding centrifugal devices (Millipore, Inc) and washed. Approximately 50–100 ng of purified DNA was cycle-sequenced using Ampli-Taq FS DNA polymerase and flourescent-labeled dNTPs in a Perkin-Elmer9600 Thermal Cycler and the fragments subsequently separated using an ABI 373-Stretch automated sequencing apparatus. Additional cyt b sequences that were previously reported by Hardman and Page (2003) were retrieved from GenBank. The numbers of individuals sequenced in the present study are listed in Table 1. Sequences generated in the present study are deposited in Genbank (12S: DQ421854 – DQ42187; 16S: DQ421872 – DQ421891; cyt b: DQ421892 – DQ421899, DQ275631 – DQ275635; CR: HQ596530 – HQ596550).

2.2 Sequence alignment, parsimony network, and sequence divergence

Sequences were aligned using MacClade vers. 4.03 (Maddison and Maddison 2001). A parsimony network based on CR data was constructed with a 95% connection limit using TCS 1.18 (Clement et al. 2000). Haplotypes separated from each other by more than 9 mutational steps could not be connected at 95% confidence. The corrected genetic distances between lineages and between species were estimated using MEGA vers. 4 (Tamura et al. 2007), and using the same program standard errors were estimated with 1000 bootstrap replicates. The nucleotide and haplotype diversities were estimated using Arlequin vers. 3.1 (Schneider et al. 2000).

2.3 Phylogenetic analyses and molecular clock

An appropriate nucleotide substitution model of sequence evolution for each data set was selected by hierarchical likelihood ratio tests (hLRTs) implemented in Modeltest vers. 3.7 (Posada and Crandall 1998). Using the cyt b sequence data, phylogenetic analyses were carried out with maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI) algorithms. As in a previously reported study, *Cranoglanis bouderius* was chosen as the outgroup (Hardman 2005). MP and ML trees were constructed using different tree searching criteria implemented in PAUP*, vers. 4.0b10 (Swofford 2002). Both ML and MP analyses were conducted using the heuristic search option, implementing stepwise addition with 100 random addition replicates and TBR branch swapping. Clade support for the MP and ML analyses were estimated using 1000 and 100 non-parametric bootstrap replicates, respectively. MrBayes, vers. 3.04 (Huelsenbeck and Ronquist 2001) was used to conduct a Bayesian approach to phylogenetic inference by running 10 million generations (10 000 burn-in) with four Metropolis coupled Markov Chain Monte Carlos (MCMC) to optimize efforts to find peaks in tree-space. Parameters were set to nst = 6 and rates = invgamma and one tree was sampled in every 100.

Table 1. Number of individuals from different drainages sequenced for the mitochondrial control region (mtCR). Number of individuals are in parentheses.

Lineage	Drainage code	Drainage	mt CR haplotypes	Reference
A	1	Battle creek, Missouri River, Montana (n = 14)	H1(3), H3(10), H4(1)	This study
	2	Black Hawk Lake, Sac County, Iowa (n = 9)	H1(7), H2(2)	This study
	3	*Wisconsin (n = 7)	H1(5), H6(2)	Hunnicutt et al. 2005
	4	*Illinois (n = 3)	H1(2), H5(1)	Hunnicutt et al. 2005
	6	Republican River Kansas (n = 2)	H1(1), H8(1)	This study
	10	*Baton Rouge, Louisiana (n = 2)	H7(2)	This study
B				
	5	Neosho River, Emporia, Arkansas (n = 20)	H15(18), H16(1), H17(1)	This study
	6	Republican River, Kansas (n = 10)	H15(10)	This study
	7	Smokey Hill River, Kansas (n = 11)	H15(4), H18(7)	This study
	8	Arkansas River, Dardanelle Arkansas (n = 18)	H9(1), H10(11), H11(6)	This study
	9	WesWatkino Lake, North Canadian River, Oklahoma (n = 9)	H10(3), H12(4), H13(1), H14(1)	This study

(*) Drainage name is not known.

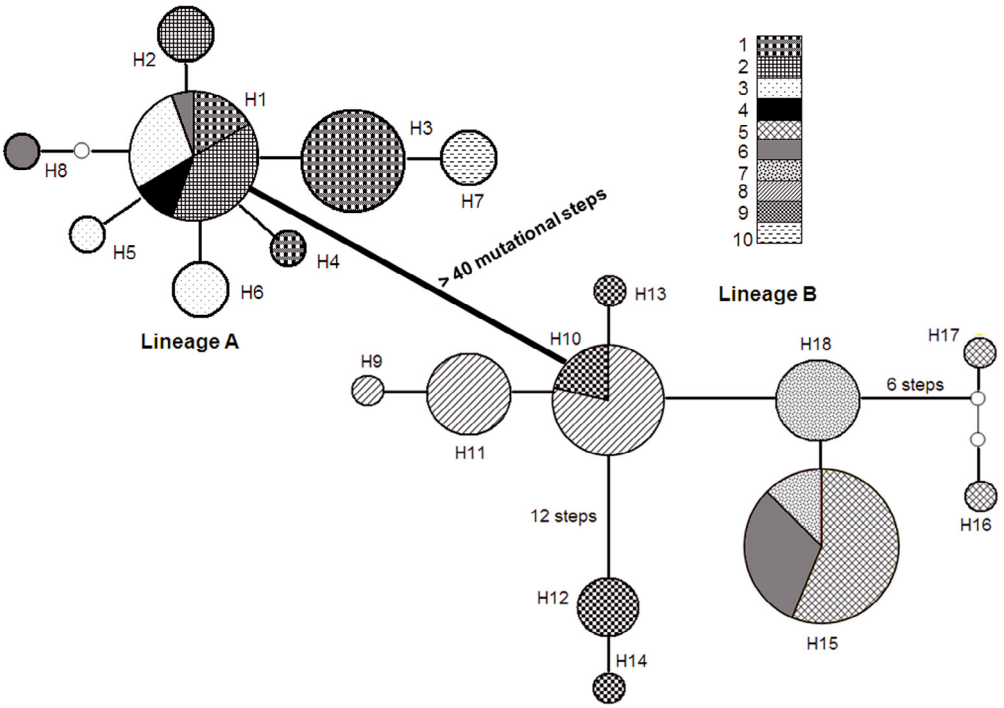

Fig. 2. Parsimony haplotype networks for lineage A and B of *A. melas*. The size of circle is proportional to the number of individuals. White circles indicate the hypothetical mutational steps that are not observed in the samples. Proportion of individuals from different regions is color coded. Lineage A and B could not be connected at 95% confidence limits. See Table 1 for drainage code.

The convergence was checked using Tracer vers. 1.4 (Rambout and Drummond 2006) and a 50% majority rule consensus tree was constructed in PAUP. The resulting trees were used to generate a majority consensus tree with posterior probability values in the BI.

Phylogenetic relationships among the bullhead catfishes were also inferred from the combined data of 12S, 16S and cyt b fragments that comprised 1350 bp using different tree building methods as described above. A molecular-clock likelihood ratio test was carried out for the combined data (Huelsenbeck and Rannala 1997). Likelihood scores of trees were generated with and without enforcing the molecular clock using PAUP. The best-fit substitution model for each data set was selected by performing hLRTs using Modeltest. A likelihood ratio test between clock enforced (L_2) and clock not enforced (L_1) scores was conducted by comparing twice the difference in log likelihood values ($2 \log \Lambda = 2 [(\log L_1 - \log L_2)]$) with degree of freedom n (number of taxa)-2 (Huelsenbeck and Rannala 1997). ML (unconstrained and constrained) was performed using the heuristic search option,

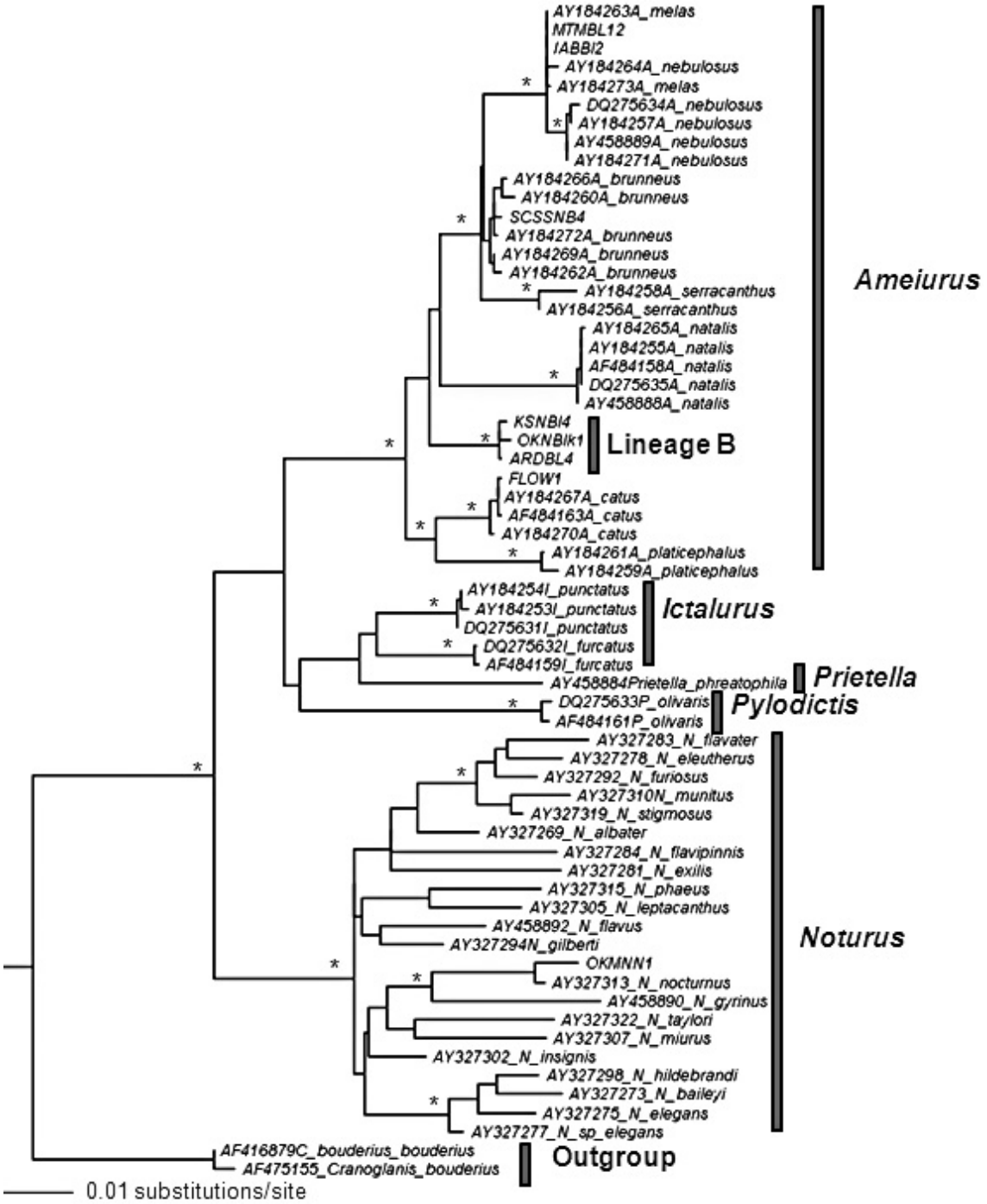

Fig. 3. Maximum likelihood tree with appropriate nucleotide substitution model inferred from partial cytochrome b data. Nodal supports with bootstrap (ML, MP) $\geqslant$ 70 and posterior probability $\geqslant$ 0.90 are indicated by asterisk. Accession numbers of sequences are mentioned. Accession number starting with "AY" and "AF" were retrieved from GenBank.

implementing stepwise addition with 100 random replicates and TBR branch swapping using PAUP.

Available fossils suggest a minimum age for the divergence of *A. natalis* and *A.catus* as 20 million years and the minimum time for the divergence between *A. nebulosus* and *A. melas* as approximately 3 million years ago (Lundberg 1975, 1992). Fossils assignable to the extant genus *Ameiurus* extend from the Miocene to late Pleistocene (Lundberg 1975, 1992). Minimal ages using the fossil record were assigned to these two nodes on the *Ameiurus* phylogeny. Since the combined dataset (12S+16S+ cyt b) revealed rate consistency ($p > 0.05$), ages of the lineages were estimated using the Langley-Fitch (LF) method (Langley and Fitch 1974) implemented in r8s vers. 1.7 (Sanderson 2003). The tree used for these analyses was a ML inferred topology using the appropriate nucleotide substitution model, with branch lengths estimated

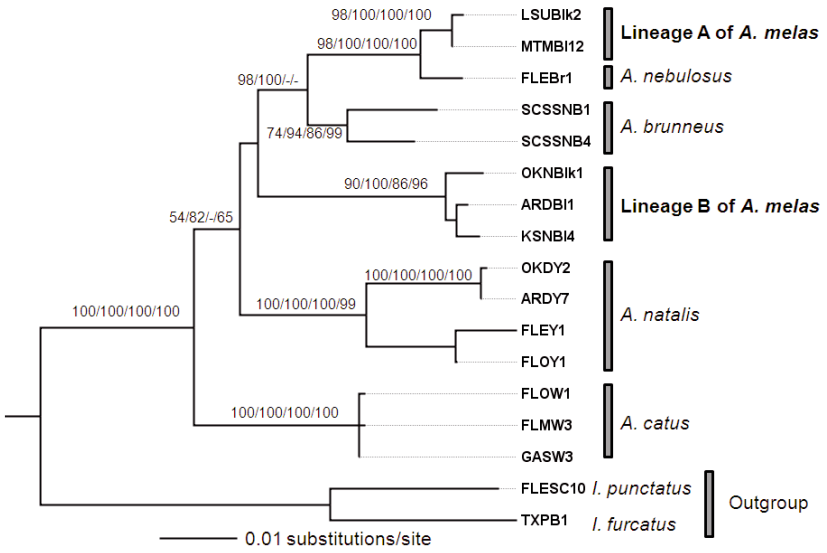

Fig. 4. ML tree of bullheads inferred from 12S, 16S and cytochrome b combined data.

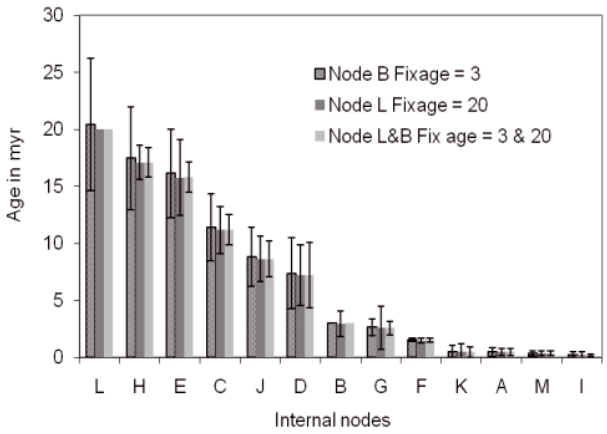

Fig. 5. Age of internal nodes estimated from the combined data ML tree using LF method implemented in r8s. The analyses differ in using one of either calibration points at 3 and 20 million years ago (mya), and estimates using both calibration points.

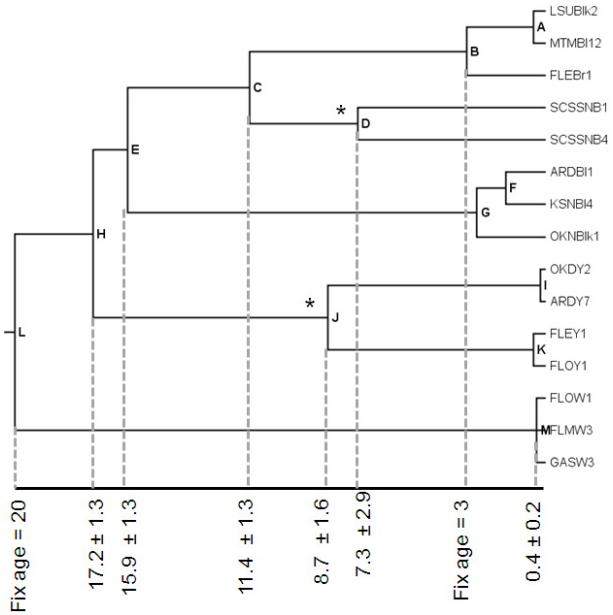

Fig. 6. ML-chronogram inferred from the combined data (12S + 16S + Cyt b) showing the approximate speciation/divergence time of each internal and external node. Asterisk indicates point at which timing of diversification is greater than the speciation event.

without enforcing a molecular clock. The sensitivity of divergence times, estimated from mtDNA sequence data, to particular fossil minimal age estimate calibration points was investigated by comparing age estimates calculated using each of the two fossil calibration points independently. Divergence time estimates were compared to determine whether the two different calibration points converged upon similar age estimates.

2.4 Historical demography

The distance-based mismatch distribution (Rogers and Harpending 1992; Rogers 1995; Schneider and Excoffier 1999) implemented in Arlequin ver 3.1 (Schneider et al. 2000) and the ML-based coalescent approach (Kuhner et al. 1998) implemented in Fluctuate vers. 1.4 (Kuhner et al. 1998) were performed to infer the population dynamics of each genetic lineage. Fu's F_S statistic (Fu 1997), which is often used as an indicator of demographic expansion and selective neutrality, was estimated using Arlequin. The ML estimates of the growth parameter (g) were estimated jointly using a MCMC implemented in Fluctuate vers. 1.4 (Kuhner et al. 1998). Transition/transversion (Ti/Tv) ratios were estimated by likelihood analyses, the Watterson estimate of θ_W was set as the starting parameter, and a random starting tree was chosen as a starting genealogy. Populations were allowed to change in size and an initial growth parameter was set to 0.01. Ten short MCMC of 200 generations each, and two long chain MCMC of 20 000 generations, were set for each run. The program was run ten times with different seeds for each lineage, and the

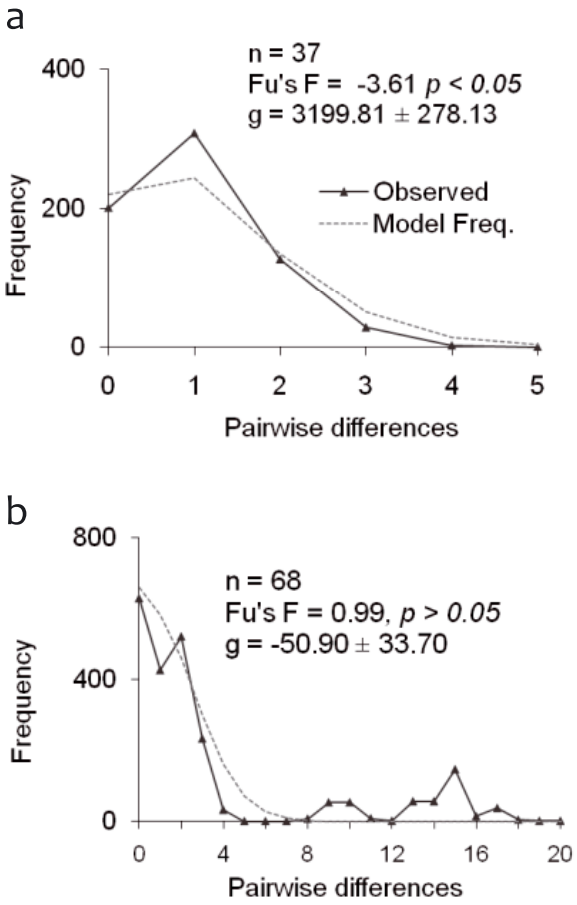

Fig. 7. Results of the pairwise mismatch distribution analyses**.** Results are shown using all mtDNA CR haplotypes from each lineage in pooled samples. Observed pairwise differences are shown by the solid line, the distribution under a model of sudden population expansion is shown as a dashed line. Fus' F and ML-based growth parameters for each group are also mentioned. Significant negative Fu's F and significant positive g values indicate population expansion. (**a**) Lineage A is distinctly unimodal and cannot be distinguished from the distribution under a model of sudden population expansion. Fu's F and g are also significant for lineage A indicating population growth. (**b**) Lineage B is apparently unimodal, though the peak is skewed to a higher pairwise difference than expected under a model of sudden population expansion, from which it cannot be distinguished. Fu's F and g are not significant for lineage B.

mean and standard deviations were estimated from these results of separate runs. Since the Fluctuate program may have been biased upwards in estimating "*g*" (Kuhner et al. 1998), a more conservative approach in testing for significance was used ($g > 3$ g[SD]).

3 Results

3.1 Parsimony haplotype network

A total of 18 CR haplotypes were recovered from 105 sequences. Parsimony network analyses revealed two distinct lineages of *A. melas* separated by more than 40 mutational steps (Fig. 2). In lineage A, there were 8 CR haplotypes defined by 8 variable sites among the 37 individuals, whereas in lineage B, 10 CR haplotypes were derived from 68 individuals with 20 variable sites. While 68 individuals of lineage B were from drainages of Oklahoma, Arkansas and Kansas, 35 individuals of lineage A were from Montana, Wisconsin, Illinois, and Louisiana (Table 1). Of the 12 total individuals sampled from the Republican river of Kansas, while ten individuals belong to lineage B, the remaining two individuals were affiliated with lineage A (Table 1; Fig. 2). While all the haplotypes in lineage A differed from each other by one or two mutational steps and connected at 95% confidence, not all the haplotypes in lineage B could be connected at the 95% limit. In addition, unlike lineage A, lineage B showed evidence of population structure. Despite the greater number of mutational steps among the Oklahoma haplotypes, all the haplotypes from Oklahoma and Arkansas (H9-H14) drainages are connected to each other, whereas all the haplotypes (H15-H18) from Kansas drainages are connected to each other (Fig. 2). The haplotype diversities (A: 0.70 ± 0.06, B: 0.72 ± 0.04) for both lineages are within the same range, whereas the nucleotide diversity for lineage A (0.0023 ± 0.0018) is at least three times lower than that observed for lineage B (0.0085 ± 0.0048). The percentage genetic distance between lineage A and B was 9.12 ± 0.23%, which exceeds the overall genetic distance between *A. melas* and *A. nebulosus* (1.62 ± 0.3%). Given such huge differences in genetic distance between lineage A and lineage B, we were curious to see the phylogenetic placement of these two lineages of *A. melas* in an ictalurid phylogeny.

3.2 Phylogenetic analyses

The TVM (Transversional Model) + I (= 1.5872) + G (= 0.5290) and TrN (Tamura-Nei) + I (= 0.6165) + G (= 0.5075) were the appropriate nucleotide substitution models for the cyt b and for the combined data, respectively. Based on the cyt b phylogeny, while representatives of lineage A were clustered with the Genbank reference sequences of *A. melas*, lineage B was placed as a separate group in the bullhead phylogeny, but not within the terminal of the *A. nebulosus-A.melas* cluster of sequences (Fig. 3). Within the branch of *A. nebulosus-A. melas* (Fig. 3), *A. nebulosus* is appeared to be paraphyletic. The combined data phylogeny is also consistent with the fact that lineage B is not within the *A. nebulosus-A. melas* group, rather it emerged as a separate group (Fig. 4). The placement of lineage B suggests a relatively long period of divergence from the most recent common ancestor shared by the other members of the clade.

3.3 Speciation timing

The timing of speciation and diversification of six extant bullhead species was assessed using the combined sequence data on 12S, 16S, and cyt b mtDNA genes. Three sets of divergence times were estimated for all nodes in the combined data phylogeny (Fig. 5). The first two used either of the two fossil calibration points, and the third used both calibration points in combination to estimate the divergence times. All

analyses converged on very similar divergence time estimates (Fig. 5). The ML-chronogram inferred from the combined data is shown in Figure 6. The rate of nucleotide substitutions for the combined data was estimated as 0.00155 substitutions per site per million years per lineage, giving a pair wise rate of 0.311% per million years. The molecular time tree revealed that the divergence times between the respective lineages of *A. natalis* and *A. brunneus* are at least two times higher than the speciation timing between *A. melas* and *A. nebulosus* (Fig. 6).

3.4 Historical demography

Comparisons of mismatch analyses, ML-based estimates of g, and Fu's F estimate for lineage A with the corresponding estimates of lineage B indicate contrasting demographic histories (Fig. 7). Evidence of demographic changes can be inferred from the results of the pairwise mismatch analyses of mtDNA CR haplotypes (Fig. 7). None of the lineages could be statistically distinguished from the distribution expected under a model of sudden population expansion, indicating that the hypothesis that these populations all result from such an expansion could not be rejected (Rogers and Harpending 1992). Lineage A is distinctly unimodal, while lineage B has a pronounced bimodal mismatch distribution. This pattern in lineage B is consistent with recovery of distinct haplotypes from Oklahoma that could not be connected at 95% confidence limits. The unimodality observed in lineage A is indicative of close genetic relationships among the individuals. In addition, significantly negative Fu's F and significant growth rates of lineage A show the typical genetic signature of population growth or sudden population expansion.

4 Discussion

Using mtDNA as a genetic marker, the present study investigates the genetic structure and historical demography of a North American freshwater catfish, *A. melas*. The analyses revealed the existence of two distinct matrilineal lineages of *A. melas*. Based on the available data, lineage B is restricted to the south-central United States, a region that never glaciated during the Pleistocene epoch (Anderson and Borns 1994). While lineage A clustered with the previously reported *A. melas* mtDNA sequences (Hardman and Page 2003; Hunnnicut et al. 2005), lineage B emerged as a distinct lineage and falls within the genus *Ameiurus* in the ictalurid phylogeny. The observation of paraphyly of *A. nebulosus* within the branch of *A. nebulosus*-*A. melas* (Fig. 3), could possibly be due to the hybridization event between female *A. melas* and male *A. nebulosus*. The possibility of hybridization between these two species is further evidenced by previous study (Hunnicutt et al. 2005). Based on cyt b sequence data, the net percentage genetic distance between lineage A and B is 9.12 ± 0.23%, which is approximately 5 to 6 times higher than the observed genetic distance between *A. melas* and *A. nebulosus* (1.62 ± 0.3%). Lineage B and the snail bullhead catfish *A. brunneus* showed the lowest percentage genetic distance (5.8 ± 1.2%) of any other species pair within the genus *Ameiurus*. A fossil-based molecular clock analysis revealed that with the exception of the *A. melas* – *A. nebulosus* species pair, the remaining extant *Ameiurus* species were estimated to have originated during the Miocene. These estimates are comparable with the previously estimated divergence times reported for *Ameiurus* species (Hardman and Hardman 2008). Based on these estimates, lineage B diverged from its common ancestor approximately 16 million years ago (MYA), which is at least 5-6 times older than the estimated divergence time between lineage A of *A. melas* and *A. nebulosus*.

The phylogenetic analyses presented here thus recovered a distinct but non-monophyletic lineage of *A. melas* from the south-central region of the United States. Despite the inconsistency in the topological placements of the species within the genus (Hardman and Page 2003), *A. nebulosus* -*A. melas* and *A. platycephalus* -*A. catus* species pairs still appear to have a sister group relationship. This suggests that the new lineage did not alter the phylogenetic relationship among the sister clades that were previously reported (Hardman and Page 2003). Due to the taxonomic conflict between the morphological and molecular data (Lundberg 1975; Hardman and Page 2003; Hardman and Hardman 2008), it is difficult to explain the cause of the paraphyletic origin of the morphologically indistinguishable lineages of *A. melas*. Nevertheless, the morphologically indistinguishable lineage B of *A. melas* could be regarded as a cryptic species. Examples of such a high degree of genetic divergence unmarked by morphological diversification are not common, but also not unusual (Musyl and Keenan 1996; Gleeson et al. 1999; Colborn et al. 2001; Grant and Grant 2002; McDaniel and Shaw 2003; Kidd et al. 2006). A detailed investigation of the morphological and anatomical traits, as well as reproductive compatibility of this matrilineal distinct lineage, is required to validate the species status.

Natural hybridization and introgression are not uncommon in teleost fish (Witz 1999; Scribner et al. 2001; Bossu and Near 2008). Recently, several studies reported evidence of hybridization and introgression events within the family Ictaluridae (Welsh and Cincotta 2004; Bossu and Near 2008; Keck and Near 2009), and more importantly between *A. melas* and *A. nebulosus* (Hunnicutt et al. 2005). This may result in the origin of new hybrid species, with the hybrid possessing the mitochondrial haplotype of the maternal parent species (Witz 1999; Shearer et al. 2002; Bossu and Near 2008). Thus, the possible existence of a new lineage of *A. melas* in an unglaciated region of North America (Oklahoma, Arkansas, and Kansas) provides the basis of an introgression scenario between male *A. melas* (or its ancestral species) and a female *Ameiurus* species that has since gone extinct, but whose mtDNA lives on in the descendents of the hybrid that has since reverted to the *A. melas* phenotype. It is also possible that the unglaciated area in which lineage B is found may have experienced large shifts in community structure and abundance that might have promoted hybridization. However, a greater number of suitable, variable, independent nuclear markers are required to test this hypothesis. Nevertheless, mtDNA clearly indicates the evidence of a new cryptic lineage of *A. melas*. Detail investigation is, therefore, required to understand the biotic and abiotic factors that are responsible for the origin and evolution of lineage B.

The observation of contrasting demographic histories and divergence patterns between lineage A and B of *A. melas* indicates a different evolutionary history for these two lineages. While all the haplotypes within lineage A are estimated to have diverged during the late-Pleistocene to Holocene periods, divergence times of haplotypes belonging to lineage B predated the early Pliocene. Although the Pleistocene glacial epoch played a dominant role in intraspecific divergence of North American freshwater fishes (Avise 2000), intraspecific divergence in several freshwater fishes also predate the Pliocene (Near et al. 2003). The dramatic growth of *A. melas* in the northern refugial areas could be associated with the availability of newly favourable habitats after the glacial retreat; whereas, the negative growth in the unglaciated region indicates a geographic subdivision of species (Lessa et al. 2003). The significant negative value of Fu's F for *A. melas* further corroborates the evidence that *A. melas* had rapid demographic expansion. By contrast, lineage B did not show any signs of demographic expansion.

In summary, the present study revealed the existence of a distinct matrilineal lineage that does not form a monophyletic group within populations of *A. melas*, thus rendering this taxon paraphyletic. Based on the estimates of a fossil-based molecular clock, this lineage is estimated to have diverged from its common ancestor approximately 16 MYA, which is at least six times older than the divergence time between *A. melas* and *A. nebulosus*. While the haplotypes of lineage A differed from each other by one or two mutational steps indicating close genetic relationships, several haplotypes in lineage B differed from each other by more than six mutational steps. Similarly, the observation of significant negative Fu's F, significant positive growth rate, and unimodal mismatch analyses in lineage A suggest population expansion, whereas the observation of negative growth rate and positive Fu's F in the cryptic lineage indicate either the population is relatively stable over time or the population is comprised of more geographically structured sub-populations. A parsimony network, however, supports the existence of two geographically structured sub-populations.

Acknowledgements. I thank Dr. Glen Collier for providing laboratory facilities and Dr. Peggy Hill and several anonymous reviewers for thoughtful comments towards improving this manuscript. I am thankful to the fisheries biologists of the respective state departments of natural resources for providing samples: Rich Caleteux (Florida), Don Harrison (Georgia), Danny Bowen (Oklahoma), Bob Limbird (Arkansas), Ron Marteney (Kansas), Mike Ruggles, Ben Davis, Pat Golden, Mark Gillum (Montana), Lannie Miller (Iowa) and David Allen (South Carolina). I thank Karen Winans for the technical assistance in DNA sequencing. The University of Tulsa's Office of Research and Sponsored Programs provided financial support for this study.

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