Post-glacial expansion into the Paleozoic Plateau: evidence of an Ozarkian refugium for the Ozark minnow *Notropis nubilus* (Teleostei: Cypriniformes)

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Genetic variation was examined within the Ozark minnow *Notropis nubilus* using complete mtDNA cytochrome *b* gene sequences from 160 individuals representing 30 localities to test hypotheses on the origin of the distribution. Phylogenetic analyses revealed three strongly supported clades of haplotypes consistent with geographic distributions: a clade from the Western Ozarks, a clade from the Southern Ozarks and a clade from the Northern Ozarks and upper Mississippi River basin. The estimated mean ages of these clades indicated that they diverged during pre-Illinoian glacial cycles extending from the late Pliocene into the early Pleistocene. Results of demographic analyses based on coalescent approaches supported the hypothesis that the Paleozoic Plateau was not a refugium for *N. nubilus* during periodic glacial advances. There is evidence of a genetic signature of northern expansion into the Paleozoic Plateau from a Southern Ozarkian refugium. Populations expanded out of drainages in the Northern Ozarks into the Paleozoic Plateau during the late Pleistocene. Subsequently, the two regions were isolated due to the recent extirpation of intervening populations caused by the loss of suitable habitat.
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INTRODUCTION

The Ozark Plateau, Ouachita Mountains and Appalachian Highlands together comprise the Central Highlands of eastern North America and contain one of the most diverse assemblages of freshwater fishes in the world (Mayden, 1988). Prior to the onset of oscillating climatic extremes, 2·6 million years before present (B.P.) (Lowe & Walker, 1997), the Central Highlands and the associated ichthyofauna were thought to be continuous (Thornbury, 1965; Wiley & Mayden, 1985; Mayden, 1988). The region was fragmented by advancing and retreating glacial fronts during the Pleistocene, which altered habitats and drainage patterns (Thornbury, 1965). Pleistocene climate change had a profound effect on Central Highland fish species, resulting in displacement or extirpation of local populations and changes in dispersion and

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dispersal abilities (Bernatchez & Wilson, 1998; Berendzen *et al*., 2008*a*). Pleistocene glacial cycles affected the entire Central Highland ichthyofauna resulting in replicate biogeographic patterns in co-distributed species (Mayden, 1985, 1988; Strange & Burr, 1997; Near *et al*., 2001; Ray *et al*., 2006; Berendzen *et al*., 2008*b*).

One biogeographic pattern that is repeatedly observed involves a discontinuous distribution of populations in the Ozark Plateau and the upper Mississippi River basin with no extant populations in the intervening habitat. This pattern is observed in multiple species of Central Highland fishes, *e.g.* large-scale stoneroller *Campostoma oligolepis* Hubbs & Greene, Ozark minnow *Notropis nubilus* (Forbes), carmine shiner *Notropis percobromus* (Cope), slender madtom *Noturus exilis* Nelson and gilt darter *Percina evides* (Jordan & Copeland) (Lee *et al*., 1980; Mayden, 1987). The northern portion of this disjunct distribution, the upper Mississippi River basin centred in south-eastern Minnesota, south-western Wisconsin, north-western Illinois and north-eastern Iowa, is unique as it was bypassed by multiple glacial advances (Fig. 1). In eastern North America, continental ice sheets advanced in stages and differed in the size and maximum extension of their southern boundaries (Burr & Page, 1986). The pre-Illinoian stage consisted of at least 10 glacial and interglacial cycles beginning 2·6 M$_{B.P.}$ in the late Pliocene and lasting until 340 000 B.P in the middle Pleistocene (Lowe & Walker, 1997; Mickelson & Colgan, 2004). West of the Mississippi River,
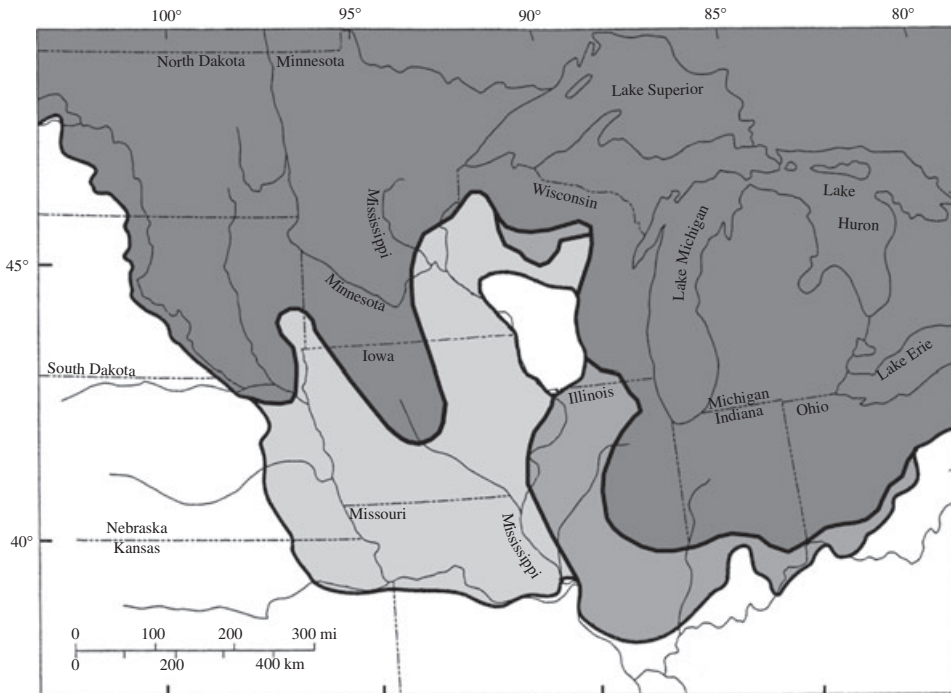

FIG. 1. Map of the upper Mississippi River basin the estimated pre-Illinoian (▢), the estimated Illinoian (▨) and the Wisconsinan maximum limits of glacial expansion (▨). The open area (▢) in south-eastern Wisconsin represents the true driftless area. [Figure modified from Hobbs (1999) with permission from the Geological Society of America.]

the pre-Illinoian glacial advances extended well into Northern Missouri terminating approximately along the modern Missouri River (Fig. 1). An area restricted to south-western Wisconsin and north-western Illinois, however, remained unglaciated during these repeated cycles (Burr & Page, 1986; Hobbs, 1999). The Illinoian, 300 000–130 000 B.P., and Wisconsinan, 80 000–10 000 B.P., glacial cycles (Lowe & Walker, 1997) had southern boundaries west of the Mississippi River that were much less extensive than pre-Illinoian cycles (Mickelson & Colgan, 2004).

Traditionally, the term driftless area has been used to describe the region of the upper Mississippi River basin centred in south-eastern Minnesota, south-western Wisconsin, north-western Illinois and north-eastern Iowa. This term, however, inaccurately suggests that the entire area was never glaciated. Although its existence is somewhat controversial (Thornbury, 1965), the true driftless area is confined to a small region of south-western Wisconsin and north-eastern Illinois, where glacial remnants have never been found (Hobbs, 1999). The region to the west of the true driftless area was not covered by the Illinoian and Wisconsinan ice sheets but contains pre-Illinoian glacial till. Together these regions are now commonly referred to as the Paleozoic Plateau, because the distinct topography of the area is underlain by Paleozoic sedimentary rock (Hobbs, 1999).

There are multiple hypotheses on why the Paleozoic Plateau was bypassed by glacial advances. One hypothesis is that the region acted like a giant sieve that removed water at the base of ice sheets, thus preventing ice advance across the plateau (Hobbs, 1999). Another is that the deep water of Lake Superior delayed ice advance contributing to the avoidance of this area (Culter *et al.*, 2001). Today, the Paleozoic Plateau contains a unique assemblage of organisms and is distinguished by steep banks and deeply dissected valleys, with clear, cool, high-gradient streams (Burr & Page, 1986).

The Paleozoic Plateau of the upper Mississippi River basin has been recognized as a refugium for a number of different organisms during glacial episodes, *e.g.* eastern chipmunk *Tamias striatus* (Rowe *et al.*, 2004), blue-spotted salamander *Ambystoma laterale* (Demastes *et al.*, 2007) and wood frog *Rana sylvatica* (Lee-Yaw *et al.*, 2008). Following glacial retreat, organisms expanded their ranges out of this refugium. The Paleozoic Plateau is also recognized as containing a unique diversity of organisms that are in need of protection. In 1989, the Driftless Area National Wildlife Refuge in north-eastern Iowa was established to help recover two federally listed species: the endangered Iowa Pleistocene snail *Discus macclintocki* and a threatened plant, northern monkshood *Aconitum noveboracense*.

It is thought that Central Highland fishes had a widespread distribution in a once continuous highland region prior to the onset of climatic oscillation (Mayden, 1988). There are two competing hypotheses for the origin of the disjunct distribution of fishes in the Paleozoic Plateau of the upper Mississippi River and Ozark Plateau. The first is that the unglaciated Paleozoic and Ozark Plateaus were both refugia for populations during glacial periods (Pflieger, 1971; Wiley & Mayden, 1985; Mayden, 1988). As ice sheets advanced southwards, a glacial vicariant event separated the ichthyofauna into the northern and southern populations. It has been suggested that aquatic organisms were able to survive in unglaciated northern pockets (Mayden, 1985, 1987). Following the retreat of the last glacial maximum, populations expanded out of these refugia into their current distribution. The disjunct populations, however, were unable to reconnect due to the alteration of the habitat between them in southern

Iowa and northern Missouri. The stream character in this region was converted to lowland habitat by the deposition of glacial loess and till (Pflieger, 1971; Mayden, 1985).

The second hypothesis is that as ice sheets advanced, populations were unable to survive in the unglaciated pockets in the north and survived only in suitable habitat in an Ozarkian refugium (Burr & Page, 1986). Following the retreat of the last glacial maximum, populations expanded out of the Ozark Plateau into the Paleozoic Plateau. Subsequently, populations in the intervening lowland areas of Iowa, Central Illinois and Northern Missouri were extirpated. While the exact cause of this extirpation is not known, the loss of suitable habitat could result from the indirect deposition of glacial loess, changing the character and water quality of streams, or from human-mediated modifications due to agriculture (Burr & Smith, 1976; Burr & Page, 1986).

These competing hypotheses result in different predictions for observed genetic variation within species exhibiting this pattern of disjunct distribution. A pattern resulting from the glacial vicariance of a once widespread distribution into isolated populations in the north and south is predicted to have monophyletic groups of haplotypes restricted to each geographical area separated by deep divergences. The demographic size of the divergent lineages should be relatively stable over time and both populations should exhibit similar levels of genetic diversity (Hewitt, 1996, 2000). A pattern resulting from recent expansion out of a southern refugium is predicted to have shared haplotypes across the disjunct geographic regions and exhibit recent expansion. Populations that expanded to the north should also exhibit lower genetic diversity than the refugial population in the south (Hewitt 1996, 2000).

Notropis nubilus is a member of the Central Highland ichthyofauna that has a disjunct distribution in the Ozark and Paleozoic Plateaus. In the Ozark Plateau of Missouri, Arkansas, Oklahoma and Kansas, there is a large, widespread population, and a much smaller disjunct population is found in the upper Mississippi River basin including Minnesota, Iowa, Illinois and Wisconsin (Fig. 2). *Notropis nubilus* is one of the most common minnows in the Ozark Plateau (Pflieger, 1975), whereas in the tributaries of the upper Mississippi River it is much less prevalent (Becker, 1983). This fish is found in streams of third order or less, with clear sandy and rocky bottoms in pools just below riffles or in protected backwaters (Lee *et al.*, 1980; Becker, 1983). They typically live in schools near the bottom (Pflieger, 1975) where there is a strong and permanent flow with little sediment disturbance (Becker, 1983). *Notropis nubilus* has declined in abundance and has been extirpated from many localities in the upper Mississippi River basin as a result of agricultural activities that increased the turbidity and siltation of the water (Becker, 1983).

The objective of this study was to examine the genetic variation across the range of *N. nubilus* to assess the roles of vicariance and population expansion in shaping the present diversity and distribution within the group. Phylogenetic and demographic patterns were inferred from a complete cytochrome *b* gene mitochondrial (mt)DNA data set. The hypothesis that demographic patterns are consistent with geological events was tested. In addition, competing hypotheses on the origin of the disjunct population of *N. nubilus* in the Paleozoic Plateau were tested. Finally, biogeographic patterns within *N. nubilus* were compared to hypotheses observed in other co-distributed taxa.
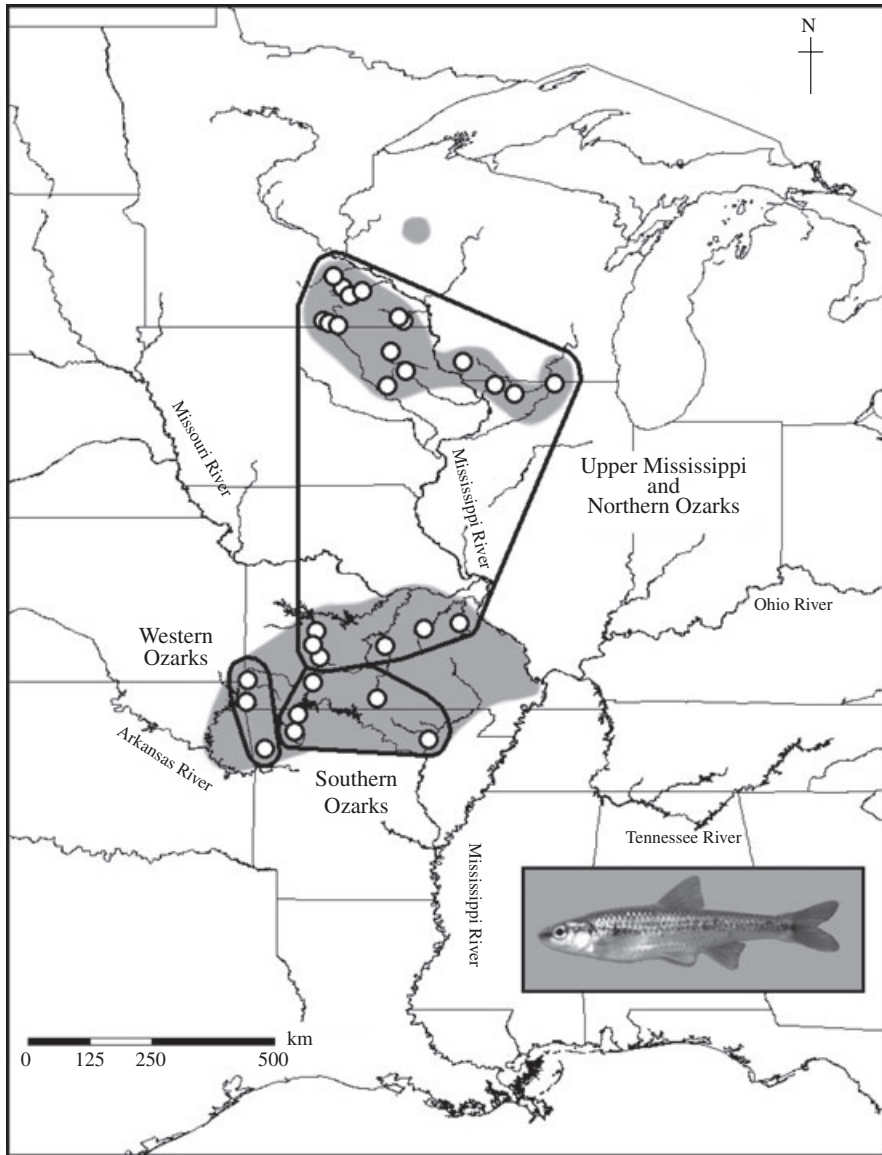
FIG. 2. Map of the central U.S.A. Grey area represents the estimated distribution of *Notropis nubilus*: sampling localities (○); ◖, major clades identified in this study. Photograph of *N. nubilus* breeding male collected from the Cedar River Drainage, Mower County, MN.

MATERIALS AND METHODS

MATERIAL EXAMINED

A total of 160 specimens of *N. nubilus* were collected from 30 localities from most of the major drainages across the range of the species (Fig. 2 and Table I). One additional *N. nubilus* sequence was downloaded from GenBank. All specimens were collected by seining with a

TABLE I. *Notropis nubilus* specimens used in this study listed by region, locality identification (ID), number of specimens from each locality (*n*), locality information, voucher number and GenBank number

Region	ID	*n*	Locality	County	State	Drainage	Latitude (N) Longitude (W)	Voucher number	GenBank number
Upper Mississippi River	CD1	4	Otter Creek	Mower	MN	Cedar River	43·506517 −92·916400	JFBM 45542	HM245443–46
	CD2	4	Lime Creek	Buchanan	IA	Cedar River	42·369800 −91·953900	JFBM 45552	HM245447–50
	CD3	6	Otter Creek	Mitchell	IA	Cedar River	43·487633 −92·942017	JFBM 45540	HM245451–56
	CD4	3	Otter Creek	Mower	MN	Cedar River	43·520672 −92·918973	JFBM 31417	HM245457–59
	ZB1	19	Salem Creek	Dodge	MN	Zumbro River	43·965633 −92·738467	JFBM 45549	HM245460–78
	ZB2	2	South Fork Zumbro River	Olmsted	MN	Zumbro River	43·924983 −92·637900	JFBM 45544	HM245479–80
	ZB3	7	North Branch Middle Fork Zumbro River	Goodhue	MN	Zumbro River	44·197833 −92·812017	JFBM 45547	HM245481–87
	ZB4	5	South Fork Zumbro River	Olmsted	MN	Zumbro River	44·142798 −92·470551	JFBM 45564	HM245488–92
	RT1	3	Riceford Creek	Houston	MN	Root River	43·604333 −91·707333	JFBM 45554	HM245493–95
	RT2	1	Riceford Creek	Houston	MN	Root River	43·604417 −91·706517	JFBM 45561	HM245496
	TK	10	Volga River	Fayette	IA	Turkey River	42·861833 −91·762350	JFBM 45550	HM245497–506

TABLE I. Continued

Region	ID	n	Locality	County	State	Drainage	Latitude (N) Longitude (W)	Voucher number	GenBank number
	MQ	8	South Fork Maquoketa River	Buchanan	IA	Maquoketa River	42·620100 −91·667100	JFBM 45551	HM245507–14
	RK1	9	Yellow Creek	Stephenson	IL	Rock River	42·251233 −89·620667	JFBM 45555	HM245515–23
	RK2	9	North Kinnikinnick River	Winnebago	IL	Rock River	42·450283 −88·934383	JFBM 45557	HM245524–32
	PT	9	Platte River	Grant	WI	Platte River	42·860617 −90·585867	JFBM 45560	HM245533–41
	AP	6	South Fork Apple River	Jo Davies	IL	Apple River	42·422667 −90·018333	JFBM 45558	HM245542–47
Northern Ozarks	GS	10	Big Piney River	Texas	MO	Gasconade River	37·218500 −92·002833	JFBM 40926	HM245548–57
	OS1	3	Pomme de Terre River	Hickory	MO	Osage River	37·935833 −93·308000	JFBM 37850	HM245558–60
	OS2	1	Pomme de Terre River	Polk	MO	Osage River	37·441333 −93·242167	JFBM 39030	HM245561
	OS3	2	Pomme de Terre River	Polk	MO	Osage River	37·671667 −93·371167	JFBM 45566	HM245562–63
	MC1	5	Big River	Washington	MO	Meramec River	38·086405 −90·685015	JFBM 45573	HM245564–68

TABLE I. Continued

Region	ID	n	Locality	County	State	Drainage	Latitude (N) Longitude (W)	Voucher number	GenBank number
	MC2	1	Reis Biological Station	Crawford	MO	Meramec River	37·971140 −91·337703	JFBM 45572	HM245569
Southern Ozarks	WT1	8	War Eagle Creek	Madison	AR	White River	36·085662 −93·707886	JFBM 30535	HM245570–77
	WT2	4	North Fork White River	Ozark	MO	White River	36·750000 −92·154333	JFBM 39476	HM245578–81
	WT3	2	James River	Stone	MO	White River	36·926167 −93·386833	JFBM 40537	HM245582–83
	WT4	8	Strawberry River	Independence	AR	White River	36·093167 −91·573500	JFBM 45571	HM245584–91
	WT5	5	Kings River	Carroll	AR	White River	36·395793 −93·641410	JFBM 30450	HM245592–96
Western Ozarks	AR1	3	Elk River	McDonald	MO	Arkansas River	36·621000 −94·587500	JFBM 45574	HM245597–99
	AR2	2	Elk River	McDonald	MO	Arkansas River	36·620833 −94·587667	JFBM 37899	HM245600–01
	AR3	1	Shoal Creek	Newton	MO	Arkansas River	37·023167 −94·556333	JFBM 45567	HM245602
	AR4	1	Lee Creek	Crawford	AR	Arkansas River	—	—	AF352265

JFBM, Bell Museum of Natural History Fish Collection, University of Minnesota.

backpack electroshocker. Fishes were preserved in 95% ethanol or frozen in liquid nitrogen for subsequent analysis in the laboratory. Other members of the subgenus *Notropis* (Bielawski & Gold, 2001) were included as out-group taxa for phylogenetic analysis using existing sequences from GenBank. Out-group taxa included: *Notropis amabilis* (Girard) (AF352269), *Notropis amoenus* (Abbott) (AF352270), *Notropis ariommus* (Cope) (AF352271), *Notropis atherinoides* Rafinesque (AF352273), *Notropis boops* Gilbert (AF352261), *Notropis candidus* Suttkus (AF352275), *Notropis chrosomus* (Jordan) (AF352262), *Notropis edwardraneyi* Suttkus & Clemmer (AF352263), *Notropis girardi* Hubbs & Ortenburger (AF352276), *Notropis jemezanus* (Cope) (AF352277), *Notropis longirostris* (Hay) (AF352264), *Notropis oxyrhynchus* Hubbs & Bonham (AF352278), *Notropis perpallidus* Hubbs & Black (AF352279), *Notropis photogenis* (Cope) (AF352281), *Notropis potteri* Hubbs & Bonham (AF352266), *Notropis scepticus* (Jordan & Gilbert) (AF352283), *Notropis shumardi* (Girard) (AF352284), *Notropis stilbius* Jordan (AF352286), *Notropis telescopus* (Cope) (AF352290), *Notropis texanus* (Girard) (AF352267) and *Notropis volucellus* (Cope) (AF352268).

MOLECULAR TECHNIQUES

Genomic DNA was extracted from muscle tissue and fin clips using QIAamp tissue extraction kits (Qiagen Inc.; www.qiagen.com) following manufacturer's instructions. The complete mitochondrial cytochrome *b* (cyt*b*) gene was amplified using polymerase chain reaction (PCR). PCR reactions were performed in a total volume of 25 µl containing 5–10 ng DNA, 1·2 µM of the forward and reverse primer: HA(16249) and LA(15058) (Schmidt *et al*., 1998), ×1 *Taq* salts, 3·0 mM MgCl$_2$, 0·4 µM deoxynucleoside triphosphate (dNTP) and 1·25 U of *Taq* DNA polymerase (GoTaq Green Master Mix; Promega Corp.; www.promega.com). The following thermal profile was used: initial denaturation at 94° C (3 min); 35 cycles of 94° C (10 s), 50° C (20 s), 72° C (20 s) and a final extension at 72° C (10 min) before termination of the reaction at 4° C. Amplification products were purified using ExoSAP-IT (USB corp.; www.usbweb.com) following manufacturer's instructions. Amplification primers were also used for sequencing. Automated sequencing of both strands was performed using Big Dye (Perkin Elmer; www.perkinelmer.com) terminator cycle sequencing on an ABI 3730xl DNA analyser at Macrogen, Seoul, Korea (www.macrogen.com). Sequences were checked for accuracy of base determination and assembled using Sequencher 4.0 (Gene Codes Corp.; www.genecodes.com). Sequences were aligned by eye. All sequences were deposited in GenBank (Table I).

SEQUENCE VARIATION, PHYLOGENETIC AND DEMOGRAPHIC ANALYSES

The number of variable, potentially parsimony-informative sites and uncorrected per cent pair-wise distances (p) for the cyt*b* sequence data set were calculated using PAUP*4.0b10 (Swofford, 2001). The number of haplotypes, number of segregating sites, nucleotide diversity and average number of pair-wise differences were determined using DnaSP 5.10 (Rozas *et al*., 2003).

Phylogenetic analyses were performed to determine the relationships among haplotypes; the data set was pruned to contain only unique haplotypes. Parsimony analyses were performed using the heuristic search option, 100 random addition sequence replicates and tree-bisection-reconnection (TBR) algorithm in PAUP*. All bases were equally weighted and maximum number of trees saved was set to 10 000. Parsimony trees were evaluated using summary values reported by PAUP*. Support for nodes was evaluated using 100 bootstrap replicates with a full heuristic search, simple step-wise addition option and TBR.

MrModeltest 2.2 (Nylander, 2004) was used to determine the model of sequence evolution (Posada & Crandall, 2001) using the Akaike information criterion (AIC) (Posada & Buckley, 2004). Maximum likelihood analyses were performed using GARLI 0.951 (Zwickl, 2006) under default search settings with the model determined by AIC. Support was evaluated using 100 bootstrap replicates with each repetition terminated after 10 000 generations without topology improvement.

Bayesian analyses were carried out using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003) using the model determined by AIC, random starting tree, uniform interval priors except base composition, which assumed a Dirichlet prior. Markov chain Monte-Carlo (MCMC) was run with four chains for 2 000 000 generations. Trees were sampled every hundred generations and branch lengths of all sampled trees were saved. The first 10 000 samples were discarded as burn-in. The burn-in, the number of trees to be ignored prior to reaching stationarity, was determined by plotting the log-likelihood scores of sampled trees against generation time. Post burn-in trees were used to calculate model variables and the posterior probabilities (Huelsenbeck & Ronquist, 2001).

Net between-group mean distances (Nei & Li, 1979) and mean sequence divergence within each group were calculated using MEGA3 (Kumar *et al.*, 2004). Distances and s.e. were estimated using 500 bootstrap replicates and the model determined by AIC.

The age of extant *N. nubilus* lineages was estimated in a Bayesian coalescent frame-work using BEAST 1.5.3 (Drummond & Rambaut, 2007). A single out-group was used, *N. volucellus*; the sister taxon to *N. nubilus* (Bielawski & Gold, 2001). There are no reliable external fossil or biogeographical calibrations to estimate divergence dates within *Notropis*. Therefore, divergence times were estimated from substitution rate estimates under a molecular clock. A likelihood ratio test was used to test if the data fit a molecular clock (Huelsenbeck & Crandall, 1997). Likelihood scores were calculated with and without a molecular clock enforced using the model determined by AIC. Significance was assessed using the likelihood ratio test statistic $[-2\log_{10}\Lambda = -2(\log_{10}L_0 - \log_{10}L_1)]$ and a χ^2-distribution (d.f. = operational taxonomic unit -2). A hypothesis of a constant DNA substitution rate among lineages could not be rejected ($P > 0.05$). There are several estimates of cyt*b* substitution rates in fishes, unfortunately, lineage-specific rate heterogeneity makes it inappropriate to apply any one of these rates to data from another lineage (Britten, 1986; Yoder & Yang, 2000). This was addressed by using a uniform prior that encompassed a range of cyt*b* substitution rates in fishes, 0·76–2·2% per million years (Zardoya & Doadrio, 1999; Bowen *et al.*, 2001; Perdices & Doadrio, 2001; Near & Benard, 2004). MCMC chains were run for 10 000 000 generations with trees sampled every 1000 generations. Model variables consisted of general time reversible (GTR) + shape variable of gamma distribution (G) substitution model with a molecular clock enforced and a coalescent constant population size tree prior. An unweighted pair-group method of arithmetic averages (UPGMA) was used to construct the starting tree. A second analysis, run for 100 000 000 generations, was run to ensure convergence and post burn-in results of both runs were pooled to calculate topology and model variables. All other model variables used uniform priors.

Several demographic variables were estimated to test if population size had changed over time in each of the *N. nubilus* clades as well as the upper Mississippi River and Northern Ozark populations separately. F_s (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) statistics were calculated using 10 000 coalescent simulations with DnaSP 5.10. Both statistics test for population growth by checking if sequences depart from neutral expectations and are generally robust to small population sizes (Ramos-Onsins & Rozas, 2002). Exponential growth rate (g) was estimated in a Bayesian coalescent framework using BEAST 1.5.3. When $g > 0$, population size has been increasing; when $g = 0$, population size is stable and when $g < 0$, population size is decreasing. As a conservative estimate of g, if the 95% highest posterior density (HPD) intervals included zero it was concluded that population size was stable. Other model and MCMC variables were the same as the divergence time analyses.

Bayesian skyline plots (Drummond *et al.*, 2005) were used to estimate changes in effective population size (N_e) through time for each lineage using BEAST 1.5.3. Ten grouped coalescent intervals were used in each case except the Western Ozarks clade where, due to the small sample size, six intervals were used. All other MCMC and model variables were the same as above. Bayesian skyline plots were visualized in Tracer 1.5 (Rambaut & Drummond, 2007).

The timing of genetic divergence between the populations in the Northern Ozarks and the Upper Mississippi River was estimated using isolation with migration (IM; Hey & Nielsen, 2004). Multiple preliminary analyses were run with the priors set broadly to determine the input values for the final run. Convergence was assumed when effective sample sizes (ESS) were greater than 100 for each variable (Kuhner & Smith, 2007). The final IM analysis was run with five chains for 20 million generations and the first 50 000 generations designated as burn-in. The range of cyt*b* substitution rates outlined above was used with the

Hasegawa-Kishino-Yano (HKY) model of sequence evolution and uniform priors. Migration rates were assumed to be equal, and the generation time was set to 1 year.

RESULTS

The complete cyt*b* gene, a total of 1140 nucleotides, was compiled for 160 individuals of *N. nubilus*. The entire data set contained a total of 419 variable sites of which 380 were potentially parsimony informative and 38 were apomorphic. Uncorrected *p*-distances within *N. nubilus* ranged from 0·00 to 5·12%. The data set was pruned to include only 69 unique haplotypes for phylogenetic analyses.

The model GTR + I + G was identified as the best model based on AIC. The consensus parsimony trees [>10 000 equally parsimonious trees, total length (TL) = 1746, consistency index (CI) = 0·3402, rescaled consistency index (RI) = 0·6909, retention index (RC) = 0·2350], maximum likelihood tree ($-\ln L = 8710·2989$) and Bayesian trees (harmonic mean $-\ln L = 21510·92$) all recovered a monophyletic *N. nubilus* (Fig. 3). *Notropis nubilus* was sister to a clade containing *N. boops* and *N. volucellus*. Results of these analyses recovered the same topologies as Bielawski & Gold (2001) and are not shown. Within *N. nubilus* three strongly supported monophyletic groups, consistent with geographic distributions, were identified: a clade containing haplotypes from the Western Ozarks, a clade containing haplotypes from the Southern Ozarks and a clade containing haplotypes from the Upper Mississippi and the Northern Ozarks (Fig. 3). The topologies of the Bayesian, maximum likelihood and parsimony trees were very similar. Minor differences occurred within each of the major clades that exhibited very short branch lengths and little resolution among haplotypes. Monophyly of these groups was strongly supported, but there was little resolution within them; haplotypes were not restricted to single populations or drainages. The net between-group mean ± S.E. sequence divergences between the Upper Mississippi and Northern Ozarks *v*. Southern Ozarks are 0·034 ± 0·008 and Upper Mississippi plus Northern Ozarks and Southern Ozarks *v*. Western Ozarks are 0·032 ± 0·007.

The time-calibrated phylogeny is presented in Fig. 4. The large confidence intervals (95% highest posterior density, HPD) surrounding key nodes reflect the large, uniform prior on substitution rates. The mean age estimate for the split between haplotypes in the Western Ozarks and the other clades was during the late Pliocene, 2·13 MB.P. The mean age estimate of cladogenesis between the Southern Ozarks and the Northern Ozarks plus Upper Mississippi clades was during the early Pleistocene, 1·49 MB.P. Molecular diversity indices of the cyt*b* data set are listed in Table II. The Western Ozarks contained the greatest amount of nucleotide diversity and the Northern Ozarks and Upper Mississippi River contain the least. Estimates of population growth, Fu's F_s and R_2 statistics, are presented in Table II. These statistics, however, can be biased because they fail to account for phylogenetic structure (Felsenstein, 1992; Pybus *et al.*, 2000; Drummond *et al.*, 2005). Therefore, Bayesian estimates of exponential growth rate (g; Table II) and Bayesian skyline plots (Fig. 5) are preferred. Growth rates were observed as increasing in the Southern Ozarks, Northern Ozarks and Upper Mississippi (Table II). The greatest population size increase was observed in the Upper Mississippi. The Western Ozarks showed no evidence of recent population expansion. Bayesian skyline plots (Fig. 5) indicated that population sizes in the Southern and Northern Ozarks increased in size from 100 000 to 50 000 B.P.,
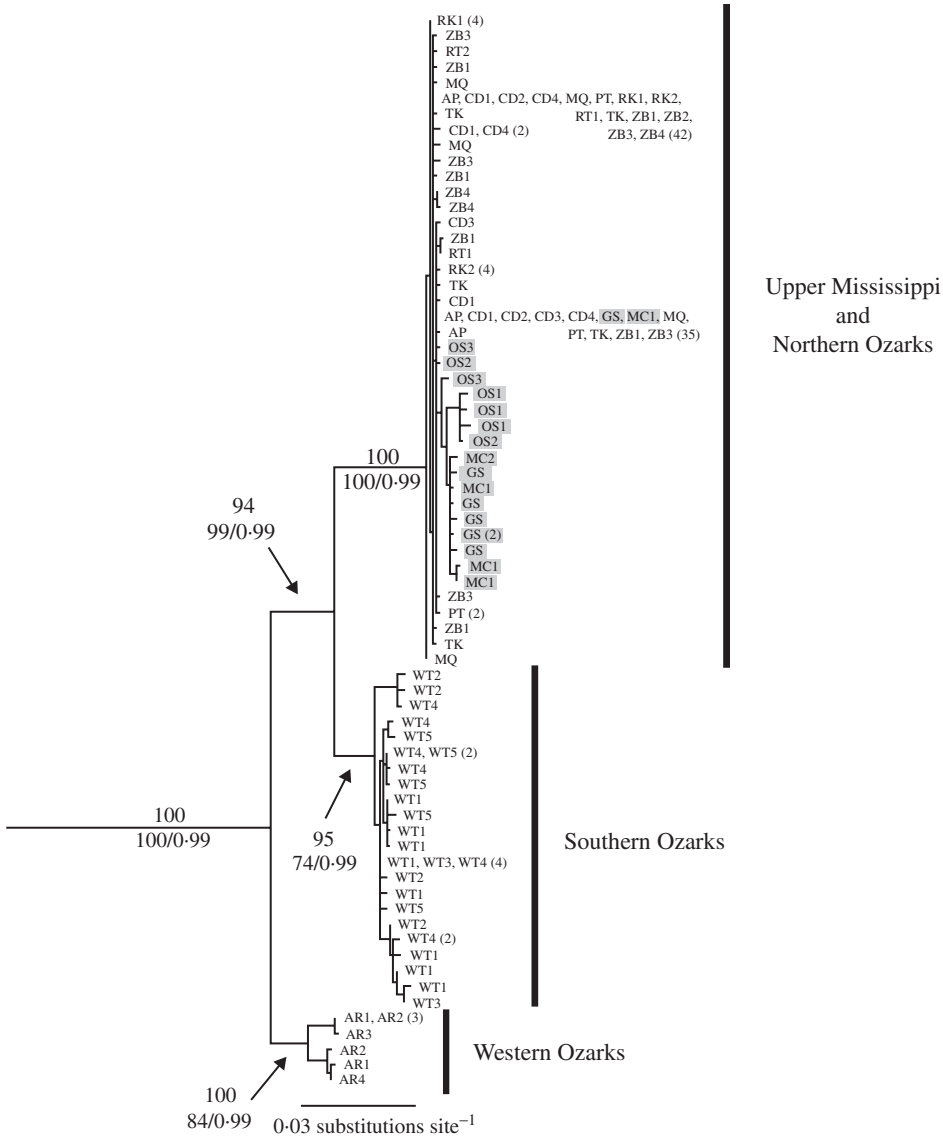
FIG. 3. Topology of *Notropis nubilus* haplotypes produced in the maximum likelihood analysis of the cytochrome *b* data set. Shaded haplotypes indicate localities in the Northern Ozarks. Numbers above nodes indicate parsimony bootstrap values. Numbers below nodes indicate maximum likelihood bootstrap values followed by Bayesian posterior probabilities. Out-groups are not shown. Locality identification is given in Table I.

which coincides with the end of the Illinoian glacial period to the beginning of the Wisconsinan glacial period. The population size was more stable up to the present. The Upper Mississippi population showed a dramatic increase in population size from 50 000 to 25 000 B.P. during the Wisconsinan glacial period, becoming more stable to the present. The population size of the Western Ozarks population remained relatively constant over time.
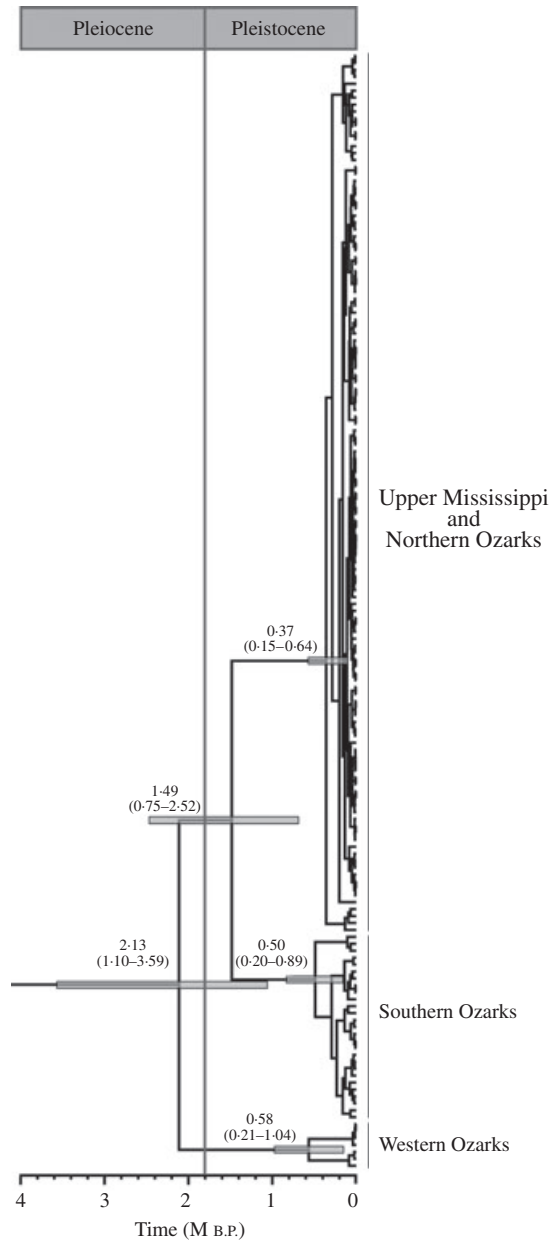
FIG. 4. Time-calibrated phylogeny showing mean divergence dates and 95% highest posterior density (HPD) intervals for major *Notropis nubilus* lineages. Time is shown in millions of years before present (MB.P.).

The estimated posterior probability distribution of the divergence time between populations in the Northern Ozarks and Upper Mississippi was clearly resolved with the posterior distribution having a single narrow peak. ESS values were >100, indicating that the posterior distribution was well sampled. The posterior distribution peaked at 74 342 YA (95% HPD 33 552–161 104 B.P.) dating to the late Pleistocene.

TABLE II. Number of individuals (*n*), number of haplotypes, number of segregating sites (S), nucleotide diversity (*π*), average number of pair-wise differences (*K*), Fu's F_s, R_2 test for detecting population growth and the exponential growth rate (*g*) with 95% highest posterior density (HPD) intervals for each *Notropis nubilus* lineage

| Lineage	*n*	Haplotypes	S	*π*	*K*	Fu's F_s	R_2	*g* (95% HPD)
Southern Ozarks	27	22	39	0·00628	6·006	−0·00755*	0·12177*	22·1 (5·1 to 43·1)
Western Ozarks	7	5	17	0·00849	8·762	0·44146*	0·18818*	−1·2 (−13·3 to 10·2)
Upper Mississippi and Northern Ozarks	126	42	47	0·00186	1·713	−0·14629	0·08900*	75·5 (20·5 to 142·3)
Upper Mississippi	105	26	28	0·00134	1·241	−0·08108*	0·09481*	218·5 (46·2 to 452·3)
Northern Ozarks	21	17	28	0·00449	4·648	0·01302	0·12959*	52·9 (8·4 to 113·3)

* $P < 0.05$.
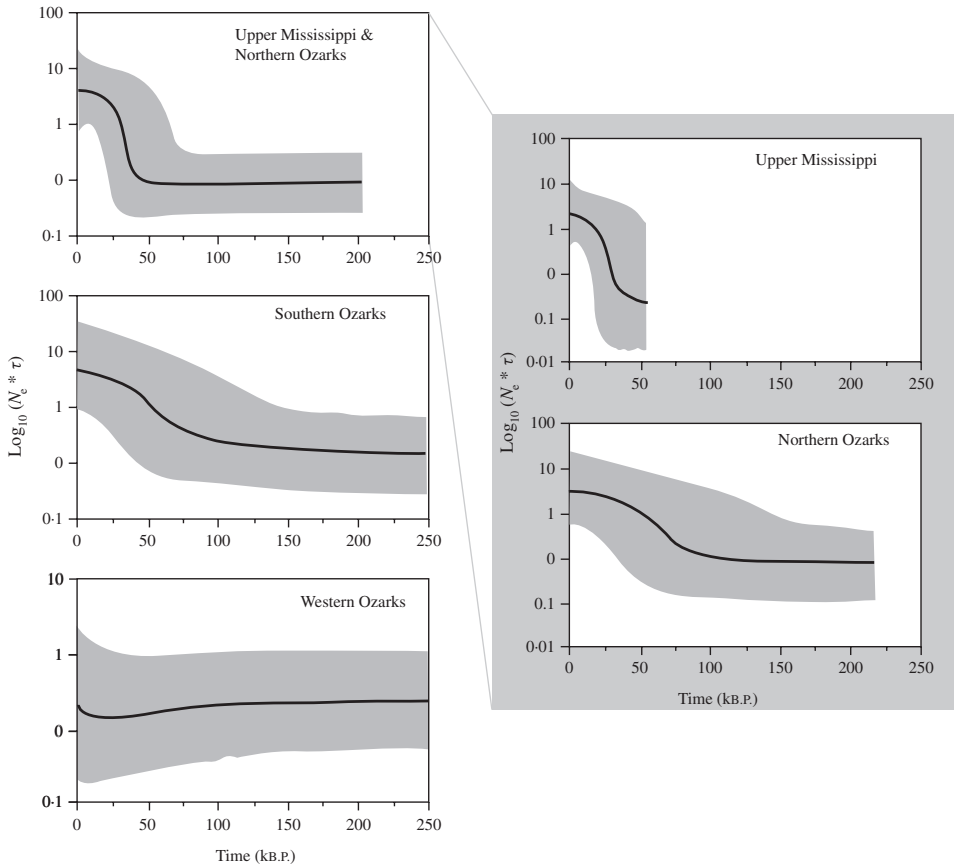
FIG. 5. Bayesian skyline plots [▬, the mean estimate of the population size ($N_e*\tau$) and the 95% highest posterior density (HPD) intervals (▨)] showing the demographic histories of major *Notropis nubilus* lineages through time (thousands of years before present, KB.P.). The Upper Mississippi and Northern Ozarks groups are also shown separately, separated by geography.

DISCUSSION

IDENTIFICATION OF INCREASED GENETIC DIVERSITY

The results of this study support the hypothesis that the current distribution of *N. nubilus* was shaped by responses to periodic glacial cycles beginning in the late Pliocene and extending through the Pleistocene. Within *N. nubilus*, three strongly supported clades exhibiting unique sets of haplotypes consistent with geographic distributions were recovered (Figs 2 and 3). Haplotypes from the Western Ozarks formed a monophyletic group that was sister to a clade containing haplotypes from the Southern Ozarks and a clade containing haplotypes from the Northern Ozarks plus Upper Mississippi River. There was deep divergence among the clades distributed in the Ozark Plateau.

The estimated ages indicated that the major clades within *N. nubilus* diverged during the pre-Illinoian glacial cycles extending from the late Pliocene to the early

Pleistocene (Fig. 4). Following the early glacial advances, the more recent Illinoian and Wisconsinan glacial cycles had less of an effect on cladogenesis of populations in the Ozark Plateau. During the Sangamon interglacial period, between the Illinoian and Wisconsinan periods, population sizes in the Southern and Northern Ozark lineages increased (Table II and Fig. 5).

The deep divergence and distinct phylogroups observed suggest that there may be cryptic species within *N. nubilus*. Additional data and analyses, however, are necessary to define and delimit this diversity. The patterns observed within *N. nubilus* are consistent with those observed in other clades of fishes and past geologic events (Near *et al.*, 2001; Hardy *et al.*, 2002; Ray *et al.*, 2006; Berendzen *et al.*, 2008*b*). Together these hypotheses can provide an understanding of the drainage history of the region (Berendzen *et al.*, 2008*a*).

WESTERN OZARK PLATEAU

Glacial advances did not directly affect the river drainages of the Ozark Plateau; however, they did affect the climate and habitat of the region. The maximum southern extent of all stages of glacial advances west of the Mississippi River terminated along the modern Missouri River on the northern edge of the Ozarks (Fig. 1). The drainage patterns observed today have maintained their basic configuration since the late Paleozoic era (Pflieger, 1971). During periods of glaciation the climate in this region was cooler and dryer, and it is probable that aquatic habitats were altered (Burr & Page, 1986). The Ozark Plateau is an uplift or dome that lies between the modern Missouri, Mississippi and Arkansas Rivers and is bounded on all sides by lowland areas (Thornbury, 1965). The drainages of the Ozark Plateau follow a discrete radial pattern (Fig. 2): drainages flow to the north into the Missouri River, to the east and south-east into the Mississippi River and to the south and south-west into the Arkansas River (Thornbury, 1965). The divergences of lineages observed within *N. nubilus* follow this radial pattern (Fig. 3).

The widespread distribution of *N. nubilus* in the Ozark Plateau is the result of both dispersal through the Mississippi and Arkansas Rivers during periods of favourable conditions, and historical connections between the various drainages of the plateau (Cross *et al.*, 1986). Today, the Western Ozarks is a distinct, isolated region of the Ozark Plateau that is drained by tributaries of the middle Arkansas River. It has been hypothesized, however, that these drainages once had a connection with rivers in the Northern and Southern Ozark Plateau, which may have facilitated dispersal among highland drainages.

The Neosho River is a tributary of the middle Arkansas River in eastern Kansas and north-eastern Oklahoma. The Spring and Elk Rivers drain the western Ozark Plateau and join the Neosho River before it enters the Arkansas River. The Neosho River is thought to have a historical connection with the Osage River in the Northern Ozarks. This connection is supported by faunal similarities, the close proximity of the headwaters of the two systems and geologic evidence (Cross *et al.*, 1986). Similarly, the Spring and Elk Rivers were thought to be connected at one time to the White River in the Southern Ozarks (Cross *et al.*, 1986). The formation of the modern Arkansas River is the result of the Old Arkansas River, a small river that cut through the western portion of the highland region, capturing rivers of the Plains Region, a preglacial drainage to the west of the Ozark Plateau that flowed southwards (Quinn,

1958; Mayden, 1985, 1988; Cross *et al*., 1986). This capture included drainages of the western Ozark Plateau.

Early climatic oscillations resulted in a vicariant event isolating haplotypes of *N. nubilus* in drainages of the Western Ozarks from the Southern and Northern Ozarks. The estimated age of this divergence is during the middle Pliocene to early Pleistocene 2·13 (1·10−3·59) MB.P. (Fig. 4). The formation of the modern Arkansas River probably resulted in isolation and divergence of populations of *N. nubilus* in the Western Ozarks from other drainages of the Ozark Plateau. This capture was thought to take place during the Illinoian glacial period (Robison, 1986), which is more recent than the estimate coalescent time of this gene within *N. nubilus*. The timing of the population split, however, may be consistent with this hypothesis. Today, the Arkansas River and its associated lowland area presumably form a barrier to gene flow maintaining isolation of populations in the Western Ozark Plateau (Mayden, 1985). This pattern was also revealed in phylogeographic studies of other clades of fishes; *e.g.* plains topminnow *Fundulus sciadicus* Cope (Li *et al*., 2009); bigeye chub *Hybopsis amblops* Rafinesque (Berendzen *et al*., 2008*b*) and *N. exilis* (Hardy *et al*., 2002).

There is evidence that *N. nubilus* was once more widespread west of the Ozark Plateau in Kansas and south in the Ouachita Mountains of Arkansas and Oklahoma. A fossil bone fragment of an *N. nubilus*, dated to the late Illinoian glacial stage, was identified from Meade County in south-western Kansas (Smith, 1963). This locality lies far west of the range of extant populations. Mayden (1985) predicted that *N. nubilus* once occurred in the Ouachita Highlands. Collections of *N. nubilus* from the Ouachita Highlands made in the 1920s were identified by Mayden (1988); however, no detailed information on these specimens was provided. Results of this study have revealed that, following the Illinoian glacial cycle, the lineage of *N. nubilus* in the Western Ozark Plateau remained relatively stable. There is no evidence of major fluctuation in effective population size (Table II; Fig. 5), and this clade contains the greatest amount of molecular diversity (Table II).

SOUTHERN AND NORTHERN OZARK PLATEAU

The second pattern of divergence in the Ozark Plateau is a vicariant event associated with the separation of haplotypes of *N. nubilus* in drainages of the Southern and Northern Ozarks. The estimated age of this divergence is during the early Pleistocene 1·49 (0·75−2·52) MB.P. (Fig. 4). The White River drainage of the Southern Ozark Plateau flows southwards, joining the lower portion of the Arkansas River just before it enters the Mississippi River in Southern Arkansas. This drainage has a shorter, more direct route to the Mississippi River and Gulf of Mexico than the drainages of the Northern Ozarks. In the Northern Ozarks, the Osage and Gasconade Rivers drain northwards into the Missouri River, which joins the Mississippi River north of St Louis, Missouri. The Meramec River drains the north-eastern portion of the Ozark Plateau and is a direct tributary of the Mississippi River, joining it just south of St Louis. There is physiographic evidence of a previous connection of the White River of the Southern Ozarks with the Gasconade River of the Northern Ozarks (Bretz, 1965). In addition, there is evidence of faunal exchanges between the headwaters of the Osage and Gasconade Rivers in the Northern Ozarks and the White River in the south (Cross *et al*., 1986). Subsequently, these drainages were isolated from one

another. Presumably the modern Arkansas and Mississippi Rivers form a barrier to gene flow in these fishes today.

The same pattern of a sister group relationship between the Northern and Southern Ozarks was observed in the phylogeographic studies of *Percina evides* (Jordan & Copeland), (Near *et al.*, 2001) and *N. exilis* (Hardy *et al.*, 2002), and in the sister species *Etheostoma tetrazonum* (Hubbs & Black) and *Etheostoma euzonum* (Hubbs & Black) (Switzer & Wood, 2002). In other groups of fishes, this pattern was common, except that haplotypes from other geographic regions were included in a Southern Ozarks clade that was sister to Northern Ozarks. Within rainbow darter *Etheostoma caeruleum* Storer haplotypes from the Meramec River were sister to a Southern Ozarks clade (Ray *et al.*, 2006). Within *N. percobromus* haplotypes from the western Ozarks were sister to the Southern Ozarks (Berendzen *et al.*, 2008*a*) and within *H. nigricans* haplotypes from the western Ozarks and Ouachita Highlands were sister to the Southern Ozarks (Berendzen *et al.*, 2003).

Beginning in the Sangamon interglacial period, populations began to increase in size in the Southern and Northern Ozark Plateau (Table II and Fig. 5). West of the Mississippi River the most recent Wisconsinan glacial advance did not extend as far south as the pre-Illinoian ice sheets (Fig. 1). Conditions during this time period presumably allowed for more favourable habitat in the Ozark Plateau, permitting populations of *N. nubilus* to expand.

PALEOZOIC PLATEAU

Results of this study revealed that the Paleozoic Plateau, including the true driftless area, did not act as a refugium for populations of *N. nubilus* during pre-Illinoian glacial advances. Haplotypes from the Paleozoic Plateau did not form a reciprocally monophyletic group with deep divergence from haplotypes in the Ozark Plateau. Deep divergences dating to this time period, however, were observed in the three lineages of the Ozark Plateau (Figs 2 and 3). Rather, haplotypes from the Paleozoic Plateau were included in a clade containing haplotypes from the Northern Ozark Plateau; haplotypes were also shared across the disjunct distribution (Fig. 3). This supports the hypothesis that as early pre-Illinoian ice sheets advanced, northern populations of *N. nubilus* were unable to survive in the unglaciated pockets and were pushed southwards into more suitable habitat in an Ozarkian refugium (Burr & Smith, 1976; Burr & Page, 1986).

Results of the IM analyses suggested that the populations in the Northern Ozark and Paleozoic Plateaus diverged *c*. 74 000 B.P. during the Sangamon interglacial period between the Illinoian and Wisconsinan glacial cycles (Lowe & Walker, 1997). There is a potential discrepancy in this dating, however, due to the influence of time dependency, the observation that rates of DNA change decline with increasing evolutionary time scales (Ho *et al.*, 2005; Burridge *et al.*, 2008). The use of interspecific substitution rates to date the divergence between the two populations is probably overestimated (Ho *et al.*, 2005). Therefore, the possibility that this expansion occurred following the retreat of the last glacial maximum 10 000 B.P. (Lowe & Walker, 1997) rather than during the Sangamon interglacial period cannot be ruled out. Regardless of the exact timing of this event, there is strong evidence of recent expansion, which is further supported by evidence of lower genetic diversity in the northern population (Hewitt, 1996) and a genetic signal of population growth

(Table II). This suggests that during the late Pleistocene, populations of *N. nubilus* expanded northwards out of the southern refugium in the Northern Ozark Plateau (Fig. 5).

Following expansion into the Paleozoic Plateau, populations of *N. nubilus* in Southern Iowa and Northern Missouri were presumably extirpated due to loss of suitable habitat resulting from the deposition of glacial loess and till (Mayden, 1985; Mason *et al.*, 1999). The streams in this region were converted from clear, cool, high-gradient streams with gravel, rock and bedrock bottoms to turbid, low-gradient streams with silt, sand and gravel bottoms (Pflieger, 1975). Interestingly, prior to 1908 a single individual of *N. nubilus* was collected near the Mississippi River at the border between Iowa, Missouri and Illinois between the Paleozoic and Ozark Plateaus (Lee *et al.*, 1980). This collection was regarded as a waif individual from the north, but unfortunately there is no additional information on this specimen. Given the near-complete absence of historical records of *N. nubilus* in this region over the last 100 years, it is unlikely that extirpation of intervening populations was entirely due to human influenced activities. The loss of habitat and extirpation probably resulted from glacial activity and led to the current distribution of *N. nubilus* in the disjunct Paleozoic and Ozark Plateaus.

The pattern of an Ozarkian refugium and post-glacial expansion into the upper Mississippi River has been repeatedly observed in phylogeographic studies of other clades of fishes; *e.g. C. oligolepis* (Blum *et al.*, 2008), *E. caeruleum* (Ray *et al.*, 2006), *H. nigricans* (Berendzen *et al.*, 2003), *N. percobromus* (Berendzen *et al.*, 2008*a*), *N. exilis* (Hardy *et al.*, 2002) and *P. evides* (Near *et al.*, 2001). This pattern is also observed in other organisms; *e.g.* ringneck snake *Diadophis punctatus* (Fontanella *et al.*, 2008) and boreal chorus frog *Pseudacris maculata* (Moriarty Lemmon *et al.*, 2007).

Previously unreported genetic diversity within *N. nubilus* that was shaped by periodic responses to climatic oscillation in the Pliocene and Pleistocene was uncovered. Prior to the onset of glaciation *N. nubilus* presumably had a widespread distribution throughout a once extensive highland region (Mayden, 1988). As ice advanced southwards during pre-Illinoian glacial cycles, populations of *N. nubilus* were pushed southwards into an Ozarkian refugium. During this time period, lineages within the Ozark Plateau were isolated by vicariant events associated with the drainage patterns within the plateau. During the Sangamon interglacial between the Illinoian and Wisconsinan glacial periods, populations in the Ozark Plateau increased in size.

The Paleozoic Plateau has been hypothesized to be a refugium for a number of terrestrial organisms. There is evidence that deciduous forests persisted in this region during the last glacial maximum (Jackson *et al.*, 2000) supporting populations of terrestrial, deciduous forest-dependent vertebrates (Rowe *et al.*, 2004). Although extant populations of freshwater fishes are distributed in the Paleozoic Plateau, this region did not act as a refugium for fishes during periodic glacial advances. Aquatic habitats in this region were not suitable for Central Highland fishes during cold climatic periods. Fish populations expanded out of drainages of the Northern Ozark Plateau into the Paleozoic Plateau at the end of the Pleistocene. Subsequently, populations between the Paleozoic and Ozark Plateaus were extirpated due to loss of suitable habitat. This study represents a phylogeographic analysis of a single mtDNA gene region; these hypotheses await further testing with additional nuclear markers.

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