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Biochemical Assessment of the Taxonomic Status of "*Rhinichthys bowersi*" (Pisces: Cyprinidae)

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***Rhinichthys bowersi* was described in 1908 from two West Virginia localities in the Monongahela River system. Subsequently, however, it was described as a *Nocomis micropogon* × *Rhinichthys cataractae* hybrid. Electrophoretic analysis of 43 enzyme-coding loci and general protein patterns of serum, muscle and eye lens revealed diagnostically useful differences in serum protein patterns and in allelic composition at seven loci. The "*bowersi*" form did not show the biochemical intermediacy expected of hybrids. In fact, "*bowersi*" alleles for two enzyme-coding loci, and the pattern of serum protein electromorphs, were diagnostically different from both of the supposed parental species. Overall genetic similarity and similarity in general protein patterns show that the two examined populations of "*bowersi*" are more similar to *R. cataractae* than to *N. micropogon.* The "*bowersi*" form is considered a valid species of *Rhinichthys* and not an F_1 hybrid.**

RHINICHTHYS bowersi was originally described by Goldsborough and Clark (1908) from Dry Fork at Harman, West Virginia, and from Shavers Fork of Cheat River at Cheat Bridge, West Virginia. Raney (1940) collected four specimens from the Cheat River in 1935 and, upon examination, redescribed *R. bowersi* as a *Nocomis micropogon* × *Rhinichthys cataractae* hybrid. Raney (1947) attributed hybridization to the use of *Nocomis* nests by *R. cataractae.* Cooper (1980) found *Nocomis* nests with *R. cataractae* eggs and larvae in them, but no hybrids. Simultaneous spawning of both species was never observed by Cooper (1980). For the purpose of this manuscript the supposed hybrid is referred to as "*bowersi.*"

Prior to 1975, 14 specimens of "*bowersi*" were known from nine collections dating to 1890. An additional 15 specimens were collected by the West Virginia Department of Natural Resources from Shavers Fork in 1975–1976. Stauffer et al. (1979) reported taking 22 additional specimens from Shavers Fork in 1976. In 1977, Stauffer et al. (1979) collected and released three additional specimens from the Tygart Valley River in West Virginia, and one from the Youghiogheny River in Pennsylvania. Hendricks (1980) reported one specimen from the Youghiogheny River in Maryland, two specimens from the Youghiogheny R. in Pennsylvania and four specimens from Snowy Creek, a tributary of the Youghiogheny R. in West Vir-

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ginia. Recently two additional specimens were identified from White Day Creek of the Monongahela River (D. A. Cincotta, pers. comm.). All reports of "*bowersi*" have been from the Monongahela River system, with the exception of two Lake Erie collections (Ross and Cavender, 1977). To date only 145 "*bowersi*" specimens are known. A recent hybrid between *R. cataractae* and a second species of *Nocomis*, *N. platyrhynchus*, was taken from the Greenbrier River of the New River system and is morphologically identical to "*bowersi*" (Stauffer et al., 1979). Esmond et al. (1981) found *N. micropogon* and *N. platyrhynchus* to be electrophoretically identical. If *N. micropogon* and *N. platyrhynchus* are the same species, this specimen could be "*bowersi*", and its presence in the Greenbrier is explained by stream capture (Lachner and Jenkins, 1971; Hocutt, 1979).

The objective of the present study was to use biochemical genetics to evaluate the taxonomic status of "*bowersi*."

Materials and Methods

Specimens were collected with a 1.5 m × 3.0 m, 0.32-cm mesh seine or electroshocking (220/DC) unit. To avoid variation in proteins due to acclimation temperature the fish were carefully removed from the seine and placed into 200-L plastic containers fitted with standard aquarium filtration-aeration systems and transported back to the laboratory where they were held in 38-L glass aquaria for 5 days at 18 C (±0.5 C). Specimens were collected from two localities in the Monongahela River drainage: 1) Horseshoe Run, Tucker County, West Virginia, along State Route 7 at distances 2.1 and 6.5 river km upstream from the confluence with the Cheat River; 2) from the Youghiogheny River drainage, Snowy Creek, Preston County, West Virginia, at the spillpool of Terra Alta Lake and 100 m downstream. Voucher specimens were deposited in the collections of the Appalachian Environmental Laboratory Fish Museum (AEL), University of Maryland. Identification of "*bowersi*" followed Stauffer et al. (1979).

Blood samples for serum protein analysis were collected by cardiac puncture with an unheparinized capillary tube. The blood was allowed to clot, then centrifuged at 12,800 rpm for 15 min, after which the serum was removed and placed in disposable culture tubes for storage in a REVCO ultra-cold freezer at −80 C.

Muscle, brain and eye lens tissue were removed from specimens after bleeding. Extracts of trunk muscle and brain were obtained by homogenizing equal volumes of tissue and distilled water in glass tissue grinders. Care was taken to remove any viscera from the tissue sample before homogenizing. Eye lenses were ground with five times their volume of 0.018% NaCl and allowed to stand for 12–18 hrs at 4 C. Homogenates of the tissues were centrifuged at 12,800 rpm for 5 min, with the supernatant fraction being retained for electrophoresis. To determine if "*bowersi*" had the same allelic composition of the putative parents, mixtures of the tissue extracts were prepared using equal parts of extracts obtained from the supposed parents.

Horizontal starch gel electrophoresis, gradient slab acrylamide and isoelectric focusing techniques were employed in this study. Both soluble protein and enzymatic staining techniques were used.

Horizontal starch gel electrophoresis essentially followed procedures outlined in Selander et al. (1971) employing 15 × 20 × 1 cm and 15 × 20 × 1.5 cm molds, Model IP-17 Heathkit power supplies, and 14% starch (Sigma Co.) gels. A constant amperage (50 ma) was used with voltage dependent on the buffer systems. Each enzyme assay was performed on 15 specimens of *Nocomis micropogon*, *Rhinichthys cataractae* and "*bowersi*" from each of the two collection localities and on mixtures of extracts from the supposed parentals.

The enzymes examined are listed in Table 1. Staining recipes were from B. A. Schaal and W. W. Anderson (pers. comm.), Allendorf et al. (1977), Shaw and Prasad (1970), Phillip et al. (1979) and Selander et al. (1971).

Precast Pharmacia acrylamide gradient gels (4–30%) were used for soluble proteins from serum, eye lens, and muscle. A Pharmacia GE-4II chamber and EPS 500/400 power supply were used for electrophoresis at 75V (initial amperage of 75 ma) and 100V (final, 80 ma) for 18 hr at 5–10 C. The buffer system was 0.09 M Tris, 0.08 M boric acid, and 0.03 M Na_2EDTA, pH 8.35.

Isoelectric focusing was done with precast LKB slab acrylamide gels of pH ranges 3.5–9.5 (buffer system—anode 1 M H_3PO_4, cathode 1 M NaOH) and 5.5–8.5 (buffer system—anode 0.4 M HEPES, cathode 0.1 M NaOH). An LKB electrophoresis chamber and Pharmacia EPS 500/400 power supply was used for isoelectric focusing runs at 200V (initial, 50 ma) to 300V (final, 6 ma) for 3 hr at 5–10 C.

TABLE 1. ENZYME SYSTEMS EXAMINED FOR *Nocomis micropogon, Rhinichthys cataractae,* AND *"bowersi."* Buffers are: IF = isoelectric focusing; R = Ridgeway et al. (1970); CT = Clayton and Tretiak (1972); TC = Tris-citrate system of Philipp et al. (1979); P = Poulik (1957) buffer; and B = Borate system of Shaw and Prasad (1970). Tissues used: M = msucle tissue; B = brain tissue.

Enzyme	E.C. number	Locus	Buffer system	Tissue
Acid Phosphatase	3.1.3.2	*ACP-A*	IF	M
		ACP-B	IF, TC	
		ACP-C	IF, TC	
Adenylate Kinase	2.7.4.3	*ADK-A*	R	M
		ADK-B		
Alcohol Dehydrogenase	1.1.1.1	*ADH-A*	R, TC, P	M
Alanine Dehydrogenase	1.4.1.1	*ALDH-A*	B	M
Aldolase	4.1.2.13	*ALD-A*	CT	M
		ALD-B		M, B
Alkaline Phosphatase	3.1.3.1	*ALK-A*	IF	M
		ALK-B	IF, TC	
		ALK-C	IF, TC	
Aspartate Aminotransferase	2.6.1.1	*AAT-A*	CT	M
		AAT-B		
Creatine Kinase	2.7.3.2	*CK-A*	R, TC	M
Diaphorase	1.6.4.3	*DIA-A*	R	M
Esterase	3.1.1.1	*EST-A*	R	M, B
		EST-B		M, B
		EST-C		M
Fumerase	4.2.1.2	*FUM-A*	R	M
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	*G6PD-A*	P, B	M, B
Glutamate Dehydrogenase	1.4.1.3	*GDH-A*	CT	M
		GDH-B	P	
α-Glycerophosphate Dehydrogenase	1.1.1.8	*αGPD-A*	TC, CT	M
		αGPD-B		
Hexokinase	2.7.1.1	*HK-A*	B, TC	M
Hexose-6-Phosphate Dehydrogenase		*H6PD-A*	B	M
		H6PD-B		
Isocitrate Dehydrogenase	1.1.1.42	*IDH-A*	CT	M
Lactate Dehydrogenase	1.1.1.27	*LDH-A*	R, B	M
		LDH-B		
Leucine Aminopeptidase	3.4.1.1	*LAP-A*	R, B	M
L-Leucine Dehydrogenase		*LeDH-A*	B	M
Malate Dehydrogenase	1.1.1.37	*MDH-A*	B, R	M
		MDH-B	B	
Malic Enzyme	1.1.1.40	*ME-A*	P	M
Mannose Phosphate Isomerase	5.3.1.8	*MPI-A*	P	M, B
Phosphoglucose Isomerase	5.3.1.9	*PGI-A*	R, B	M, B
Phosphoglucomutase	2.7.5.1	*PGM-A*	R	M
Sorbitol Dehydrogenase	1.1.1.14	*SoDH-A*	R	M
Superoxide Dismutase	1.15.1.1	*SOD-A*	R	M
		SOD-B		
Xanthine Dehydrogenase	1.2.1.37	*XDH-A*	P	M

Soluble proteins from serum, eye lens and muscle tissue were stained with 0.5% Buffalo Black NBR in 7% acetic acid for 4–8 hr. Following staining, the gels were destained by successive changes of 7% acetic acid until the background was clear.

Data derived from soluble proteins (eye lens, serum and muscle protein) stained via Buffalo Black NBR were treated differently than data derived from the enzymatic staining techniques. For gradient slab electropherograms, direct comparisons were made within a gel for

mobility and distribution of proteins. Models of soluble protein patterns for each species and for each tissue were constructed. All samples from that set (tissue-species combination) were compared to the model. Proteins were then coded as present (1) or absent (0). If a protein occurred in a gel and not in the model, then that protein was added to the model and all gels reexamined for that protein; however, the model gel was scored as 0. Jaccard's coefficients, S_j (Sneath and Sokal, 1973), were calculated for each set of proteins within a species-tissue combination and was followed by comparison among species-tissue combinations.

For each enzyme locus, the mobility of the most common electromorph of *Rhinichthys cataractae* was designated 100. Other electromorphs were assigned values representing proportionate mobility (×100) relative to that of the standard. Enzyme and locus terminology follows that used in previous cypriniform studies (Rainboth and Whitt, 1974; Buth, 1978). Electromorphs of identical mobility were assumed to be visualizations of genetically identical proteins. For two enzymes (ADK-A and EST-C), *N. micropogon* showed no activity whereas the other two forms did; in this case, *N. micropogon* was assigned a unique homozygous genotype.

RESULTS

Isozyme variability.—Extracts from each specimen (110 individuals) of *Nocomis micropogon*, *Rhinichthys cataractae*, "*bowersi*" and a mixture of the parental extracts, from both populations were analyzed for gene products at 43 enzyme loci. A total of 53 presumptive alleles were resolved. Relative mobilities of allelic products of the polymorphic loci and monomorphic loci, with interspecific variation, are illustrated in Fig. 1. Allelic frequencies are listed in Table 2. Of the 43 enzyme loci, 36 were monomorphic with no interspecific variation.

Several loci with diagnostically useful alleles were resolved for both species and "*bowersi*" (Table 2; Fig. 1). Two loci, *EST-B* and *αGPD-A*, are very important in the determination of the taxonomic status of "*bowersi*." If "*bowersi*" is a hybrid, one would expect the mixture of the two supposed parental extracts to be identical in allelic content to "*bowersi*." The alleles resolved from both supposed parents for *EST-B* and *αGPD-A* were not found in "*bowersi*." "*bowersi*" was also found to have alleles not found in either *N. micropogon* or *R. cataractae*. Isozymes

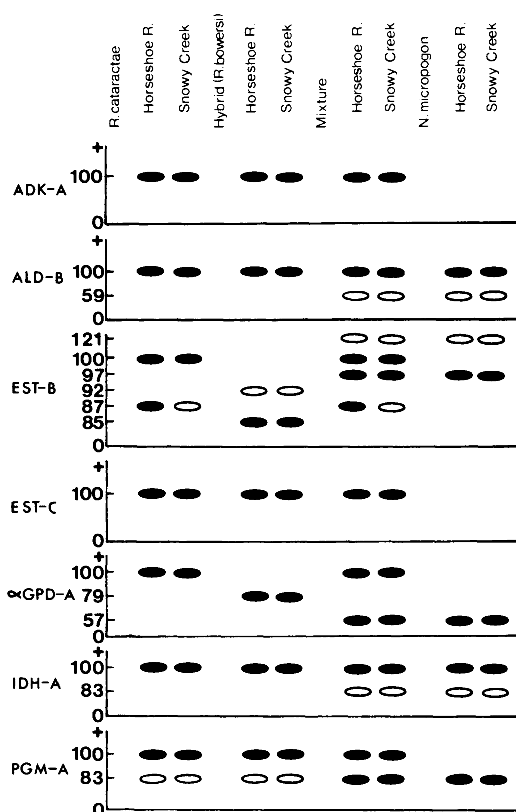

Fig. 1. Electrophoretic mobilities of enzymatic allelic products in *Rhinichthys cataractae, Nocomis micropogon,* and "*bowersi.*" Diagrammatic representations of electromorphs represent relative mobilities of relevant homopolymers and do not include heterozygotes or heteropolymers. Solid symbols = frequencies ≥0.50. Hollow symbols = frequencies <0.50.

encoding for *AKD-A* and *EST-C* were resolved for *R. cataractae* and "*bowersi,*" but not resolved for either population of *N. micropogon.*

Estimates of genetic variability at 43 loci show very low interpopulational differences for both species and "*bowersi,*" whereas interspecific differences were evident. The average heterozygosity per locus ($\bar{H}$) of *R. cataractae* was 0.015 for both populations with a percent polymorphism of 4.6. "*Bowersi*" had a $\bar{H}$ of 0.023 and 0.022 for the Horseshoe Run population and Snowy Creek population, respectively, with a percent polymorphism of 4.6. The two populations of *N. micropogon* showed a $\bar{H}$ of 0.031 for the Horseshoe Run population and 0.033 for the Snowy Creek population, with a percent polymorphism of 7.0 for both populations. The effective number of alleles per locus was 1.02

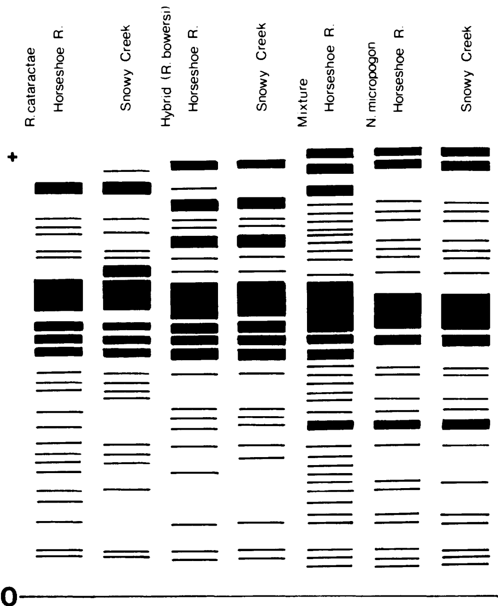

Fig. 2. Models of general soluble serum proteins for *Rhinichthys cataractae, Nocomis micropogon,* "*bowersi*" and the mixture of *R. cataractae* and *N. micropogon* extracts.

for *R. cataractae,* 1.04 for "*bowersi*" and 1.05 for *N. micropogon.* Nevo (1978) reports that fish have an average polymorphism of ≃15% and mean heterozygosity of ≃0.050 which is considerably higher than the values found in this study.

General soluble proteins.—A total of three soluble eye lens proteins, and five muscle proteins was consistently observed on gradient slab acrylamide gels for all samples; there was no interspecific variation for either eye lens or muscle proteins. Serum protein patterns showed interspecific variation (Fig. 2) on gradient slab acrylamide gels.

Analysis of the model serum proteins (Fig. 2) shows *R. cataractae* sharing four bands with "*bowersi,*" and four bands with *N. micropogon.* "*Bowersi*" has three serum protein bands in common with *N. micropogon.* Six similar serum protein bands occurred in all three forms. Nine bands were unique to *R. cataractae,* five to *N. micropogon* and five to "*bowersi.*" Intraspecific variation was observed for both species and "*bowersi.*" The mixture of *R. cataractae* and *N. micropogon* serum yielded all serum protein bands found in either species. The five bands unique to "*bowersi*" were not expressed in the mixture. The mean number of serum protein bands was as follows: *R. cataractae* 22.5 (N = 4) for Horseshoe Run, 19.5 (N = 4) for Snowy Creek; *N. micropogon* 23.3 (N = 4) for Horseshoe Run, 22.8 (N = 5) for Snowy Creek; "*bowersi*" 21.0 (N = 6) for Horseshoe Run, 17.6 (N = 7) for Snowy Creek; and 34.6 (N = 5) for the mixture of the two supposed parentals.

Phenetic relationships.—Phenetic relationships among the two populations of *R. cataractae,* "*bowersi*" and *N. micropogon* were expressed through comparison of Nei's (1972) index of genetic similarity (I) for enzymatic data and Jaccard's similarity coefficient (S_j) for the soluble serum protein data (Table 3). Inspection of Table 3 reveals that the "*bowersi*" form is more similar to *R. cataractae* than to *N. micropogon.* Jaccard's similarity coefficient based on serum proteins shows the same pattern (Table 3). The mixture of extracts from *R. cataractae* and *N. micropogon* was more similar to *N. micropogon* than *R. cataractae*; this is because *N. micropogon* contributed more serum protein bands than *R. cataractae* (Fig. 2).

Discussion

Raney (1940) hypothesized that specimens of the "*bowersi*" form, which he considered products of ongoing hybridization, may have resulted from "a fortuitous fertilization as *R. cataractae* spawned over a *Nocomis* nest." Stauffer et al. (1979), on the basis of a hybrid index, reported that specimens of "*bowersi,*" were indeed, intermediate in morphometric and meristic characters between *N. micropogon* and *R. cataractae.* Goldsborough and Clark (1908) noted in their original diagnosis of "*bowersi*" that it was intermediate in some characters between *N. micropogon* and *R. cataractae.*

However, Stauffer et al. (1979) state that, except for two records, "*bowersi*" is known only from the Monongahela River system, while the two supposed parental species occur sympatrically over a wide geographic area (Jenkins et al., 1972; Denoncourt and Cooper, 1975; Stauffer et al., 1977). The only two collections outside the Monongahela were from Lake Erie drainages. These records can be explained zoogeographically because the modern day Monongahela River system once flowed northward beyond the present Pittsburg, Pennsylvania into the ancestral Laurentian drainage which in-

TABLE 2. ENZYME ALLELE FREQUENCIES OF *Rhinichthys cataractae*, *Nocomis micropogon*, AND "*bowersi*" WITH 2× THE STANDARD ERROR OF THE FREQUENCIES.

	R. cataractae		Hybrid (*R. bowersi*)		*N. micropogon*	
Enzyme	Horseshoe Run	Snowy Creek	Horseshoe Run	Snowy Creek	Horseshoe Run	Snowy Creek
ADK-A^{100}	1.00	1.00	1.00	1.00	—	—
ALD-B^{100}	1.00	1.00	1.00	1.00	0.67	0.53
					(0.17)	(0.18)
ALD-B^{59}	—	—	—	—	0.33	0.47
					(0.17)	(0.18)
EST-B^{121}	—	—	—	—	0.30	0.37
					(0.17)	(0.18)
EST-B^{100}	0.50	0.53				
	(0.18)	(0.18)	—	—	—	—
EST-B^{97}	—	—	—	—	0.70	0.63
					(0.17)	(0.18)
EST-B^{92}	—	—	0.47	0.37	—	—
	—	—	(0.18)	(0.18)		
EST-B^{87}	0.50	0.47	—	—	—	—
	(0.18)	(0.18)				
EST-B^{85}	—	—	0.53	0.63	—	—
			(0.18)	(0.18)	—	—
EST-C^{100}	1.00	1.00	1.00	1.00	—	—
αGPD-A^{100}	1.00	1.00	—	—	—	—
αGPD-A^{79}	—	—	1.00	1.00	—	—
αGPD-A^{57}	—	—	—	—	1.00	1.00
IDH-A^{100}	1.00	1.00	1.00	1.00	0.57	0.63
					(0.18)	(0.18)
IDH-A^{83}	—	—	—	—	0.43	0.37
					(0.18)	(0.18)
PGM-A^{100}	0.93	0.93	0.60	0.57	—	—
	(0.09)	(0.09)	(0.18)	(0.18)	—	—
PGM-A^{83}	0.07	0.07	0.40	0.43		
	(0.09)	(0.09)	(0.18)	(0.18)	1.00	1.00

cluded the Lake Erie area (Hocutt, 1979). Another possibility for the two Lake Erie records is that they do indeed represent hybrids of *N. micropogon* × *R. cataractae*, but separate from "*bowersi*" (Stauffer et al., 1979); however, their morphometric and meristic data were consistent with the Monongahela "*bowersi*." The record of *N. platyrhynchus* × *R. cataractae* collected from the Greenbrier River, may also be "*bowersi*." Esmond et al. (1981) found that *N. micropogon* and *N. platyrhynchus* were electrophoretically identical.

The biochemical data for "*bowersi*" does not follow that reported for other hybrids. "*bowersi*" showed no evidence of inheritance of soluble serum proteins or isozymes from the supposed parentals. Comparisons of "*bowersi*" with the mixture of tissue extracts of *R. cataractae* and *N. micropogon* were not identical, which would be expected if "*bowersi*" were indeed a F_1 hybrid of these parentals (Fig. 1, 2). In fact, "*bowersi*" had unique electromorphs for *EST-B*, *αGPD-A* and serum proteins which were not present in either of the supposed parental species. Electromorphs of *AKD-A*, *ALD-B*, *EST-C* and *IDH-A* (Fig. 1), Nei's genetic similarity and the analysis of soluble serum protein (Table 3) suggest that "*bowersi*" is more similar to *R. cataractae* than *N. micropogon*. This supports Goldsborough and Clark's (1908) original description in which "*bowersi*" was recognized as a species in the genus *Rhinichthys*.

An unsuccessful attempt was made to induce spawning of "*bowersi*" in the laboratory. This is not necessarily surprising because neither Buynak and Mohr (1981) nor Cooper (1980) could

TABLE 3. COMPARISON OF NEI'S (1972) GENETIC SIMILARITY COEFFICIENTS [I] FOR ISOZYMIC DATA (ABOVE DIAGONAL) AND JACCARD'S SIMILARITY COEFFICIENTS [S_j] FOR SOLUBLE SERUM PROTEIN DATA (BELOW DIAGONAL) FOR *Rhinichthys cataractae, Nocomis micropogon,* AND *"bowersi."*

	R. cataractae		*"bowersi"*		*N. micropogon*	
	Horseshoe Run	Snowy Creek	Horseshoe Run	Snowy Creek	Horseshoe Run	Snowy Creek
R. cataractae						
Horseshoe Run	0.90*[1]	1.00	0.96	0.96	0.89	0.89
Snowy Creek	0.67	0.90*	0.96	0.96	0.89	0.89
"bowersi"						
Horseshoe Run	0.45	0.32	1.00*	1.00	0.90	0.90
Snowy Creek	0.50	0.30	0.86	0.96*	0.90	0.90
N. micropogon						
Horseshoe Run	0.33	0.29	0.35	0.33	0.99*	1.00
Snowy Creek	0.21	0.26	0.34	0.31	0.96	0.99*

[1] Values are Jaccard's similarity coefficients where * appears on diagonal, showing variation in soluble serum proteins when compared to model (see text).

induce one of the supposed parent species, *N. micropogon,* to spawn in captivity. Many species have not, at present, been observed spawning in the field, and in fact original descriptions of fishes do not require data on reproductive isolation, although it is usually inferred. Collections of *"bowersi"* from the upper Cheat River represented four distinct age classes based on scale examination (Stauffer et al., 1979). Two large specimens of *"bowersi"* captured from Shavers Fork were dissected (Stauffer et al., 1979). One had small ovaries with eggs approximately the same size as those of *R. cataractae* females collected at the same time; the other had normal testes. Recently, two males of *"bowersi"* collected from Snowy Creek had definite breeding tubercles, and another female had eggs apparently close to being ripe. *"bowersi"* appears to have characteristics of a species, even though meristic and morphometric characteristics are clearly intermediate between *N. micropogon* and *R. cataractae.*

Intermediacy of characters does not necessarily indicate hybrid origin (Aspinwall and Tsuyuki, 1968). Both *N. micropogon* and *R. cataractae* are quite similar biochemically (Figs. 1, 2), but they do have species specific isozymes, as does *"bowersi."* There are records of at least four distinct age classes of *"bowersi"* and the taxon apparently has persisted from at least 1899 to the present. The morphometric and meristic characteristics (Stauffer et al., 1979) and biochemical characters of *"bowersi"* are at least as consistent as those of the supposed parents. It appears that *"bowersi"* has the characteristics expected of a species. It is persistent in nature, occupies a distinct range, and has a unique combination of meristic, morphometric and biochemical characters. We recommend that *"bowersi"* be resurrected as a valid species of the genus *Rhinichthys.*

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