Mitochondrial DNA Variation in Johnny Darters (Pisces: Percidae) from Eastern Kentucky Supports Stream Capture for the Origin of Upper Cumberland River Fishes

Rex Meade Strange

American Midland Naturalist, Vol. 140, No. 1. (Jul., 1998), pp. 96-102.

Stable URL:
http://links.jstor.org/sici?sici=0003-0031%28199807%29140%3A1%3C96%3AMDVIJD%3E2.0.CO%3B2-A

American Midland Naturalist is currently published by The University of Notre Dame.

Am. Midl. Nat. 140:96–102

Mitochondrial DNA Variation in Johnny Darters (Pisces: Percidae) from Eastern Kentucky Supports Stream Capture for the Origin of Upper Cumberland River Fishes

REX MEADE STRANGE[1]
Department of Zoology, Southern Illinois University, Carbondale 62901

ABSTRACT.—The Poor Fork of the Cumberland River has been interpreted as an area of secondary contact and hybridization between *Etheostoma nigrum* and the closely related *E. susanae*. I examined mitochondrial DNA (mtDNA) diversity among *E. nigrum* populations in the adjacent Kentucky River drainage, *E. susanae* populations in the Cumberland River drainage, and the population of *E. nigrum* occurring in the putative hybrid zone of the Poor Fork. Fifteen restriction enzymes revealed the presence of four mtDNA haplotypes, two each in the Kentucky and Cumberland rivers. *Etheostoma nigrum* from the Kentucky River had two haplotypes (K1 and K2), which differed by a single restriction site. All *E. susanae* from the Cumberland River had a distinctive haplotype (S1), while *E. nigrum* from the upper Poor Fork had a unique haplotype (CN1). Johnny darters from the upper Poor Fork of the Cumberland River were phenotypically and genetically more similar to *E. nigrum* populations in tributaries of the upper Kentucky River than to *E. susanae,* and were probably introduced by stream capture. Implications of this distribution pattern include: (1) that more than one event or mechanism was responsible for the isolation of fishes in the upper Cumberland River, and (2) that conservation efforts for *E. susanae* should focus on habitats occurring immediately above Cumberland Falls rather than in the contact zone in the Poor Fork.

INTRODUCTION

The johnny darter, *Etheostoma nigrum* Rafinesque, is widely distributed across North America, occupying pools and raceways of small headwater streams with silt or sand substrates (Page, 1983). The species is present in most major drainages in Kentucky, with the exception of the Cumberland River (Burr and Warren, 1986). This river system is occupied by the Cumberland johnny darter, *Etheostoma susanae* (Jordan and Swain) which is restricted to the area above the Cumberland Falls in eastern Kentucky (Burr and Warren, 1986; O'Bara, 1991). The relationship between these two species is unclear.

Etheostoma susanae differs from *E. nigrum* by having a broken preorbital stripe, an interrupted preoperculomandibular canal and the absence of scales on the top of the head, opercle, and along the midbelly (Jordan and Swain, 1883; Starnes and Starnes, 1979; Page, 1983). *Etheostoma susanae* was described by Jordan and Swain (1883), and later reduced to a subspecies of *E. nigrum* (Kuhne, 1939). Kuehne and Bailey (1961) examined biogeographic relationships between fishes of the Kentucky and Cumberland rivers and reported geological evidence for the capture of a Cumberland River tributary by a stream in the Kentucky River system. These authors suggested that *E. susanae* entered the upper Cumberland River before the formation of the Cumberland Falls. Squamation patterns in *E. nigrum* from the Kentucky River were used by Starnes and Starnes (1979) to support this hypothesis. More recently, Burr and Page (1986) compiled faunistic evidence that contradicted Kuehne and Bailey's (1961) hypothesis and suggested a Kentucky River origin for the upper Cumberland River fish fauna, including *E. susanae*. Krotzer (1990) reported evidence to support the hypothesis of Burr and Page (1986) in her morphological study of *E. nigrum*. 'About

[1] Present address: Department of Biological Sciences, University of Alabama, Tuscaloosa 35487-0344

TABLE 1.—Haplotype data for populations of *Etheostoma nigrum* and *E. susanae.* Numbered site localities correspond to those in Figure 1

Site	Sample size	Haplotype	Frequency
Etheostoma susanae			
1. Bunches Creek, Cumberland River	2	S1	1.0
2. Laurel Creek, Cumberland River	17	S1	1.0
3. Kilburn Fork, Cumberland River	15	S1	1.0
4. Poor Fork, Cumberland River	3	S1	1.0
Etheostoma nigrum			
5. South Fork, Kentucky River	7	K1	1.0
6. Red Bird River, Kentucky River	12	K1	1.0
7. Middle Fork, Kentucky River	4	K1	1.0
8. Clemons Fork, Kentucky River	7	K1, K2	0.8, 0.2
9. Poor Fork, Cumberland River	13	CN1	1.0

half' (number not given) of the 15 specimens she examined from the Poor Fork of the Cumberland River (not included in Starnes and Starnes, 1979) were morphologically intermediate between *E. nigrum* and *E. susanae.* Etnier and Starnes (1993) suggested that these may be interspecific hybrids.

Strange (1994) re-elevated *Etheostoma susanae* to specific status on the basis of distinctive mitochondrial DNA (mtDNA) haplotypes in either drainage. However, only three specimens were collected from the Poor Fork, and were indistinguishable genetically and morphologically from typical *E. susanae.* I collected additional specimens during subsequent trips to the Poor Fork that were identified as *E. nigrum* on the basis of a continuous preorbital stripe and preoperculomandibular canal.

Stream capture occurs as the result of natural erosional processes whereby streams cut across drainage divides and divert or capture tributary streams in an adjacent drainage. It is possible that numerous stream capture events in eastern Kentucky resulted in the transfer of fish populations both into the Kentucky River drainage from the Cumberland River drainage and *vice versa.* Although this form of vicariance may be quite common in fishes (Banarescu, 1990), it rarely has been documented through genetic analyses (but *see* Waters *et al.,* 1994). Analysis of mtDNA distribution patterns may elucidate the historical events and mechanisms responsible for the distribution of natural populations (Avise *et al.,* 1987). In this paper I examine mtDNA distribution patterns among *Etheostoma nigrum* and *E. susanae,* summarizing and supplementing data previously reported but not published in Strange (1994).

MATERIALS AND METHODS

I collected a total of 80 darters from nine localities in the upper Cumberland and Kentucky rivers (Table 1; Fig. 1). None of the specimens were obvious hybrids and were identified as either *E. nigrum* or *E. susanae* on the basis of morphological characters described by Starnes and Starnes (1979). Fish were brought to the laboratory on ice where mitochondrial DNA was isolated by CsCl-propidium iodide gradient centrifugation. Fifteen restriction enzymes were used to digest mtDNA molecules following the manufacturer's (Promega) recommendations: *Apa* I, *Bam*H I, *Bcl* I, *Bgl* II, *Bst*E II, *Dra* I, *Eco*R I, *Eco*R V, *Hind* III, *Hpa* I, *Nco* I, *Pst* I, *Pvu* II, *Sca* I, and *Xba* I. Details of mtDNA extraction and storage are found in Billington and Hebert (1988) and Strange (1995). Each restriction enzyme recognizes a specific six base-pair sequence. Mitochondrial DNA fragments were then end-

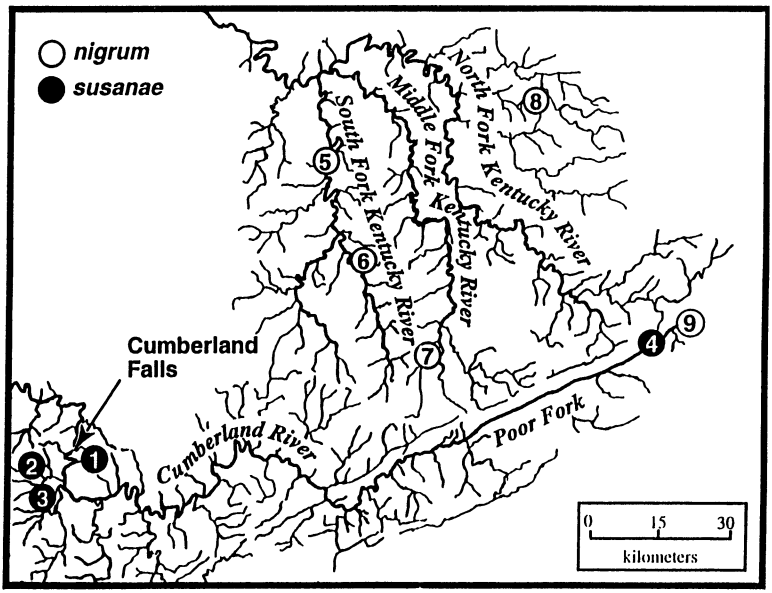

FIG. 1.—Sample localities for *Etheostoma nigrum* (open circles) and *E. susanae* (closed circles). Numbers correspond to sites listed in Table 1

labeled with ^{32}P-dNTPs using Klenow large fragment DNA polymerase I. Labeled fragments were separated by electrophoresis through 1.2% agarose vertical gels, and visualized by autoradiography. The sizes of mtDNA restriction fragments were estimated with reference to the mobilities of fragments of lambda-DNA digested with *Eco*R I and *Hind* III following Billington and Hebert (1988).

All fragment patterns could be related to each other by assuming a gain or loss of one or more restriction-sites for most enzymes. Questionable fragment patterns were 'run' alongside known fragments to assure fragment homology. Each mtDNA haplotype was then characterized by a composite alphabetical code (reported in alphabetical order), corresponding to the specific patterns revealed by each restriction enzyme. However, to avoid ambiguity in determining site homology, restriction-site maps were inferred for each restriction enzyme from a series of double digestions. A parsimony network of the relationships between individual haplotypes was constructed from the presence or absence of mapped restriction sites. This analysis was implemented using the MIX algorithm of PHYLIP 3.5 (Felsenstein, 1992) which estimates the minimum number of site changes (gain or loss) necessary to explain the observed relationships among haplotypes. Relative sequence divergence among each haplotype pair was assessed in terms of distance (p, the estimated number of substitutions per site) calculated from matrices of shared restriction sites following Nei (1987, equation 5.41). Distances provide a basis to estimate the proportion of all base pairs that have undergone substitution since the two haplotypes had a common ancestor.

RESULTS

The 15 restriction enzymes revealed 57 restriction sites which I identified from fragment pattern profiles and double digestions (Table 2). Thirty-nine sites (68%) are invariant and, of the remaining polymorphic sites, eight (14%) are unique to specific haplotypes. The size

TABLE 2.—Fragment patterns and sizes (kilobase pairs) of *Etheostoma nigrum* and *E. susanae* mtDNA for restriction enzymes revealing polymorphisms or cutting more than once, and total length for the mtDNA genome as determined for each enzyme where appropriate

Enzyme	Patttern	Cuts	Fragment sizes	Total
Apa I	A	5	6.71 5.77 2.12 1.13 0.96	16.69
	B	5	7.78 3.59 2.12 1.70 0.96	16.15
Bam H I	A	2	12.23 4.47	16.70
	B	1	16.70	16.70
Bcl I	A	2	12.66 4.24	16.90
	B	1	16.90	16.90
Bgl II	A	3	10.72 5.17 0.79	16.68
	B	3	10.37 5.17 1.18	16.72
	D	2	11.51 5.17	16.68
Bst E II	A	2	11.40 5.55	16.65
Dra I	A	3	8.90 5.36 2.20	16.46
	B	4	8.90 5.36 1.39 1.23	16.88
Eco R I	A	3	10.00 5.11 1.58	16.69
	C	2	10.00 6.69	16.69
Eco R V	A	1	16.60	16.60
	B	2	13.53 3.07	16.60
Hind III	A	6	5.63 4.26 2.24 1.74 1.65 1.41	16.93
	B	5	5.63 4.26 3.98 1.65 1.41	16.93
Hpa I	A	5	6.38 4.83 2.77 1.23 1.21	16.42
	B	4	6.38 4.83 2.77 2.44	16.42
Nco I	A	4	7.91 3.87 3.16 1.43	16.37
	B	5	7.01 3.87 3.16 1.43 0.97	16.44
Pst I	A	5	8.55 3.41 2.30 1.91 0.45	16.62
	B	5	7.70 5.31 2.30 0.88 0.45	16.64
Pvu II	A	2	12.90 3.90	16.80
	B	1	16.80	16.80
Sca I	A	2	12.86 4.04	16.90
Xba I	B	4	8.17 4.37 2.61 1.74	16.89
	C	3	8.17 4.37 4.35	16.89
Mean length				16.68 (±0.20)

of the mtDNA genome is approximately 16.68 (±0.2) kilobase pairs according to the independent fragment profiles, which is similar to that reported for other percids (Billington and Hebert, 1988; Strange and Burr, 1997). In all, the restriction sites represent 342 base pairs, or 2.1% of the mtDNA genome.

The 18 variable restriction sites define four mtDNA composite haplotypes which are unique to either the Cumberland or Kentucky rivers. In the Cumberland River, all 37 specimens of *Etheostoma susanae* had the S1 haplotype (AAAB.ABAA.BBBB.BAB), whereas all 13 specimens of *E. nigrum* from the Poor Fork had the CN1 haplotype (BABD.ABCA.AABA.AAC). The other two haplotypes were found among *E. nigrum* from the Kentucky River, K1 (BDBA.AAAB.AAAA.AAC) and K2 (BDBA.AAAB.AABA.AAC), and are separated by a single *Nco* I restriction site.

The (Wagner) parsimony network (Fig. 2) indicates that a series of 14 mutational steps are required to explain the relationship of the *Etheostoma susanae* (S1) haplotype and the *E. nigrum* K1/K2 haplotype pair. Six steps separate Kentucky River haplotypes from CN1.

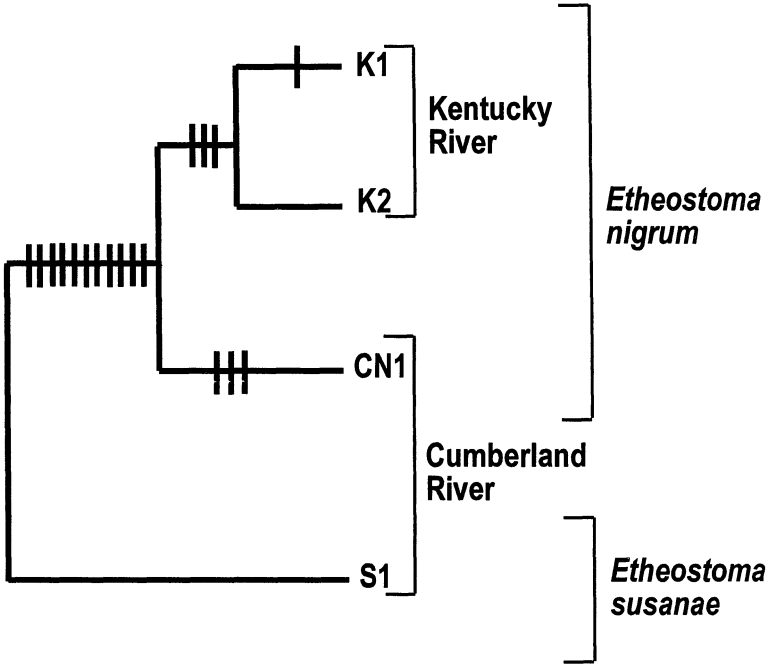

FIG. 2.—Parsimony network separating *Etheostoma nigrum* and *E. susanae* mtDNA haplotypes. Slash marks along branches indicate number of restriction-site differences between haplotypes

In turn, the S1 and CN1 haplotypes differ from one another by 14 restriction sites. Estimated sequence divergence between the *E. nigrum* Kentucky River haplotypes and S1 is 3.23%, while the divergence between *E. susanae* and CN1 is 2.25%. Estimated sequence divergence between CN1 and the Kentucky River haplotypes is 1.19%.

DISCUSSION

The only studies of mtDNA haplotype diversity in darter populations are Strange (1994) and Strange and Burr (1997). The geographic distribution of *Etheostoma nigrum* and *E. susanae* mtDNA haplotypes in eastern Kentucky differ from these data in two respects. First, the degree of divergence (2.25%) between the *E. susanae* haplotype (S1) and the Cumberland River *E. nigrum* haplotype (CN1) is an order of magnitude larger than reported (0.00–0.33%) for co-occurring conspecific darters. This suggests a recent contact after the breakdown of a long-term barrier to dispersal and gene flow (*sensu* Avise *et al.*, 1987). Haplotype diversity within darter populations is typically low and limited to single restriction site differences (Strange and Burr, 1997). On the other hand, the divergence (1.19%) between the *E. nigrum* CN1 haplotype and the Kentucky River haplotypes (K1 and K2) is similar to the divergence (1.20%) between *Etheostoma sagitta sagitta* (Cumberland River) and *E. sagitta spilotum* (Kentucky River) populations (Strange and Burr, 1997).

Second, darter populations typically possess haplotype assemblages that are reciprocally monophyletic with haplotype assemblages of other populations (Strange and Burr, 1997). Based on the presence or absence of restriction sites, the CN1 haplotype is most closely related to the Kentucky River haplotypes (*E. nigrum*) than it is to the *E. susanae* haplotype,

agreeing with the morphological identifications of these specimens. Taken together, these data support species-level distinction between *E. susanae* and *E. nigrum* (Strange, 1994).

Stream capture (Banarescu, 1990) and transportation by humans (Bermingham, 1990) are major sources of fish dispersal and gene flow over drainage divides. Darters are not used as bait by fishermen, so 'bait bucket' introduction is unlikely. Stream capture is common in upland and mountainous areas such as eastern Kentucky, and it is likely that introductions following such drainage shifts have occurred in this area. The capture of a Kentucky River tributary by a Cumberland River stream is the best explanation for the present distribution of *Etheostoma nigrum* phenotypes and mtDNA haplotypes in the upper Poor Fork.

Although the geographic distribution of *Etheostoma nigrum* mtDNA haplotypes confirms Burr and Page's (1986) stream capture hypothesis in general, it also suggests that the origin of the upper Cumberland fish fauna cannot be attributed to a single isolation event. My data indicate at least two separate events occurred; an unknown event responsible for introducing *E. susanae* (or its progenitor) above the Cumberland Falls, and another introducing *E. nigrum* from the Kentucky River. As mentioned before, the estimated sequence divergence between *E. sagitta sagitta* and *E. sagitta spilotum* mtDNA haplotypes is similar to the divergence estimate for CN1 and K1/K2. If the rate of mtDNA sequence evolution is similar in these darters, it is possible that *E. nigrum* (CN1) was introduced into the Cumberland drainage in the same event as the *E. sagitta sagitta* haplotypes. Another fish, *Ericymba buccata,* may have also invaded the Cumberland from the Kentucky since it does not occur below the Cumberland Falls or in adjacent drainages.

Fishes occurring above the Cumberland Falls that do not have closest relatives known to inhabit the Kentucky River system include *Phoxinus cumberlandensis* and *Etheostoma kennicotti,* each with closest relatives or conspecifics in the upper Tennessee River (Page and Smith, 1976; Starnes and Jenkins, 1988). *Etheostoma baileyi,* like *E. sagitta* subspecies, is limited to the Cumberland and Kentucky rivers, suggesting a similar route into the upper Cumberland River. However, it is the only snubnose darter (subgenus *Nanostoma*) species occupying a former Teays River tributary (*e.g.,* the Kentucky River) and its phylogenetic relationships are unknown. This species may have entered the upper Cumberland River via the middle Cumberland River, and later entered the Kentucky River system. A similar mechanism could be proposed for *E. susanae* but, like *E. baileyi,* its phylogenetic relationships are not known. Thus, the upper Cumberland River fauna may have origins from at least three different drainages and cannot be attributed solely to the Kentucky River (*contra* Burr and Page, 1986; Starnes and Jenkins, 1988).

Conservation implications.—Etheostoma susanae was believed to be the only johnny darter occurring above the Cumberland Falls (Burr and Warren, 1986; O'Bara, 1991). I found two separate populations existing within this drainage, each readily distinguishable morphologically and genetically. Such differences typically result from long-term barriers to gene flow and are indicative of species-level distinction. Future conservation efforts should consider that the Poor Fork is an area of secondary contact between *E. susanae* and *E. nigrum,* and the relocation of individuals should not be undertaken between these distinctive populations. Instead, habitat protection should be the conservation priority, and concentrated on the area immediately above the Cumberland Falls.

Acknowledgments.—I would like to thank R. Cicerello and E. Laudermilk of the Kentucky State Nature Preserves Commission for sharing their unpublished data, recommendations, personal comments, and assistance in the field. B.M. Burr assisted in the field collections, offered suggestions, and was generally supportive throughout this and other endeavors. K. Cook helped collect darters during two separate trips to the Cumberland River. I also thank N. Billington and Roy Heidinger of the Southern Illinois

University at Carbondale Cooperative Fisheries Laboratory, in whose laboratory the research was performed. This project was funded by the U.S. Fish and Wildlife Service.

LITERATURE CITED

AVISE, J. C., J. ARNOLD, R. M. BALL, E. BERMINGHAM, T. LAMB, J. E. NEIGEL, C. A. REEB AND N. C. SAUNDERS. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.*, **18**:489–522.

BANARESCU, P. 1990. Zoogeography of freshwaters, Vol. I. AULA-Verlag, Wiesbaden, Germany. 511 p.

BERMINGHAM, E. 1990. Mitochondrial DNA and the analysis of fish population structure, p. 197–221. *In*: D. H. Whitmore (ed.). Electrophoretic and isoelectric focusing techniques in fisheries management. CRC Press, Boca Raton, FL.

BILLINGTON, N. AND P. D. N. HEBERT. 1988. Mitochondrial variation in Great Lakes walleye (*Stizostedion vitreum*) populations. *Can. J. Fish. Aq. Sci.*, **45**:643–654.

BURR, B. M. AND L. M. PAGE. 1986. Zoogeography of the fishes of the lower Ohio-upper Mississippi River basin, p. 287–324. *In*: C. H. Hocutt and E. O. Wiley (eds.). Zoogeography of North American freshwater fishes. John Wiley and Sons, New York, NY.

——— AND M. L. WARREN. 1986. A distributional atlas of Kentucky fishes. Kentucky Nature Preserves Commission Scientific and Technical Series No. 4, Frankfort, KY. 398 p.

ETNIER, D. A. AND W. C. STARNES. 1993. The fishes of Tennessee. University of Tennessee Press, Knoxville. 681 p.

FELSENSTEIN, J. 1992. PHYLIP manual. Vers. 3.5. Distributed by the auther Univ. of Washington, Seattle.

JORDAN, D. S. AND J. S. SWAIN. 1883. List of the fishes collected in the Clear Fork of the Cumberland, Whitely County, Kentucky, with descriptions of three new species. *Proc. U.S. Natl. Mus.*, **6**:248–251.

KROTZER, M. J. 1990. Variation and systematics of *Etheostoma nigrum* Rafinesque, the johnny darter (Pisces: Percidae). Ph.D. Dissertation, University of Tennessee, Knoxville. 192 p.

KUEHNE, R. A. AND R. M. BAILEY. 1961. Stream capture and the distribution of the percid fish *Etheostoma sagitta*, with geologic and taxonomic considerations. *Copeia*, **1961**:1–8.

KUHNE, E. R. 1939. A guide to the fishes of Tennessee and the mid-South. Tennessee Department of Conservation, Division of Game and Fish, Nashville. 124 p.

NEI, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York, N.Y. 512 p.

O'BARA, C. J. 1991. Current distribution and status of the upper Cumberland River johnny darter, *Etheostoma nigrum susanae*. *J. Tenn. Acad. Sci.*, **66**:9–11.

PAGE, L. M. 1983. Handbook of darters. T.F.H. Publications, Neptune City, N.J. 217 p.

——— AND P. W. SMITH. 1976. Variation and systematics of the stripetail darter, *Etheostoma kennicotti*. *Copeia*, **1976**:532–541.

STARNES, W. C. AND R. E. JENKINS. 1988. A cyprinid fish of the genus *Phoxinus* (Pisces: Cypriniformes) from the Tennessee River drainage with comments on relationships and biogeography. *Proc. Biol. Soc. Wash.*, **101**:517–529.

——— AND L. B. STARNES. 1979. Taxonomic status of the percid fish *Etheostoma nigrum susanae*. *Copeia*, **1979**:426–430.

STRANGE, R. M. 1994. Reconsideration of the taxonomic status of the Cumberland johnny darter, *Etheostoma nigrum susanae*: mtDNA analysis and the importance of speciation concepts for conservation biology. Report submitted to the U.S. Fish and Wildlife Service, Asheville, N.C. 30 p.

———. 1995. Intraspecific phylogeography of North American highland fishes. M.S. Thesis, Southern Illinois University. Carbondale, Ill. 99 p.

——— AND B. M. BURR. 1997. Intraspecific phylogeography of North American highland fishes: a test of the Pleistocene vicariance hypotheis. *Evolution*, **51**:885–897.

WATERS, J. M., M. LINTERMAN AND R. W. G. WHITE. 1994. Mitochondrial DNA variation suggests river capture as a source of vicariance in *Gadopsis bispinosis* (Pisces: Gadopsidae). *J. Fish Biol.*, **44**: 549–551.

SUBMITTED 12 AUGUST 1996 ACCEPTED 17 MARCH 1997