Endangered Species Identification Along Corridors in WV using GIS

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16. Abstract

Since the late 19th century, the exploitation of oil, gas, timber, and mineral resources in Appalachia has been associated with the production of an extensive transportation system and with a concurrent decline in the environmental quality of aquatic systems. This project will establish the infrastructure for a web delivered, interactive mapping system developed from satellite imagery and integrated with Geographical Information Systems that will locate all endemic, threatened, or endangered species relative to transportation systems in WV. The project will incorporate a detailed case study along the proposed Corridor H of the Appalachian Highway System to establish the interactive mapping system criteria and processes, The case study will also address the authentication of a specific rare Appalachian fish as an endangered species or a rare hybrid developed as a result of previous transportation related environmental disturbances in this area.

The Cheat minnow, Rhinichthys bowersi, is a rare fish that has been reported almost exclusively from streams in the Monongahela River system of West Virginia and Pennsylvania. The status of the Cheat minnow is currently controversial. There have been reports that the Cheat minnow has unique genetic characters, is reproductively isolated from other minnows, has a limited distribution, and should be considered a valid species.

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FINAL REPORT

Endangered Species Identification along Corridors in WV using GIS

ATI TRP 99-10

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EXECUTIVE SUMMARY

The Cheat minnow, *Rhinichthys bowersi*, has been designated a species of concern by the West Virginia Department of Natural Resources. Mitochondrial DNA analysis (Gladwell 2002) supports the origin of *R. bowersi* from hybridization between cyprinids *R. cataractae* and *Nocomis micropogon*. The Cheat Minnow, which has been designated as a species of concern by both Federal and State agencies, is found in streams along the direct, proposed routes for construction of Corridor H. In addition, the Cheat Minnow has a unique relationship to the environment and to the influences of transportation and associated extraction industries. Previous investigators have hypothesized a relationship between fish hybridization and environmental disturbance. The habitat of *R. bowersi* has experienced severe environmental degradation from mineral and timber extraction and associated transportation systems.

In this study, environmental quality was compared between stream systems in which parental species of *R. bowersi* were found with and without resulting hybridization and an extensive analysis of mitochondrial DNA was conducted to determine whether or not the Cheat Minnow was a distinct species or merely a hybrid between two cyprinid species.

In this study, environmental quality indicators included biotic diversity and stream structure indices. Data from this analysis were expressed in a geospatial model and analyzed for significant differences. No significant pattern of differences were found between streams with or without the presence of Cheat Minnows, and the data did not support the hypothesis that relates hybridization frequency and production of *R. bowersi* to environmental disturbance. An intermediate level of disturbance with slightly elevated levels of biodiversity was found in watersheds yielding the Cheat minnow. Mitochondrial DNA analysis indicated that the Cheat Minnow is a hybrid between two cyprinid, parental species. Because hybridization within *Cyprinidae* similar to that which produced the Cheat Minnow is relatively common, and there is no evidence of a distinct reproductive population of *R. bowersi,* the status of the Cheat Minnow as a reproductively viable species is suspect.

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{Gladwell's Thesis}
mtDNA - mitochondrial deoxyribonucleic acid
G3PDH - glycerol-3-phosphate dehydrogenase
EST-B and EST-C - esterase
ALD - alcohol dehydrogenase
ADK-A - adenylate kinase
ALD-B - aldolase
IDH - A - isocitrare dehydrogenase
PCR - polymerase chain reaction
hyb1, hyb2, hyb3 and *R. bowersi – Rhinichthys bowersi*
nm1, nm2 and *N. micropogon - Nocomis micropogon*
Rcat1, Rcat2 and *R. cataractae - Rhinichthys cataractae*
Ratra1, Ratra2, RA and *R. atratulus - Rhinichthys atratulus*
CA - *Campostoma anomalum*

{ Schlenker's Thesis}
ANOVA – Analysis of Variance
ArcGIS – Environmental Systems Research Institute Geographic Information System Software
DA – Discriminant Analysis
EPT – Ephemeroptera, Plecoptera, Trichoptera Taxa
ESRI – Environmental Systems Research Institute
GIS – Geographic Information System
GPS – Geographic Positioning System
Intersect Function – GIS Function for Creating Points at Line Intersections
Map Algebra – Mathematical Functions Performed on GIS Files
N. micropogon – Nocomis micropogon
Polyline – GIS File for Line Geometry
Polygon – GIS File for Geometrical Shapes (Polygons)
R. bowersi – Rhinichthys bowersi
R. platyrhynchus – Rhinichthys platyrhynchus
RBP – Rapid Bioassessment Protocol
R. cataractae – Rhinichthys cataractae
Spatial Join – GIS Technique to Spatially Link Data
US EPA – United States Environmental Protection Agency
WV DNR – West Virginia Department of Natural Resources

INTRODUCTION

Since the late 19th century, the exploitation of oil, gas, timber, and mineral resources in Appalachia has been associated with the production of an extensive transportation system and with a concurrent decline in the environmental quality of aquatic systems. Although road and railway construction and use have occurred concurrent with environmental degradation, the extent to which environmental degradation can be directly attributed to transportation and not to other anthropogenic events, remains unclear.

Appalachian systems that may have been negatively impacted by transportation systems have also been exposed to potentially harmful effects of acid rain, acid rock drainage from abandoned mine lands, eutrophic effects of untreated wastes, stream channelization, and siltation from mining and agriculture. To date, many investigators have documented an overall decline in environmental quality and biotic diversity but have not carefully related these losses to anthropogenic events directly linked to transportation systems.

The Cheat minnow, *Rhinichthys bowersi*, is a rare fish that has been reported almost exclusively from streams in the Monongahela River system of West Virginia and Pennsylvania. Since its initial discovery in 1908, fewer than 200 specimens have been collected from fewer than 50 sites. Investigators have variously described the Cheat minnow as either a valid, self-perpetuating species or as a hybrid between two widely distributed species, longnose dace, *R cataractae,* and river chub, *Nocomis micropogon.*

The purpose of the following project is to determine whether the Cheat Minnow is rare Appalachian species threatened by transportation development in West Virginia or whether the fish is a rare hybrid that is possibly produced in response to environmental disturbances. A second goal of the project is to determine whether environmental disturbances in the habitat of the Cheat Minnow have resulted from transportation development and use.

These goals will be accomplished by first, using mitochondrial DNA base sequences in order to determine whether the Cheat Minnow is a valid species or a hybrid. Second, the project will locate where the Cheat Minnow is found using GPS technology and then map the occurrences of the Cheat Minnow with an GIS interactive mapping system in order to see if these locations are proximate to transportation systems. Third, the project will assess those locations to determine whether there is a relationship between anthropogenic stress and occurrence of the Cheat Minnow.

The long-term goal of the project is the construction of an infrastructure using GIS, ArcView and ER Mapper that will allow queries of a database of rare, endangered or endemic species which links known biotic and abiotic indicators of environmental quality and the proximity of transportation systems.

BACKGROUND

In West Virginia, the Heritage Program, a division of the WV Department of Natural Resources (DNR), supports and regulates the study of rare and threatened species. Because the Cheat minnow is rare in nature and has a questionable origin, it has been a species of special concern of the Heritage Program. If *R. bowersi* is established to be a valid species, West Virginia DNR state officials have recommended that the species be considered as possibly threatened under the Endangered Species Act (Dan Cincotta, WV DNR, personal communication).

The status of the Cheat minnow is currently controversial. Goodfellow et al. (1984) report that the Cheat minnow has unique genetic characters, is reproductively isolated from other minnows, has a limited distribution, and should be considered a valid species. They argue that if the Cheat minnow is a hybrid between longnose dace and river chub, then it should be produced wherever these species are found together. Because these two species are syrnpatric over much of eastern and central United States, the Cheat minnow should be commonly found throughout the region of sympatry. It is not, however, a counter argument has been proposed that longnose dace and river chubs hybridize primarily in the Monongahela River system, because of the extreme environmental degradation of West Virginia streams. The relationship between environmental degradation and fish hybridization in tributaries of the Monongahela River has not been thoroughly studied.

If *R. bowersi* is a hybrid, is produced in areas of environmental degradation, and this environmental degradation is associated with the development or maintenance of transportation systems, then present transportation activities in the Monongahela system could seriously affect it. The proposed path of Corridor H passes across a number of *R. bowersi* populations in the middle range of the Cheat River near Parsons, WV. If current litigation by some environmental groups results in existing highway enhancement, instead of Corridor H development, then a number of highways will be expanded that either cross *R. bowersi* habitat or parallel it. To date, a thorough mapping of *R. bowersi* locations and the proximity of existing populations of *R. bowersi* to railways and roads have not been determined.

Either the construction of Corridor H or the enhancement. of existing highways could severely impact *R. bowersi* in either of two ways. If *R. bowersi* is a valid, self-perpetuating species, then any further environmental degradation from any activity obviously threatens it. However, if *R. bowersi* is in fact a hybrid that is produced consequent to human activity, then the unique situation may exist where human activity actually results in its production.

The impact of transportation systems and associated environmental degradation on Appalachian fishes has been little studied. The historic and present extraction of mineral and woodland resources has resulted in often catastrophic alterations of fish habitat. The Cheat minnow, a rare Appalachian fish, has a unique relationship to stream environmental quality and anthropogenic effects associated with land and water use. This fish serves as an effective indicator of the relationship between environmental disturbance, quality of habitat, and distribution of this rare minnow.

The Cheat minnow, *R. bowersi,* is described as either a valid species or hybrid longnose dace and river chub. Previous investigators have hypothesized that environmental disturbance has increased the likelihood of hybridization, resulting in the Cheat minnow.

The fish was discovered during an investigation initiated because of a decline in stream quality, specifically fish biomass. The Monongahela River system, specifically the Cheat River system, is the primary habitat for the Cheat minnow and is also an area marked by historic and present environmental impacts. Due to the limited geographic area of the Cheat minnow and the extensive environmental degradation thereof, *R. bowersi* is often assumed to be a product of anthropogenic stress on the Cheat River system.

Description of Cheat Minnow

The Cheat minnow is a rare fish found almost exclusively in the streams of the Monongahela River system of West Virginia and Pennsylvania. Fewer than 200 specimens of *R. bowersi* have been collected from less than 50 sites since its discovery in 1908. The minnow is described as either a valid species or hybrid between longnose dace, *R. cataractae,* and river chub, *N. micropogon*. The West Virginia Department of Natural Resources (WV DNR) Heritage Program, which supports and regulates the study of rare and threatened species, has designated the Cheat minnow as a species of concern due to the rarity of specimen collections and the undefined origin of the fish. If *R. bowersi* is determined to be a valid species, the WV DNR recommends the fish be considered threatened under the Endangered Species Act (Dan Cincotta, WV DNR, pers. comm. 2002).

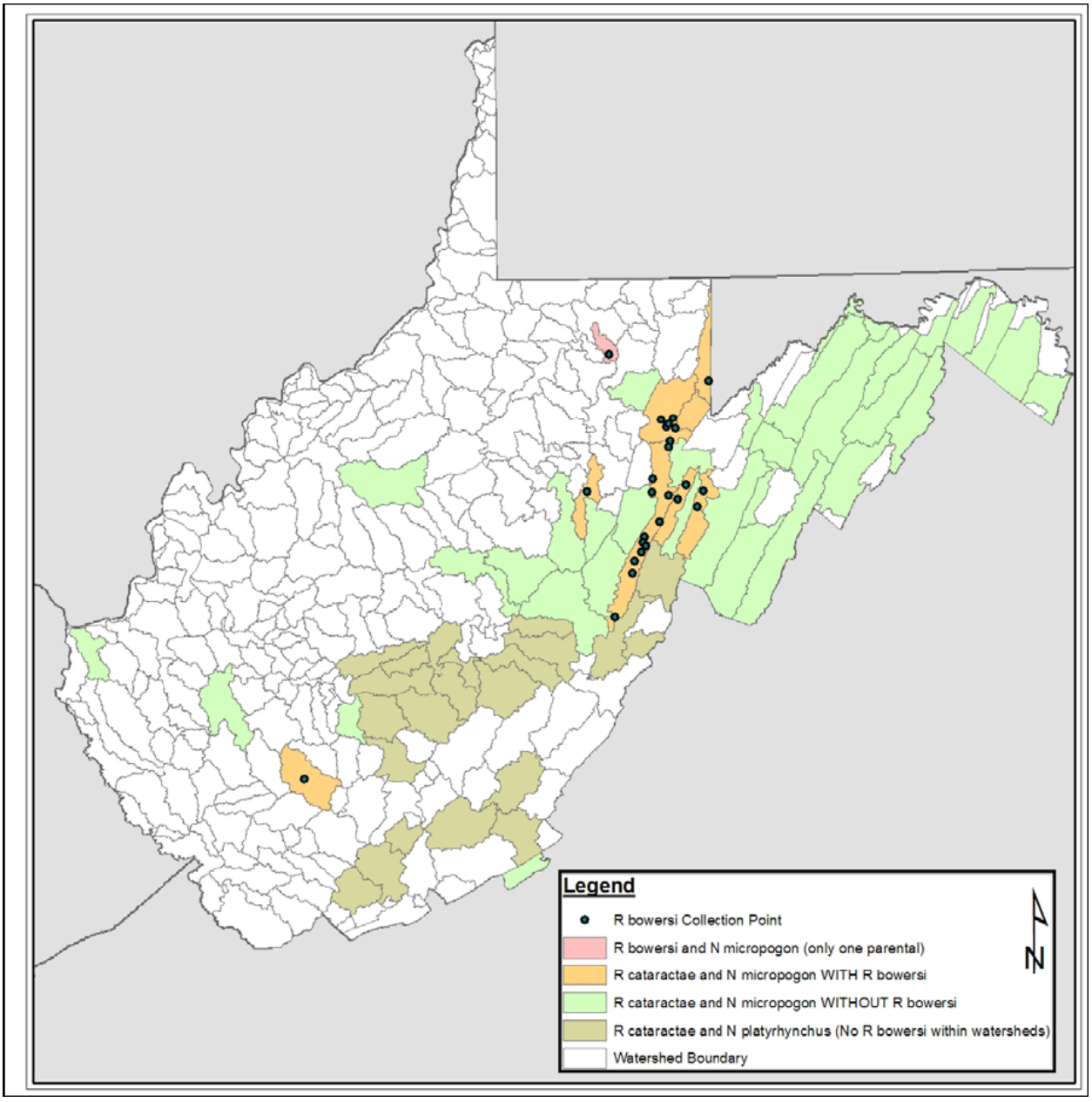
Habitat and Ecology of the Cheat Minnow

Edmund Lee Goldsborough and H. Walton Clark first described the Cheat minnow as a species and designated it as *R. bowersi* in 1908 (Goodfellow et al., 1984). A decline in aquatic life was noted in the streams of northeastern West Virginia, specifically the Monongahela and upper Potomac basins, initiating research in the early 1900's. Prior to this time, streams in the region were frequented and well known for abundant fish populations. After collections were made from the Cheat Bridge area of Shaver's Fork in 1940, E. C. Raney redescribed the minnow as a hybrid *N. micropogon* and *R. cataractae* (Raney, 1947). Because *R. bowersi* had unique genetic characters, reproductive isolation, and limited distribution, Goodfellow et al. (1984) argued for the consideration of *R. bowersi* as a valid species.

Mitochondrial DNA studies indicate the minnow is the result of hybridization of *R. cataractae* and another cyprinid species, most likely *N. micropogon* (Gladwell, 2002). The status of *R. bowersi* as a species continues to be a topic of debate. Hybridization is a common contributing factor to the demise of many native species and is a major concern for the biodiversity of the freshwaters of North America (Perry et al., 2002). Freshwater fishes hybridize in nature, often a result of degradation of the environment. The frequency of hybridization increases as populations are forced into close proximity and is inversely correlated with species diversity. Hybridization is often the result of the intergradation of the environment, as the habitats of historically allopatric species are rendered intermediate (Hubbs, 1955).

R. bowersi occupies an intermediate habitat to that of the probable parental species. *N. micropogon* is a widely distributed species, typically found in riffles, runs, or pools comprised of a rocky substrate. *R. cataractae* is typically found in small to medium, swift, rocky streams with high gradients (Stauffer et al., 1995). Collections of *R. bowersi* have historically occurred in watersheds where these two species coincide:

Figure 1. Fishes of West Virginia Collection Locations by Subwatershed



Although *R. bowersi* is typically found in watersheds with *R. cataractae* and *N. micropogon*, it has not been recorded in watersheds with the congener of *N. micropogon*, *N. platyrhynchus*. *N. platyrhynchus* is thought to be genetically identical to *N. micropogon*, but isolated geographically. For this reason, *R. cataractae* is assumed to have the same likelihood of hybridization with *N. platyrhynchus* as with *N. cataractae*. However, *R. bowersi* collection records do not indicate that this relationship occurs. Because the Cheat minnow is not found in watersheds with *R. cataractae* and *N. platyrhynchus*, the argument has been made that *R. bowersi* is a valid species.

The morphology of the Cheat minnow is also intermediate to that of the parental species. *R. cataractae* has smaller, more numerous scales than *N. micropogon*. Similar to other hybrid cyprinids, *R. bowersi* scales are intermediate in size and number to that of the parental species. As is consistent with other cyprinid hybrids, *R. bowersi* more closely resembles one of the parental species, *N. micropogon*. Lower jaw and scale structures of *R. bowersi* closely resemble that of *N. micropogon*, while most other characteristics are intermediary:

Figure 2. Nocomis micropogon, Rhinichthys bowersi, and Rhinichthys cataractae (top to bottom)

PROBLEM STATEMENT

We are presently examining the mitochondrial DNA of *R. bowersi* and through interpretation of preliminary results, believe that it is a hybrid. We propose that it is produced when isolating mechanisms fail and individuals of *R. cataractae* and *N. micropogon* spawn simultaneously over the same "nests" (nest association). We also propose that the "unique" distribution of *R. bowersi* primarily in the Monongahela drainage results from the extreme environmental degradation of streams in this area, alternation in reproductive habitat, and consequent failure of normal isolating mechanisms (see Poly and Sabaj, 1998). We also propose that the development and use of transportation systems facilitate this process.

To date, no investigator has carefully examined the relationship between *R. bowersi* and environmental degradation. To determine whether a rare fish, *R. bowersi,* is produced in response to environmental degradation and this environmental degradation is associated with the development and maintenance of transportation systems, we propose a study that will accomplish four goals:

1. Use GPS technology to locate precisely all known populations of *R bowersi.*
2. Use ArcView and ER Mapper software to develop a GIS system that locates all known populations of *R bowersi* and determines their proximity to all historic and current railway and road sites.
3. Use basic water quality analysis, stream structure analysis, and toxicity testing to determine present environmental quality of *R. bowersi* and whether stresses to current populations of *R. bowersi* are most likely attributed to transportation systems or other sources of anthropogenic stress.
4. Use fish and benthic bioindicators to compare environmental quality between areas that contain *R cataractae, N micropogon,* and *R bowersi* and control areas that contain *R cataractae* and *N micropogon* but lack *R. bowersi.*

In this study, standard measures of environmental quality are used within geospatial systems to relate the distribution of *R. bowersi* to environmental disturbance generally and to impacts from transportation systems specifically. This system consists of an analysis of relative environmental factors (concentration of stream crossings per sub-watershed, benthic macro-invertebrate populations, substrate composition, stream sinuosity, and width to depth ratios) linked within a Geographic Information System (GIS) that relates stream quality, stream structure, and aquatic diversity to environmental disturbance associated with transportation systems. The system will produce a data model of transportation systems correlated to discriminating environmental factors in the Cheat minnow habitat.

HISTORY OF RELEVANT RESEARCH

From 1890 until 1975, 14 specimens of *R. bowersi,* also known as the Cheat minnow, were identified from nine collections in West Virginia. Edmund Lee Goldsborough and H. Walton Clark first collected specimens of *R. bowersi* from Dry Fork, Harman, West Virginia; and Shavers Fork, Cheat River, at Cheat Bridge, West Virginia. From these collections, they first described this nominal species in 1908 (Goodfellow, 1984). Their research was spurred by a noticeable decline of aquatic life, especially fishes, in the streams of northeastern West Virginia,

specifically in the Monongahela and upper Potomac basins. Until this time, streams in this region were well known and visited because of the abundant fish life found in them. Investigations by Goldsborough and Clark indicate that the streams were being impacted by logging and mining operations during the industrial development of railroad systems, thus injuring and nearly destroying the aquatic life that lived in these streams (Goldsborough and Clark, 1929).

In 1940, E.C. Raney (1940a) collected specimens of the Cheat minnow from the Cheat Bridge area of Shavers Fork and re-described it as a hybrid *N. micropogon* and *R. cataractae* (Raney, 1947). From 1975 until 1976, the West Virginia Department of Natural Resources collected 15 specimens of *R. bowersi* (Dan Cincotta, personal communication). In 1976, Stauffer collected 22 additional specimens from Shavers Fork (Stauffer, 1979), then collected and released three others from Tygart Valley River, West Virginia, and one from the Youghiogheny River, Pennsylvania. Hendricks (1980) reported one specimen from the Youghiogheny River, Maryland; two from the Youghiogheny River, Pennsylvania; and four from Snowy Creek, a tributary of the Youghiogheny River in West Virginia. Two additional specimens were collected in the 1990s from White Day Creek of the Monongahela River by the West Virginia Department of Natural Resources. As of 1984, 145 specimens of the cheat minnow were known, all of which were caught from the Monongahela River, except for two collections from Lake Erie in 1977 (Goodfellow, et al., 1984). Also, there is record of collection of a *N. platyrhynchus* x *R. cataractae* that also could be a *R. bowersi* in that *N. platyrhynchus* and *N. micropogon* are electrophoretically identical (Esmond, et. al., 1981).

Since the cheat minnow's description by Raney in 1940, its taxonomic status has been disputed in a series of publications, and the state of West Virginia has recently designated *R. bowersi* as a candidate for protection under the Endangered Species Act (Dan Cincotta, WVDNR, personal communication). In this study, mtDNA base pair polymorphism is analyzed to determine the status of *R. bowersi* as an introgressive hybrid. Mitochondrial DNA has become a powerful tool in evolutionary studies of animals (Wilson et al., 1985; Moritz et. al., 1987; Avise et. al., 1987). Mitochondrial DNA is presently used as a phylogenetic marker that is useful because of its maternal inheritance, haploidy, lack of introns, and predictable rate of evolution (Moritz, et. al., 1987; Moore, 1995). The mtDNA genome (Figure 1) comprises two ribosomal RNA (rRNA), 22 transfer RNA (tRNA) and 13 protein genes that code for enzymes functioning in electron transport or ATP synthesis (Anderson et. al., 1981; Chomyn et. al., 1986; Digby et. al., 1992). Ribosomal 12s RNA was chosen for this study because it is relatively conserved among taxa and its rate of evolution is predictive of the mtDNA genome (Simon, et. al., 1990). It has been useful in phylogenetic studies discriminating families (Simon and Mayden, 1998; Parkinson, 1999) and congeneric species (Gillespie, et. al., 1994).

Habitat, Distribution, and Reproduction

The morphological and meristic characteristics of *R. bowersi* are similar to and intermediate of possible parentals *R. cataractae* and *N. micropogon*. *N. micropogon* has a long and broad body that is somewhat round. It has a very deep caudal peduncle and has tubercles on the top portion of its head. Its mouth is slightly subterminal and almost horizontal, and it has one row of pharyngeal teeth. The upper lip protrudes beyond the lower lip, and barbels can be found on each side of the mouth in the groove formed where the upper and lower jaws connect. The head of *N. micropogon* is large and triangular with a long, bluntly rounded snout. The eye is located

dorsolaterally and is very small, its diameter contained several times in the snout length. The body shape of *R. cataractae* is long and cylindrical. It is a rather large minnow with a flat head on the ventral surface. Its mouth is inferior, horizontal, and small and extends up to the posterior nostril. It includes a frenum, but is not protractile. A small, thin barbel is present at the posterior end of the maxillary. The eye is small in diameter, and the snout is long and fleshy and projects past the mouth (Stauffer, et al., West Virginia Fisheries; Goodfellow, et al., 1984). Some characteristics that distinguish *R. bowersi* from *N. micropogon* and *R. cataractae* are listed in Table 1 (Stauffer et. al., Fishes of West Virginia).

Table 1. Species Characteristics

	R. bowersi	N. micropogon	R. cataractae
Lateral lines	44-55	38-43	57-70
Pharyngeal tooth formula	Typically 1, 4-4, 1	4-4	2, 4-4, 2
Scales	Basilateral corners; lack basal radii	Basilateral corners; lack basal radii	Basil radii
Lower jaw	Dentary elements meet at acute angle	Dentary elements meet at acute angle	Dentary elements nearly form straight line at union

Most of the identifying characteristics of *R. bowersi* are between the two intermediate suspected parental species, but do not overlap. *N. micropogon* has few but large scales, whereas *R. cataractae* has many small scales. It also has been observed in other minnow hybrids that the hybrid would be an intermediate in the size and number of scales present, which describes *R. bowersi*. Also, other cyprinid hybrids have proven to more closely resemble one parental specie more than another. This is the case with *R. bowersi*, as shown in the table above (Stauffer et. al., Fishes of West Virginia).

For the most part, *R. bowersi* has been found in the Cheat drainage of West Virginia, with the exception of four collected fish, one from Youghiogheny River; Pennsylvania; one from Youghiogheny River at Hoyes Run, Maryland; another was found Youghiogheny River at Connellsville, Pennsylvania; the fourth was found in a Lake Erie drainage near the Ohio River system. The fish is found in deep runs over rubble substrate (Stauffer et. al., Fishes of West Virginia).

N. micropogon is a widely distributed species occurring from Susquehanna River drainage in New York to the James River drainage in Virginia, with a few reports in southern rivers. It also has been found in the Great Lakes and Ohio River basin. It is found throughout the state of West Virginia, although absent from the New River drainage. It is found in riffles, runs, or pools that have a rocky substrate (Stauffer, et al., Fishes of West Virginia). *R. cataractae* is usually found in the rocky bottoms of small to medium size streams characterized by swift waters and high gradients (Stauffer et al., Fishes of West Virginia). It is widely distributed throughout North America, including parts of northern Mexico. It is most abundant from the Great Lakes to the Appalachians and to the Rocky Mountains. In West Virginia, it is found in the Atlantic Slope,

New River, Monongahela River, and the lower part of the Kanawha River drainage, as well as Twelvepole Creek (Stauffer et al., Fishes of West Virginia).

The breeding patterns of *N. micropogon* were also studied in Mill Creek, a Michigan tributary to the Huron River. Nest building and spawning of *N. micropogon* occurred from mid-April through late May in water temperatures of 15 ^{o} – 20.5^{o} C. Nests were found in waters of one to two feet in depth with a moderate current and a gravel bottom. The site is chosen by males that dig a pit by removing gravel from the site. After spawning occurs, the male covers the nest with a dome-shaped pile of gravel. The pit is usually about one foot wide with a center of three to six inches in depth. The completed dome of gravel has an average diameter of four feet. A male will invest approximately 20-30 hours in building the nesting site. The peak time for spawning of *R. cataractae*, as observed by Bartnik (1970), occurs in mid-May. This fish spawns over cobble and boulders in swift water with temperatures of about 16^{o}C.

R. bowersi has been hypothesized to be a distinct species of a hybrid origin. Although little is known about the spawning behavior of this fish, Stauffer et. al., (1997) inferred that it was a fertile species because the females had mature eggs, and males had well-developed testis. It is still unknown whether or not gametes are viable. Several unsuccessful attempts have been made to reproduce spawning in a lab setting (Goodfellow et. al., 1984).

In 1940, Raney (1940b) concluded that *N. micropogon* and *R. cataractae* have the potential to hybridize in nature when he found that *R. cataractae* spawned over the nest of *N. micropogon*, referred to as nest association. According to Jenkins and Burkhead (1994), this type of spawning is common in North American minnows and most likely accounts for most of the observed hybrid combinations. Raney's conclusion was further validated by Cooper's recordings in 1980, stating that eggs of *R. cataractae* were found in many of the *N. micropogon* nests, but simultaneous spawning was not observed. However, when he studied the development of these eggs, he concluded that indeed spawning occurred at about the same time (Poly, 1998).

The habitat of the Cheat minnow has experienced severe environmental degradation. Early investigations indicated logging and mining operations were impacting the streams, specifically having a severe impact on aquatic life (Goldsborough and Clark, 1908). Significant environmental disturbances such as these lead to changes in ecological community in a process known as succession. Alterations in biomass, productivity, diversity, and niche breadth are factors that indicate succession (Connell, 1977). These changes relate to the level of environmental disturbance such that the highest diversity is found in areas of intermediate disturbance. Species diversity is inversely correlated to hybridization, in that hybridization is more likely to occur in areas of low species diversity (Hubbs, 1955).

Connell's intermediate-disturbance hypothesis states that diversity is a nonequilibrium state and will decrease within a community if disturbance decreases (Connell, 1978). Areas of low disturbance are more favorable to competitive species which may eliminate other species by garnering and maintaining resources. Highly disturbed areas are typically more favorable to colonizing species, where growth and/or dispersal rates are high. At intermediate levels of disturbance, competitive species may locally eliminate colonizing species, while colonizing

species are able to take advantage of newly available resources in recently disturbed areas (Miller, 1982).

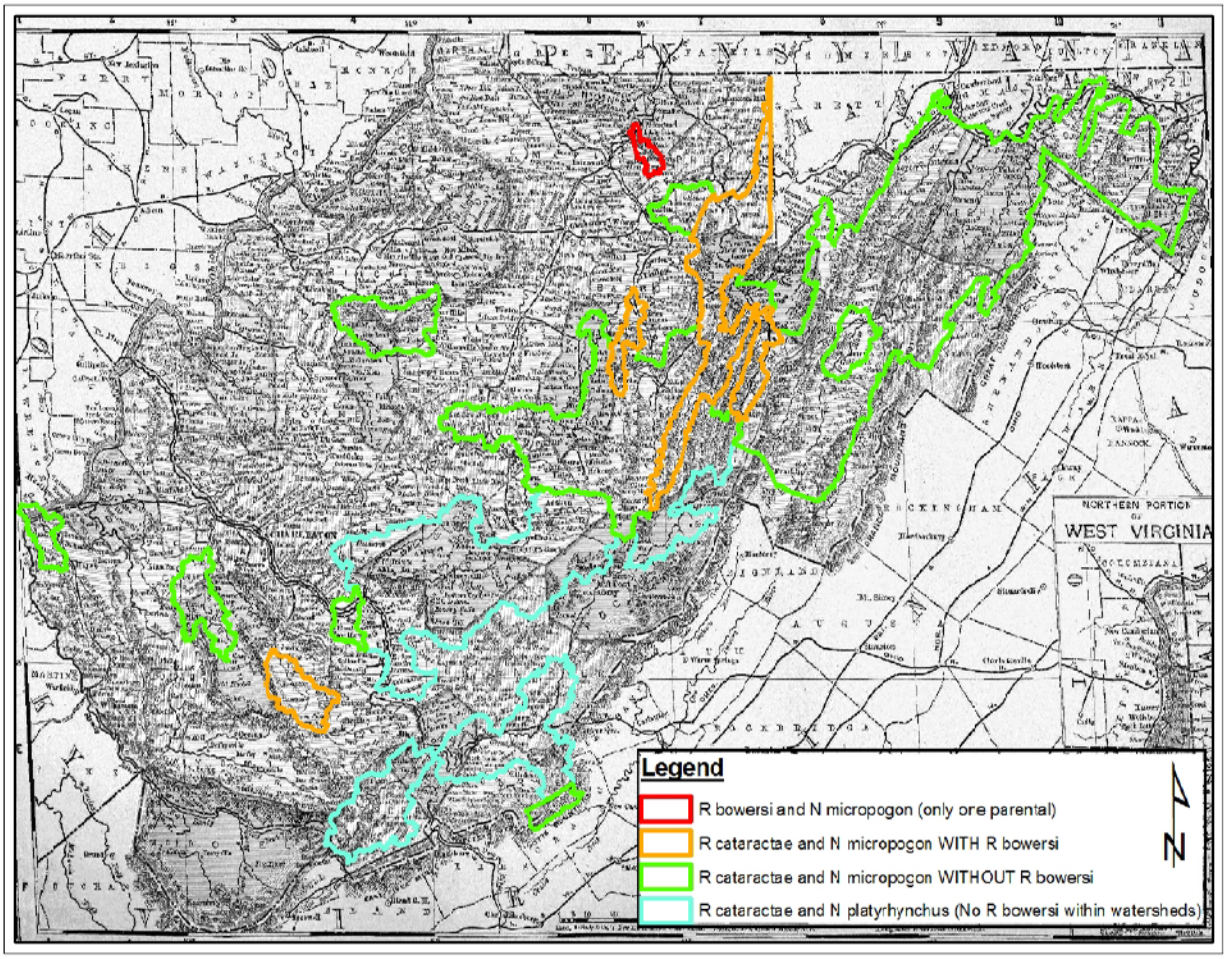
The size of disturbance plays an important role in community succession by changing the time frame for within-patch dynamics. Larger disturbances tend to have longer persistence times, favoring colonizing species by providing a longer time for reproduction. Smaller areas of disturbance support competitive species by providing greater access to new resources. Both small and large patches of disturbance peak in diversity at an intermediate rate, though large disturbances peak at a lower rate due to differences in species response (Miller, 1982).

The development of transportation systems has degraded environmental quality in a number of watersheds generally and negatively impacted fish habitat specifically. The streams of the Cheat minnow occupy an area marked by environmental disturbance dating back to mineral and timber extractions in the 1800's. The minnow was initially identified and designated *R. bowersi* by Edmund Lee Goldsborough and H. Walton Clark in 1908 (Goodfellow et al., 1984). These investigations, sparked by reports of stream degradation, indicated logging and mining operations were impacting the streams, specifically the aquatic life that had historically been abundant (Goldsborough and Clark, 1908).

Roads are acknowledged as indicators of loss of ecological health (Trombulak, 2000). Described as a significant and the most widespread modification of natural landscape in the past century (Trombulak, 2000). Roads effect ecology in seven primary ways: mortality from road construction, mortality from collision with vehicles, modification of animal behavior, alteration of the physical environment, alteration of the chemical environment, spread of exotics, and an increased use of areas by humans. At a minimum, physical characteristics of environment such as soil density, temperature, soil water content, light, dust, surface-water flow, patterns of run-off, and sedimentation are altered (Trombulak, 2000). High concentrations of suspended solids may directly kill aquatic organisms and impair aquatic productivity. The effects of roadways propagate many kilometers upstream and downstream of road crossings with the effects lasting decades after road use is discontinued (Richardson, 1975). Ground-water flow paths are intercepted by roads, diverting flows to surface-water systems at road crossings. These effects are more often noted at smaller streams, creating and destroying wetlands throughout the process (Wemple et al., 1996).

Transportation systems are a common link between environmental impacts on the Cheat minnow habitat. Both mineral and timber extraction required an extensive network of roads throughout the watersheds occupied by the Cheat minnow. Figure 3 depicts classified watershed areas overlaid on a map of West Virginia produced in 1895 (Rand, 1895):

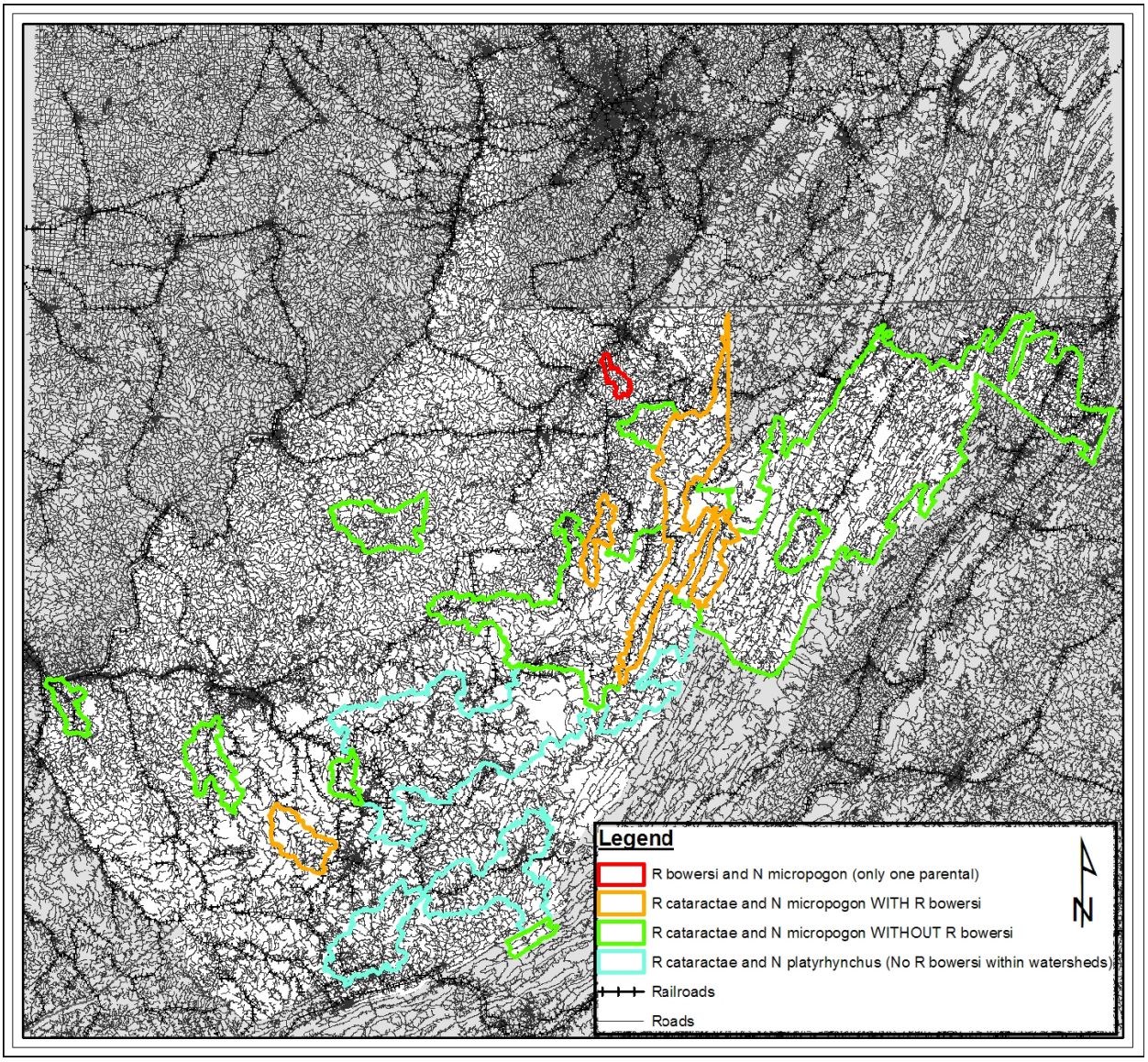
Figure 3. West Virginia, 1895, Relative to Cheat Minnow Watersheds



Source: Rand, McNally and Co. 1895. New 11x14 Map of West Virginia.

The effects of these transportation systems have carried through decades with lasting impacts. Many roadways have been expanded and extended, increasing the impact on the environment. Figure 4 depicts current roadways as singular lines in order to demonstrate relative densities within each watershed category.

Figure 4. Current Transportation Systems Relative to Cheat Minnow Watersheds



The Cheat River system covers most records of *R. bowersi* and has been subjected to an extended history of fish habitat degradation. The habitat of the Cheat minnow covers an area seriously impacted by mineral and timber extraction. As a result, the area is highly divided by roadways and therefore assumed to have significant environmental impacts (Trombulak, 2000). The relationship between environmental disturbance and the distribution of *R. bowersi* is poorly understood. The Cheat minnow has been impacted by environmental degradation and may well have been impacted by the development of transportation systems.

In this study, we will initially map and assess environmental quality from approximately 40 sites. These will include one site on each of the Potomac and Greenbrier Rivers that contain *R. cataractae* and *Nocomis* species but lack *R. bowersi* and up to 36 sites that have been reported with *R. bowersi.* These sites are restricted to major tributaries of the Ohio River in Pennsylvania and West Virginia.

The final report will include a database containing all water quality and biotic data, distribution of bioindicator species throughout all sites, a GIS system that relates all biotic and abiotic data to historic and current transportation systems. Final report will also clearly analyze the relationship among the frequency of fish hybridization, deterioration of aquatic environments, and the development of transportation systems. Final report will also analyze taxonomic status of R. bowersi relative to ongoing mitochondrial DNA analysis, which is in part funded by WV DNR and presently underway in the Biotechnology Unit at Marshall University.

The collecting of fish was done by the use of a Smith-Root SR12 Barge with a 7.5 gpp electrofisher (Appendix Figure 2). After inserting the electrofisher into a body of water, the power was adjusted to archive an output of 2.5 – 3.0 amperes. We also used a Honda generator that was used as a backpack shocker (Appendix Figure 3). The electrofisher was maneuvered in a zigzag pattern across the stream with two or three netters using the backpack unit, and three to five netters used the barge [electrofisher]. Quick identification on each fish was performed as they were collected. Only the fish species needed were placed in a live well unit and were kept alive until the river sweep was complete. Upon completion, all fish collected were identified once more and separated by species, then put on dry ice for holding until reaching a -20°C freezer. All *R. bowersi* were collected and identified by Dan Cincotta of the West Virginia Department of Natural Resources' Fish and Wildlife Division. DNA tissue was then extracted from the fish, and amplification and sequencing were performed. Three *R. bowersi* specimens were collected from Shavers Fork River (Appendix Figure 4).

The genomic DNA was extracted from muscle tissue by the use of Qiagen/Qiamp tissue kit (catalogue #29304), following the manufacturer's instructions with slight modifications. The total 12s gene was amplified using the Klentaq LA DNA polymerase (Sigma) under conditions recommended by the manufacturer. Also 1M of Betaine (Sigma) was added to the reaction to help lower the melting point of the DNA. Amplification primers PHEa and 16sd (Table 2 and Figure 5) were used to obtain the 12s gene. The amplification was from genomic DNA in total volume of 50ul, which was performed on a GeneAmp 9700 PE Applied Biosystems. The PCR profile was denatured at 94°C for a five-minute cycle; 94°C 45-second denaturation; 55°C one minute annealing; 72oC one minute extension for 30 cycles followed by 72°C extension for three minutes for one cycle and 4°C for infinity. Amplification was checked on 1% gel of Sea Kem GTG agarose (FMC) (Appendix Figure 6).

All PCR products with amplification of one band were cleaned with Qiagen PCR cleanup following the manufacturer's instructions. One bowersi specimen had been preserved in ethanol and formaldehyde at different stages of its preservation, producing multiple bands in amplification. Also, another bowersi specimen and two *N. micropogon* specimens had multiple bands. The correct size was cut out of the 1% gel GTG low agarose (FMC) and was gel purified using a Qiagen gel extraction kit (Qiagen). All samples were cloned into Clonetech Advantage PCR cloning kit. A single colony was chosen and was cultured for 24 hours; then a plasmid mini-prep was performed. A restriction digest (Appendix Figure 7) was performed using EcoRI to check for correct insert (New England Biolabs).

All plasmids with the correct inserts were sequenced in five steps using universal primer T7 and sequencing primers PHEa, 12sa, 12sd, and 12sc (Table 2). Automative sequencing, BigDye Chemistry (Perkin-Elmer Applied Biosystems) was performed on an ABI 377 sequencer (Appendix Figure 8).

The software program Sequencer was used to align all five chromatographs from each species, which, once aligned, would give a complete 12s mtDNA sequence. The sequence would then be aligned with all nine species of fish to show the mismatch bases. Then the sequencing data would be used to develop 10 phylogenetic trees. First, it would be taken into Clustalx (Kimura, 1980; Higgins, 1989). Sequences aligned would be bootstrapped to create multiple data sets (Felsenstein, 1985), and others would be nonboostrapped. Then they would be run through three Distance Matrix programs: Neighbor Joining, FITCH, and KITSCH (Felsenstein, 1981a, 1981b, 1982, 1983, 1984, 1988; Fitsch and Margoliash, 1967; Nei, 1987; Saitou, 1987), which would provide a phylogram. Also from the aligned sequence, DNA pairs would be looked at using TreeView (Page, 1996), which would show a cladogram.

Table 2. Primers Used in Amplifications and Sequencing

Primers used in amplification and sequencing			
Name	**Sequence (5'- 3')**	**Strand**	**Reference**
12Sa	AAACTGGGATTAGATACCCCACTA	L	Kocher et al.1989
12Sc	GGAAAGAAATGGGCTACA	L	Simons et al. 1997
12Sd	GGGTTGGTAAATCTCGTGC	H	Titus & Larson, 1995
PHEa	AAAGCACAGCACTGAAGATG	L	Titus & Frost, 1996

METHODS

Habitat Mapping

The morphology of each study site will be characterized through surveying with a total station theodolite. A GPS unit will be used to determine the geo-reference position where the theodolite is to be setup and to establish the direction of UTM north. Each study site is planned to be 100 meters in length along the stream course. A series of points will be 'shot in' that will be used to characterize the morphology of the stream channel. The survey will consist of transects across the stream at intervals of 10 meters. The distance and angle measurements taken in the field are digitally stored in the theodolite for later downloading into a computer where the data are subsequently reduced into three dimensional, geo-referenced, coordinates. The coordinates can then be used to create topographic profiles and maps of the study sites in the software SURFER and ARCVIEW. Pertinent features of the stream channel can also be overlain on the profiles and maps. Three dimensional models of the study sites can also be generated by the above mentioned programs.

Assessment of Stream Geomorphology and Stream Bed Structure

In a recent study of the New/Kanawha River Systems, investigators from the United States Geological Service (USGS) found overall environmental quality to be highly correlated with bed and bank material characterization (unpublished data from Douglas Chambers, USGS). The most efficient technique for characterizing streambeds is the Wolman Pebble Count (1954). Using this method, particles are first classified using the Wentworth size scale, in which particle size doubles with the addition of each class. This method involves the following procedures:

- A reach of stream is selected that includes the transect that will be sampled. Stream reach must include riffles and pools in a proportion consistent with the total study site. In this case, the study site will be a .1 kIn section of stream encompassing the previously reported site for *R. bowersi.*
- A transect will be initiated from a randomly chosen site within the reach, at a bankfull elevation.
- The intermediate axis will be measured for each embedded particles found along the transect. Process will be continued until the requisite number of particles (100 or more) is measured.
- Data are plotted by size class and frequency.

Stream Banks will be characterized by sieve analysis and erosion pins-

- Sieve analysis will be conducted on 25 lb. soil samples according to methods in U. S. Soil Conservation Service, Soil Survey Handbook (1982).
- Bank Erosion Pins will be used along repeated cross-section and longitudinal stream sections. Erosion will be quantified as change in pin exposure over time.

Assessment of Benthic Biodiversity

The following bioindicators will be used to assess the level of benthic species richness and diversity:

- Sieve analysis will be conducted on 25 lb. soil samples according to methods in U. S. Soil Conservation Service, Soil Survey Handbook (1982).
- Taxa Richness is the total number of taxonomic groups (mostly orders) based on gross examination. This is a good overall indicator of stream quality depending on type of stream and its location.
- EPT Richness is the number of taxa from each of the Insect Orders: Ephemeroptera. Plecoptera and Trichoptera. These orders are generally regarded as sensitive to water quality changes. Identification down to the level of family gives a better index of water quality
- Organism Density Per Sample is calculated as the density of each replicate = [(Total # of squares in grid) x (Total # organisms picked)]/(Total # of squares picked). This parameter gives an indicator of density of organisms in a stream. It is best used to make comparisons
- Percent Composition of the Major Groups is calculated as the % Composition = (Average density by group)/(Total average density of the sample) and is calculated for the following taxa; Order Ephemeroptera, Order Plecoptera, Order Trichoptera, Order Diptera (Family Chironomidae, Family Tipulidae, Other Families), Order Odonata, Order

Megaloptera, Order Coleoptera, Order Amphipoda, Order Isopoda, Order Decapoda, Class Gastropoda, Class Pelecypoda, Phylum Annelida, Class Hirudinea.

- EPT to Chironimidae Ratio is the ratio of the total number of Ephemeroptera, Plecoptera, and Trichoptera to the total number of Chironimidae counted in a particular sample. This ratio gives an indication of pollution because members of the EPT orders are generally more sensitive to environmental degradation/alteration than the members of the Chironimidae family (order Diptera). A minimum ratio of 0.75 is used by the Vermont Department of Environmental Conservation as an indication of an unaltered stream habitat; a stream with an EPT/Chironomidae of below 0.75 is considered altered.
- Number of organisms per functional feeding group per square meter. Functional feeding group designations are Shredders, Collectors, Scrapers, and Predators.

Remote Sensing, GIS Mapping, and Database Development

The development of the GIS database will begin with the acquisition of recent aerial imagery. The imagery selected for this project will consist of digital orthophoto quads (DOQs). This format of imagery has been prepared by the United States Geological Survey (USGS) from data gathered by the National Aerial Photography Project (NAPP). The images have approximately the same aerial extent as one 7.5', 1 :24000 topographical map (there is a little extra area on the edges to aid in mosaicking two or more images). The NAPP imagery can be obtained in either black-and-white or color infrared (CIR) media, distributed via CD-ROMs. For purposes of this project, the CIR data will be obtained to perform multi-spectral analysis of the watershed areas surrounding the specific study sites. CIR imagery can be broken into three different band widths- one in the green region of the spectrum, one in the red and one in the near infrared (NIR). By utilizing the full spectrum of data in CIR images, one can study environmental phenomena not visible to the human eye or easily reached due to remote locations. Some examples of situations are easily and concisely studied with remote sensing/digital image processing techniques. These include acid mine drainage, sediment plumes in aquatic environments, discovering locations of point source pollution and analyzing vegetation types and condition.

Another aspect covered in the GIS will be generating up-to-date smaller scale maps of the study area. These maps will be generated using the CIR imagery. The nature of the imagery and their one-meter pixel resolutions will allow for the differentiation of several types and layers of data. The layers in these maps will include all transportation right-of-ways, hydrography and watersheds, any particular landmarks and areas of past or present activities that may affect the study area (i.e. construction, mines, timber operations, development). By having an accurate, up-to-date map of the study area, findings at the sites can be supplemented and correlated by occurrences in the watershed. Therefore, a full cause and effect relationship can be noted and studied.

The programs to be used will be ESRI ArcView, Spatial Analyst, 3D Analyst and ER Mapper. ArcView will be primarily used to perform mapping and databasing processes of generating the GIS. ArcView is very efficient at representing spatial data and the attributes of that data. ER Mapper will be the primary image processing and 3 dimensional modeling program to which the ArcView database will be dynamically linked. This linking ability allows small area or point data to be dynamically displayed it concert with the spatial model to provide corroborative quantitative evidence.

ER Mapper can be used to separate the bands of the CIR imagery, radiometrically enhance, mosaic geometrically corrected images and merge with digital elevation models for 3 dimensional analysis and modeling by being using the combination of spatial and statistical algorithms such as principle component analysis and clustering algorithms. This also allows for feature extraction of problem areas.

Environmental Assessment Site Selection

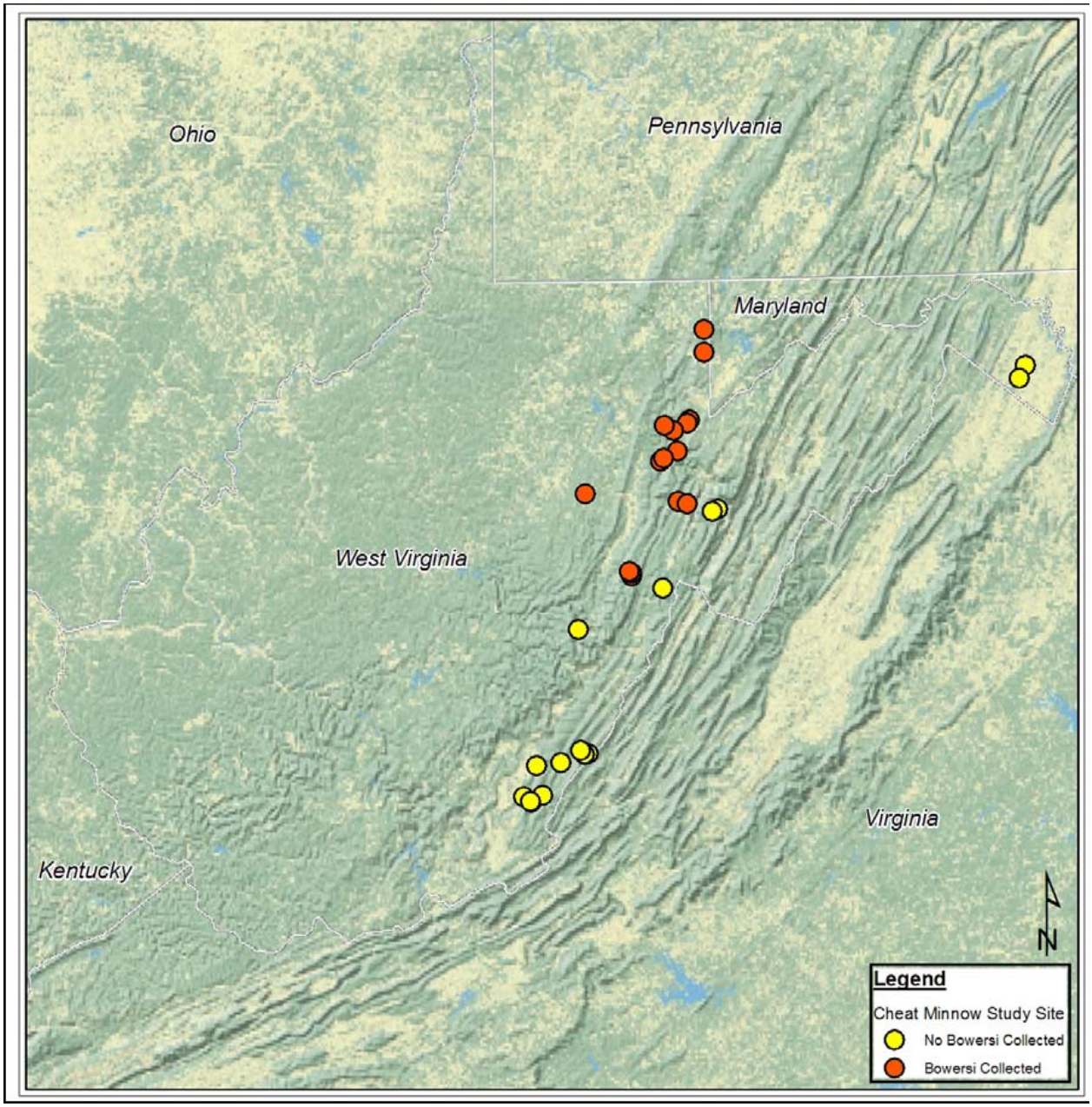
Sites were selected based on historical collections of *R. bowersi* and the potential parental species, *R. cataractae* and *N. micropogon* (or its congener, *N. platyrhynchus*). Control sites were selected from those displaying similar habitat characteristics and yielding the potential parental species without *R. bowersi*. U. S. Environmental Protection Agency (EPA) methods for Rapid Bioassessment Protocols: For Use in Streams and Wadeable Rivers were utilized to administer a preliminary habitat assessment to ensure similarities among control and study sites (Barbour et al., 1999). Parameters such as stream sinuosity, width-to-depth ratio, substrate composition, and surrounding vegetation were recorded in the field and compiled into an Access database. A Geographic Positioning System (GPS) was used to collect coordinates for each site. These coordinates were stored in the project database and used to map site locations into an Environmental Systems Research Institute (ESRI) GIS. Site location information is listed in Table 3 and shown in Figure 5 as a general site location map. A detailed site location map can be found in the Appendices.

Table 3. Environmental Assessment Site Locations

	Stream	Site	Latitude	Longitude
No collections of R. bowersi	Anthony Creek	below Whites run	4196857.93	569001.26
	East Fork of Greenbrier	below camp Pocahontas	4269569.13	611284.37
	Greenbrier River	at Caldwell	4182576.71	553646.51
	Greenbrier River	above Anthony	4195612.74	559018.22
	Harts Run	above mouth	4180395.01	556641.63
	Howard Creek	city park	4183211.63	561377.49
	Howard Creek	interstate intersection	4180686.28	556431.22
	Meadow Creek	upper	4200493.17	580358.22
	Meadow Creek	middle	4200263.17	578667.03
	Meadow Creek	lower	4201805.53	577111.14
	Opaquon Creek	at Leetown	4361981.00	762105.78
	Opaquon Creek	below Rt 51 bridge	4356703.48	759621.58
	Seneca Creek	at old camp ground	4302416.21	633805.62
	Seneca Creek	above mouth Whites Run	4301558.33	631705.29
	Slaty Fork	below bridge	4252102.91	576322.47
R. bowersi site	Black Fork	at Hambleton	4326365.02	617354.21
	Glady Fork	above Rt 33 bridge	4305590.68	617617.41
	Horseshoe Run	at Leadmine(upper)	4339474.21	622150.14
	Horseshoe Run	above Mikes Run	4338181.04	621258.21
	Horseshoe Run	above mouth (horseshoe1)	4334773.97	615715.81
	Laurel Fork	above Rt 33 bridge	4304697.64	621435.58
	Middle Fork	at Ellamore	4308688.22	579033.95
	Minear Run	above mouth	4337166.91	611915.63
	Pheasant Run	above mouth	4322433.75	610197.77
	Shavers Fork	above Cheat Bridge	4274637.64	598201.88
	Shavers Fork	below mouth Pheasant Run	4323566.96	611615.45
	Shavers Fork	above Red Run	4276058.65	598297.08
	Shavers Fork	below Red Run	4276633.31	597347.29
	Snowy Creek	above bridge	4376962.23	628132.85
	Snowy Creek	below bridge	4367369.31	628211.88

NOTE: Lat/Long listed in NAD 83, UTM Zone 17 (meters)

Figure 5. Environmental Assessment Sites



Data and Database Management

Data were recorded on field and laboratory sheets and compiled in a Microsoft Access database. Forms were constructed for general site information, benthic macroinvertebrate counts, Wolman pebble counts, EPA Rapid Bioassessment Protocol forms, and water chemistry data. Sites were assigned identification strings used to link all forms into a relational database. Complete datasets were exported to spreadsheets for calculations with Microsoft Excel.

Stream Sinuosity

Stream sinuosity, or the meander ratio of the stream, was calculated using georeferenced aerial photographs within the GIS. Stream sinuosity values are associated with aquatic diversity such that lower sinuosity values are typically noted in areas of lower aquatic diversity due to the relative decrease in diversity and quantities of habitat provided by the stream (Barbour et al. 1999). A measured stream distance of 1,000 meters was compared to the relative valley distance in order to determine the sinuosity upstream and downstream of the site location. The resulting ratios of stream to valley distance were averaged for each site to best represent the geology and stream morphology of the sub-watershed.

Width-to-Depth Ratios

Width-to-depth ratios were collected in the field as bankfull surface width and bankfull mean depth measurements in meters, according to Rosgen's stream classification protocol (1996). These dimensions were then transferred into the project database where width-to-depth ratios were calculated for each site. A high width-to-depth ratio would indicate a shallow, wide stream, while a low ratio would indicate a deep, narrow stream.

Stream Substrate Composition

Wolman pebble counts were conducted at each site to determine substrate composition (1954). Three sets of 100 substrate size measurements were conducted at each site and recorded to the nearest tenth of one centimeter. Measurements were taken in a zigzag pattern across the stream, recording substrate size at each random boot-toe position, according to standard protocol (Wolman, 1954). Field measurements were recorded in a digital voice recorder and transcribed into the project database. Wentworth calculations were performed for each set of 100 measurements in order to classify the substrate size measurements into groups (1922). The resulting counts of measurements for each size class were averaged for each site in order to best represent the substrate of the site and subwatershed.

Benthic Macroinvertebrate Sampling Methods

Benthic macroinvertebrates were collected as composite kicknet samples of natural substrate using 500-600μm mesh nets and preserved with a solution of 70-percent ethyl alcohol, according to Rapid Bioassessment Protocols (Barbour, 1999). In order to reduce intersample variation and detect generalized habitat impairment, three 1-meter samples were collected at each site and combined as a composite sample. Samples were collected from a riffle-run sequence with intentional bias to areas with highest expected benthic macroinvertebrate populations. Benthic macroinvertebrate sample field processing consisted of removing only large organic debris and stones from the sample in order to reduce the risk of specimen loss.

During laboratory processing, a random sub-sample was taken from each composite sample using a numbered grid frame and a random number generator to remove a minimum of 100 organisms. Organisms were removed from the debris and sorted to taxonomic order using a fluorescent magnifier light at 1.75X magnification. Sorted organisms were identified to taxonomic family using 4.5X magnification and 180 Watt illumination. Organism counts were recorded in the laboratory and compiled in the project database as number of organisms per family (please see the Appendices). Taxa richness, number of Ephemeroptera, Plecoptera, and Trichoptera taxa (EPT), percent EPT individuals, percent Chironomidae individuals, and percent dominant in the two most common families were calculated for each site.

GIS Data Model of Stream Crossing Densities in *R. bowersi* and Control Watersheds
An ArcGIS data model was built as a tool within the GIS to calculate the densities of stream crossings in watersheds relevant to the study (Figure 6). Base layers of sub-watershed boundaries and Fishes of West Virginia collection locations for R. *bowersi, R. cataractae, N. micropogon,* and *N. platyrhynchus* were spatially joined to calculate fish collection densities per sub-watershed for potential parental species with and without the presence of *R. bowersi*.

The numbers of collections for each species of fish are applied to sub-watershed boundaries in order to produce a sub-watershed polygon which holds a count of the number of collections for that species. The sets of sub-watershed polygons are spatially joined in order to produce sub-watershed polygons that represent areas with potential parental species in the presence of *R. bowersi* and separate sub-watershed boundaries for those with potential parental species in the absence of *R. bowersi*. These results are shown in Figure 7.

Figure 6. GIS Data Model of Stream Crossing Densities Relative to *R. bowersi*

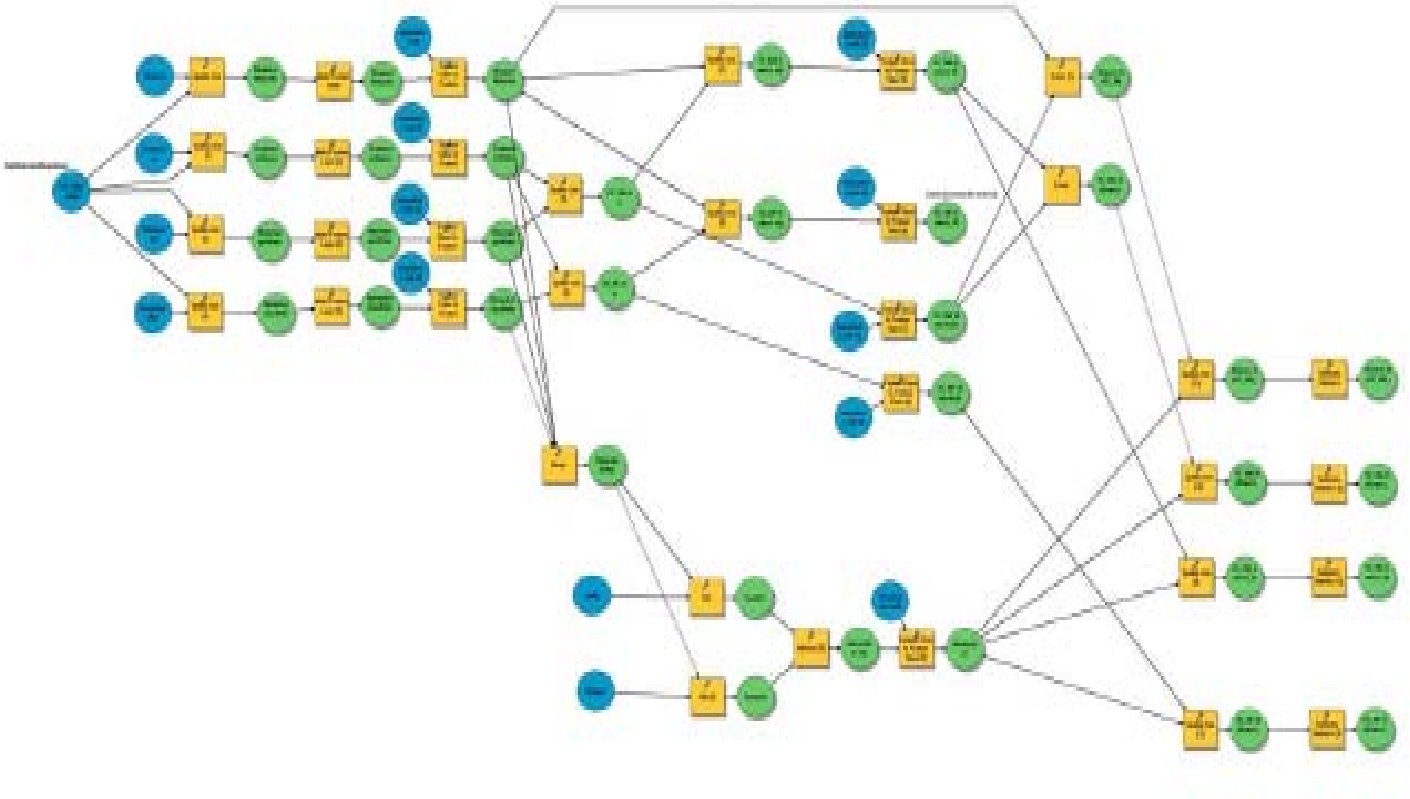
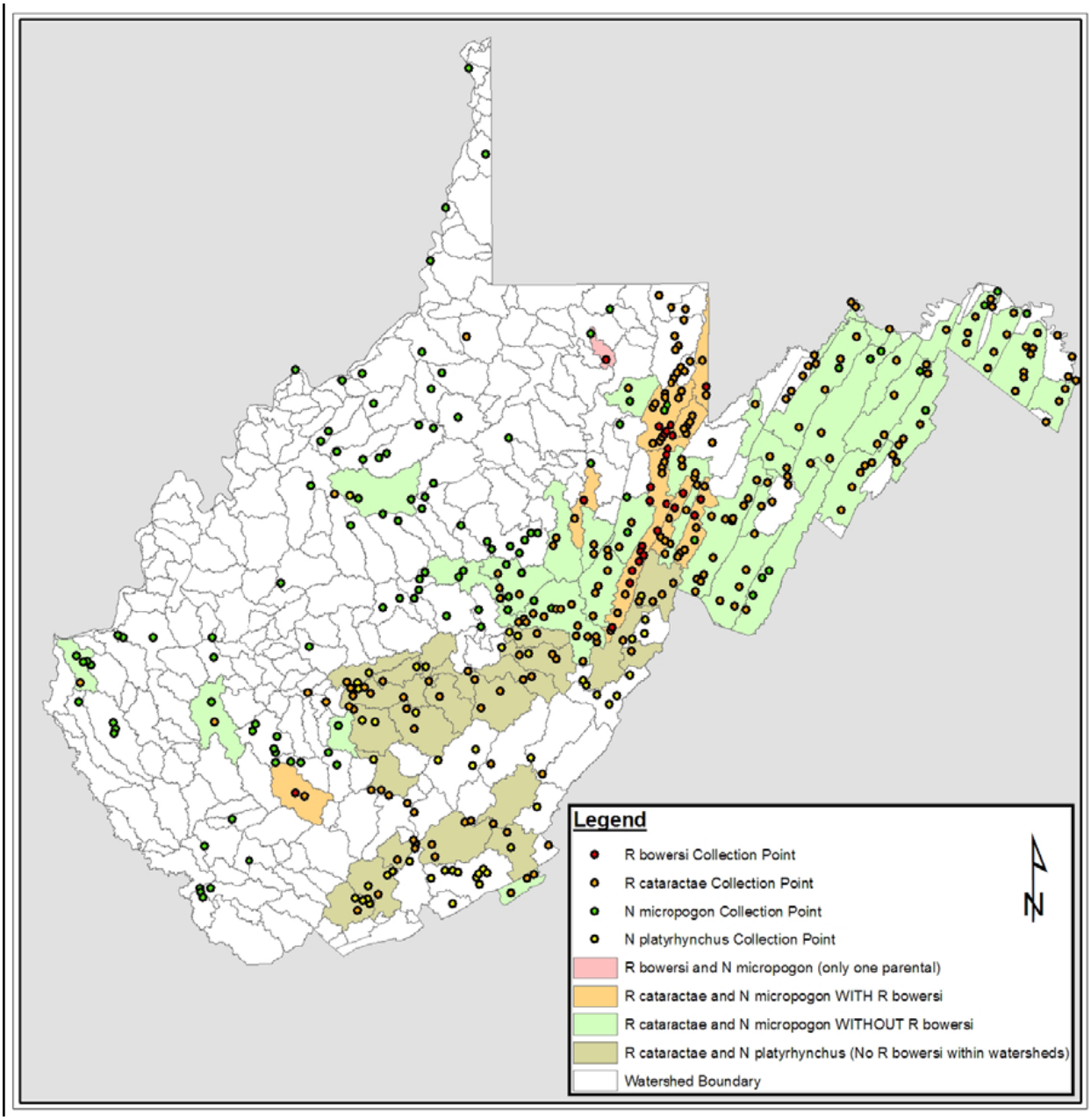


Figure 7. Fishes of West Virginia Collection Points and Sub-Watershed Boundary Classifications



CONCLUSIONS AND FINDINGS

Results: Mitochondrial Analysis

A complete section (958bp) of 12s rRNA was sequenced for each of the nine specimens. All nine sequences, plus two other sequences from GenBank, were aligned with variable sites marked (Figure 9). Sequences of three specimens previously identified as *R. bowersi* (designated as hyb1, hyb2, and hyb3) were compared to nearest neighbor sequences, with hyb1 in node with specimens of *R. cataractae*, hyb3 in node with *N. micropogon* and hyb2 in a node with a GenBank sequence of *Campostoma anomalum* (Appendix Figure 10). Specimens hyb1 had three variable sites from *R. cataractae* (Rcat2) and one variable site from *R. cataractae* (Rcat1) (Appendix Figure 11). Also, specimens hyb2 had 19 variable sites from *C. anomalum* from GenBank (Appendix Figure 12), with hyb3 having one variable site from *N. micropogon* (NM1) and two variable sites from *N. micropogon* (NM2) (Appendix Figure 13). The distance matrix from each specimen is shown in Table 3. Ten phylogenetic trees (Appendix Figures 10 and 14-22) were developed using Neighbor Joining, FITCH, and KITSCH software programs. The three different hybrids went into different clusters. This shows the cross works with both male and female of *R. cataractae* and *N. micropogon*. Cladistic and phylogenetic relationships of the three *R. bowersi* in this study show a relatedness to *R. cataractae*-(hyb1), *N. micropogon*-(hyb3), and *C. anomalum*-(hyb2), but do not constitute a phylogenetic group.

Discussion

The classification of cyprindid fishes historically has been based on breeding behavior, nuptial coloration, and meristic and morphometric characters such as numbers or size of pharyngeal teeth, body scales, fin rays, and tooth and scale counts. These characteristics have been used to sort cyprinids into traditional Linnaean taxonomic categories based most often on similarity of morphological characteristics. The integration of cyprinid morphology into a series of dichotomies has resulted in the identification of 57 species of cyprinids in West Virginia, representing 22 genera (Stauffer, et. al, 1995). The application of traditional taxonomic criteria to the status of *R. bowersi* is complicated by its likely origin through introgressive hybridization; *R. bowersi* is likely to be of hybrid origin developed in sympatry. This complicates any resolution of its status by traditional, morphological analyses of either morpho, metric or meristic characteristics.

The determination of whether *R. bowersi* is a reproductively isolated and consequently genetically and ecologically distinct species is also complicated by its origin through hybridization. Historically, biologists have identified species through criteria that originated in the "biological species" concept (described by Mayr, 1982, and others). Mayr described a process in which species develop unique morphological, physiological, and behavior traits in allopatry from other, similar populations and maintained these unique characteristics through reproductive isolation. In the biological species concept, the shared characteristics within a population that distinguish them from other populations occur through such genetic events as drift, neutral selection, and/or as adaptations to environmental stress. In contrast, *R. bowersi* has been proposed to be of hybrid origin, based on intermediacy of morphology, uniqueness of some protein polymorphisms, and susceptibility to parasites. The manner in which hybrid individuals would develop into reproductively isolated, ecologically viable species while sympatric with parental species is not well defined.

In 1979, Stauffer, et. al., reviewed the status of *R. bowersi*, which was previously thought to be a hybrid, and reported unique morphological characteristics that would classify it as a valid species. A previous analysis of chromosome structure had failed to find discriminating characters between *R. bowersi* and its suspected parentals. *R. bowersi* and its two parental species had very similar karyotypes with 2N=50 chromosomes, consistent with all other North American cyprinids (Campos and Hubbs, 1973). Also, chromatid lengths of all chromosomes were similar and could not be used to determine the inheritance of chromosomes from parentals to offspring.

Because this study proved only that *R. bowersi* had the same diploid number of chromosomes as its presumed parents (as well as that of most North American cyprinids), its taxonomic status remained undetermined (Morgan, et al., 1984). Hybridization has played a role in the evolution of other species. Hybridization can be defined as the interbreeding of individuals from two populations, or likewise groups, that are distinguishable based upon one or more heritable characters (Harrison, 1993). Introgression is the incorporation of genes from one set of differentiated populations into another permanently. This is often due to the incorporation of alien genes into a new, reproductively integrated population system (Rieseberg and Wendell, 1993).

One case paralleling that of *R. bowersi* is that of the *Canis lupus* (red wolf). For decades, the taxonomic status of *C. lupus* has been debated. Some have considered it a species, some a subspecies of *C. lupus*, and others a hybrid or cross-breed of *C. latrans* (coyote) and *C. lupus* (Roy, 1996). Debates about its taxonomic status prompted studies to trace its ancestry back to its origin (Dowling et. al., 1992). Analyses of its mitochondrial and nuclear DNA markers strongly indicate that *C. lupus* is a hybrid. Also, documentation shows hybridizations for many cyprinids combinations (Mir et al., 1988; Jenkins and Birkhead, 1994).

Goodfellow et. al., (1984) stated that *R. bowersi* was a valid species and not an F1 hybrid. They found that *R. bowersi* had unique alleles at two protein coding loci and patterns of general serum proteins that were diagnostically different than the parental species. Of 43 enzyme loci that were screened, only two, glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8) and esterase (EST-B, EC 3.1.1) were taxonomically informative. The pattern of EST-B and G3PDH was not typical of what was expected of a hybrid in that the alleles found for the parental species were not present in *R. bowersi*, and the alleles for *R. bowersi* were not present in either of the parents. Also, the data for the serum proteins showed *R. bowersi* being more closely related to *R. cataractae* in that they shared four bands alike; however, *R. bowersi* shared only three with *N. micropogon*. There were five unique bands that were present only in *R. bowersi* that were not expressed in either of the parental species (Goodfellow, et al., 1984). The isozymes of AKD-A, ALD-B, EST-C, and IDH-A, along with analyses of the soluble serum proteins, showed more similarity to *R. cataractae* than *N. micropogon* (Goodfellow, et. al., 1984).

Stauffer, et. al., (1997) recommended that *R. bowersi* be identified as a species developed through introgressive hybridization and named as *Pararhinichthys bowersi*. This renaming of *R. bowersi* was based on its recorded persistence in nature for more than 100 years and the presence of sexually mature males and females. Also, they argued that the diagnostic electromorphs for two genetic loci were unique for *R. bowersi*, which was runs contrary to expectation if *R. bowersi* was a F1 hybrid." Stauffer et. al., also analyzed nine morphometric and meristic

characteristics and showed that six were intermediate, three were closely related to *R. cataractae*, and two closely related to *N. micropogon*. The computer analysis of the scale shape among the species show that *R. bowersi* and *N. micropogon* have basilateral corners, no radii, and similar shapes relative to those of species of Rhinichthys (Stauffer et al., 1997).

Cloutman (1988) showed parasites as a useful way to identify hybrids. Stauffer stated that if *R. bowersi* is a hybrid, it would have parasites present from both parental species; however, it has only the parasites that infect *R. cataractae* (Stauffer et al., 1997). Stauffer et. al., (1997) also noted that *Dactylogyrus reciprocus* (a monogenean parasite) was found in *R. bowersi* and *N. micropogon*. However, this finding offers little support as verification for true species validation because there are no unique or specific parasites for *R. bowersi* (Poly and Sabaj, 1998). Poly and Sabaj (1998) also note that *R. bowersi* only occurs in sympatry with both suspected parental, whereas each parental occur in the absence of the other.

In 1998, Poly and Sabaj argued that the biochemical evidence and data of Goodfellow et. al., (1984) were flawed. Goodfellow, et. al., reported two unique alleles for loci glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8) and esterase (EST-B, EC 3.1.1) and unique general protein patterns from blood serum of *R. bowersi* but were lacking in the mixture of the parental extracts. Poly and Sabaj (1998) noted that G3PDH is dimeric and would show up as a hybrid enzyme just by mixing the parental extracts. This same method was demonstrated with hemoglobins from *Chaenobryttus gulosus* and *Lepomis cyanellus* or *L. macrochirus* by Maxwell, et. al., 1963. Poly and Sabaj also note that, Goodfellow, et. al., (1984) found enzymes in certain tissues of diploid cyprinids that have not been previously reported from tissues of cyprinid fishes (Buth et. al., 1991). Goodfellow et. al., (1984) reported alcohol dehydrogenase in muscle tissue, the first reporting of this enzyme to occur in this particular tissue type for cyprinids. Also, labeling isozymes from one diagnostic locus, EST-B, is difficult because its quaternary structure is not easily recognized and because esterase mobility is affected by many posttranslational modifications (Poly, 1997).

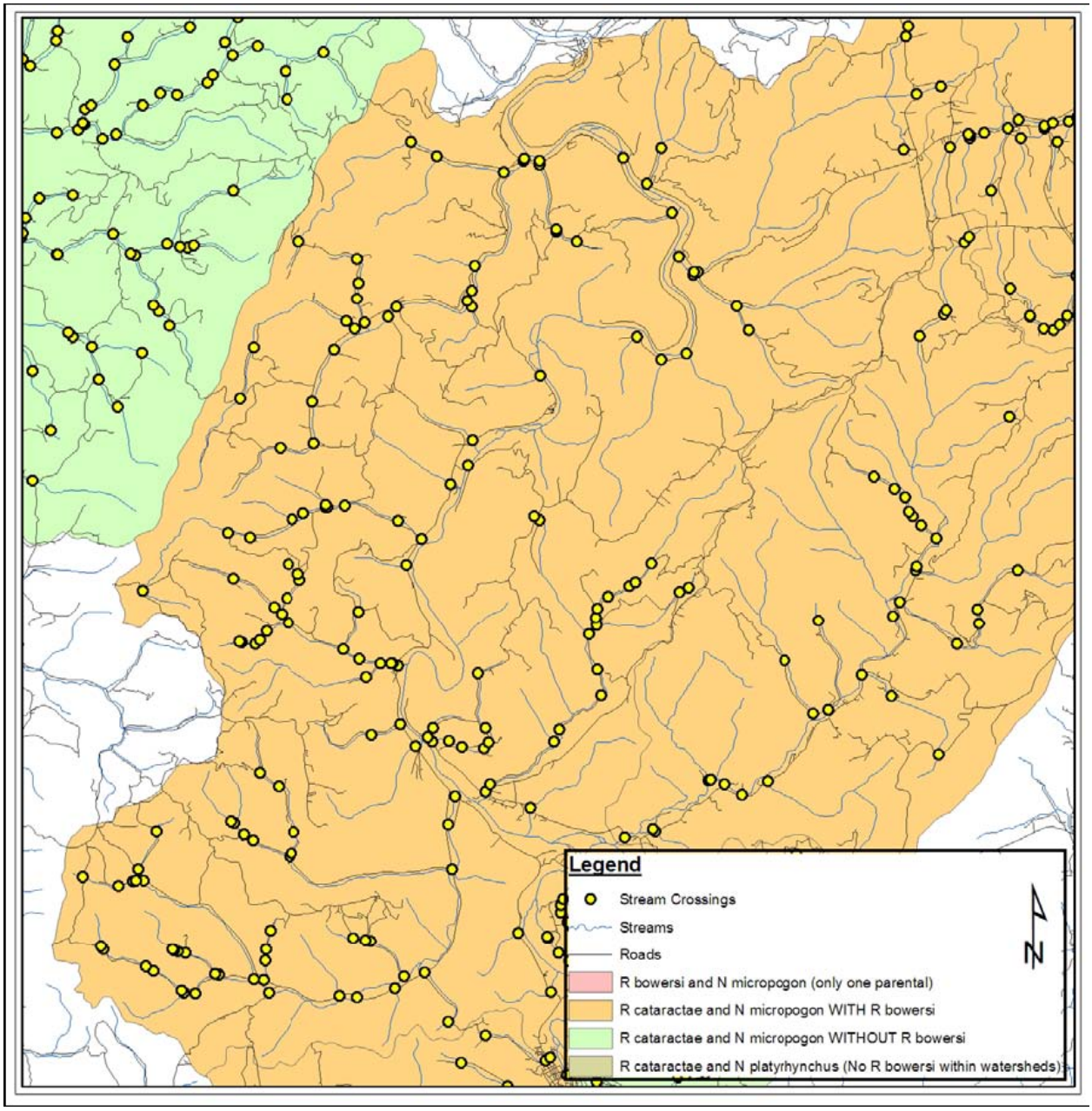
In this work, three specimens of cyprinid fishes from Shavers Fork (Figure 23 and 24, Cheat River Drainage) were examined for meristic and morphometric characters known to be descriptive of *R. bowersi* and were initially identified as cyprinids of hybrid origin, morphologically consistent with *R. bowersi*. Two specimens (labeled as hyb1 and hyb3) were collected from riffle/run stream sections of Shavers Fork above Cheat Bridge and above the mouth of Red Run, respectively. The specimen hyb2 was a preserved specimen from Shavers Fork and was obtained from the West Virginia Department of Natural Resources. Mitochondrial sequences for the 12S gene (958 bp) of these three fish were then compared to those of specimens of proposed parental species *R. cataractae* and *N. micropogon*, and an outgroup of three specimens from congeneritor (to *R. bowersi*, and *R. cataractae*) *R. atratulus*, and from GenBank sequences of two species of cyprinid fishes found in Shavers Fork, *R. atratulus* and *Campostoma anomalum*. These sequences were included in phylogenetic analysis because the former is congeneric to both *R. cataractae* and *R. bowersi* and the latter is common in Shavers Fork and known to participate in hybrid reproduction with other cyprinids. Phylogenetic relationships are represented in Appendix Figures 10 and 14-22.

Differences between mitochondrial DNA sequences among three hybrid individuals and generic (*R. cataractae*) and congeneric (*Nocomis micropogon* and *Campostoma anomalum*) specimens to *R. bowersi* were analyzed by Neighbor Joining (NJ), FITCH, and KITSCH software programs. These programs utilized different algorithms for generating phylogenetic trees that are derived from distance matrices. Mitochondrial sequences were aligned (Higgins, et. al., 1989) and analyzed by both cladistic and phylogentic methods bootstrap resampled alignments were also analyzed to estimate confidence in tree topologies (Felsenstein, 1985). Clades represent the relative similarity (i.e. the clustering or branching orders) between sequence (and the individuals that produce them) without reference to genetic distance. Cladograms are useful in establishing groups or clades but can be somewhat misleading because branching within clades does not accurately represent genetic distance, although visually appears to do so. Because tree topography can be affected by the order in which data are entered, data were subjected to jumble analysis, which randomized entry order of sequences and retrieves the most common tree topology. Parsimony analyses produced trees free of evolutionary distance with branching, indicating only shared ancestry. Consequently, the orders of taxa are informative, but distances are not.

After examination through Nearest Neighbor, FITCH, and KITSCH software programs for native and resampled alignments, and parsimony-based treeing programs, hyb1 consistently clustered with *R. cataractae*, hyb2 consistently clustered with *C. anomalum*, and hyb3 branched as a node including *N. micropogon*. When the 12S mtDNA sequence of hyb2 was compared to other cyprinids through GenBank (Simmons and Mayden, 1997), its 12s sequence was similar to that of cyprinid *Campostoma anomalum* (<2.0% differences). Because *R. cataractae* also hybridizes with *Campostoma anomalum* and hybrid specimens from this cross have been collected at several sites in the Cheat Drainage (Clover and Horseshoe Runs), GenBank DNA sequences of *C. anomalum* from a western population (Simmons et. al., 1997) was included in phylogenetic analysis. Hybrid specimen hyb2 did cluster with the genebank *C. anomalum* sequence in all analyses. However, the *C. anomalum* sequence from genebank was from populations distant from Shavers Fork conspecific populations and the >2.0% difference in base sequence between *C. anomalum* and may result from interspecific or from interpopulation differences. Consequently, the formation of a node of hyb2 with *C. anomalum* was not informative and the species involved in the formation of hybrid hyb2 are unknown.

The morphology of the three hybrid individuals in this study, indicate that each is produced by a cross of *R. cataractae* and another cyprinid species. The specimen hyb1, hyb2, hyb3 all have the lip structure, scale structure and number, and frenum indicating genetic influence of *Rhinichthys.* The head length, coloration, and body shape of the three hybrids are all consistent with *R. cataractae*, not *R. atratulus*. This indicates that each of the three hybrid fish had *R. cataractae* as a parent. If the maternal inheritance of the mitochondrial chromosome is included in this analysis, then the parental species that produced these three hybrids can be inferred. Because hyb1 has the maternal mitochondrial DNA of *R. cataractae*, the other parent must be of some other cyprinid species, most likely *N. micropogon*. Specimen hyb3 has maternal mitochondria DNA of *N. micropogon* and must therefore inherit *R. cataractae* characteristics from a paternal source. Specimen hyb2 has maternal mitochondrial DNA from a cyprinid other than *R. cataractae* or *N. micropogon*, possibly *C. anomalum*. This suggests that characteristics of *R. cataractae* in hyb2 were also contributed from a paternal *R. cataractae* source.

Figure 8. Stream Crossings within the Cheat River Drainage

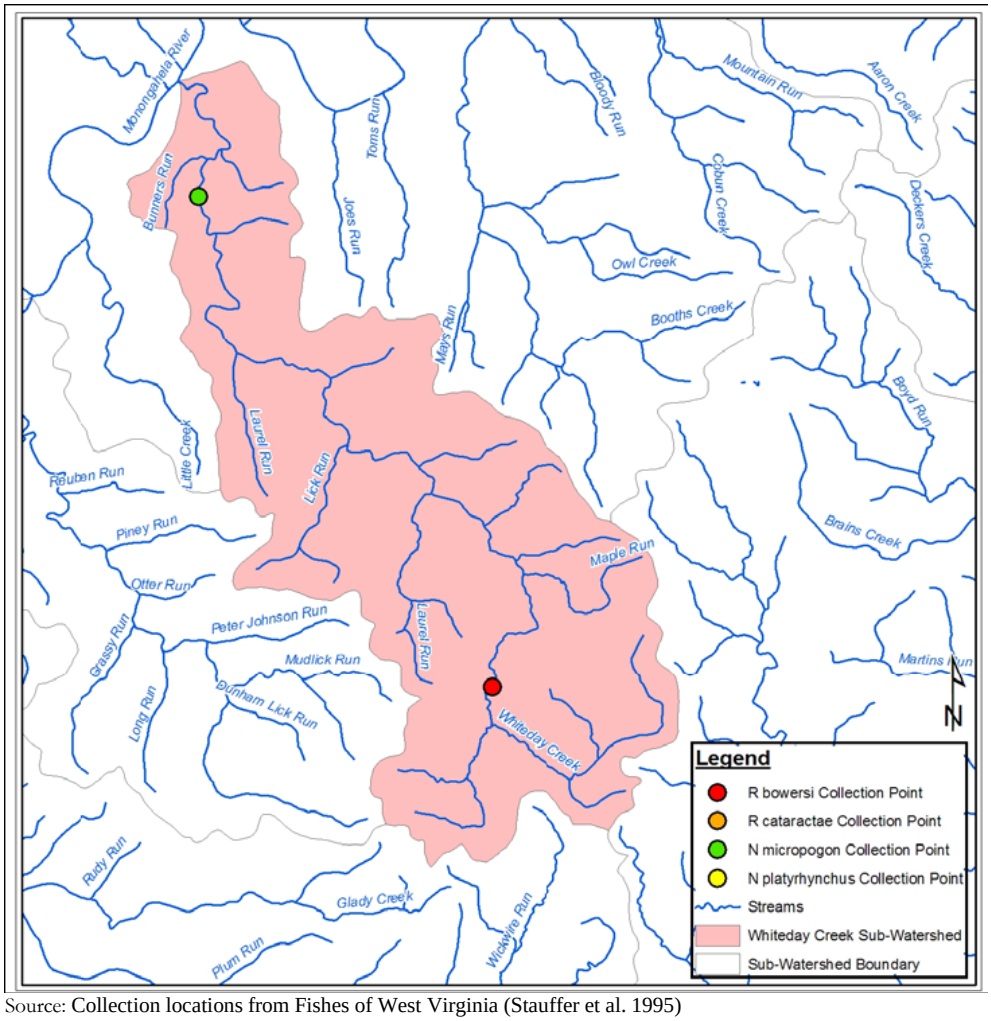


RESULTS

Distribution of *R. bowersi* in West Virginia Streams

Results of the GIS data model indicate that *R. bowersi* occurs within sub-watersheds with *R. cataractae* and *N. micropogon* with one exception. According to Fishes of West Virginia records, one collection of *R. bowersi* was made from the Whiteday Creek system within the Monongahela River drainage. While one collection of *N. micropogon* has occurred in this subwatershed, *R. cataractae* has not been collected from the Whiteday Creek system according to Fishes of West Virginia data.

Figure 9. Distribution of R. bowersi and N. micropogon in Whiteday Creek Sub-Watershed of the Monongahela River Drainage



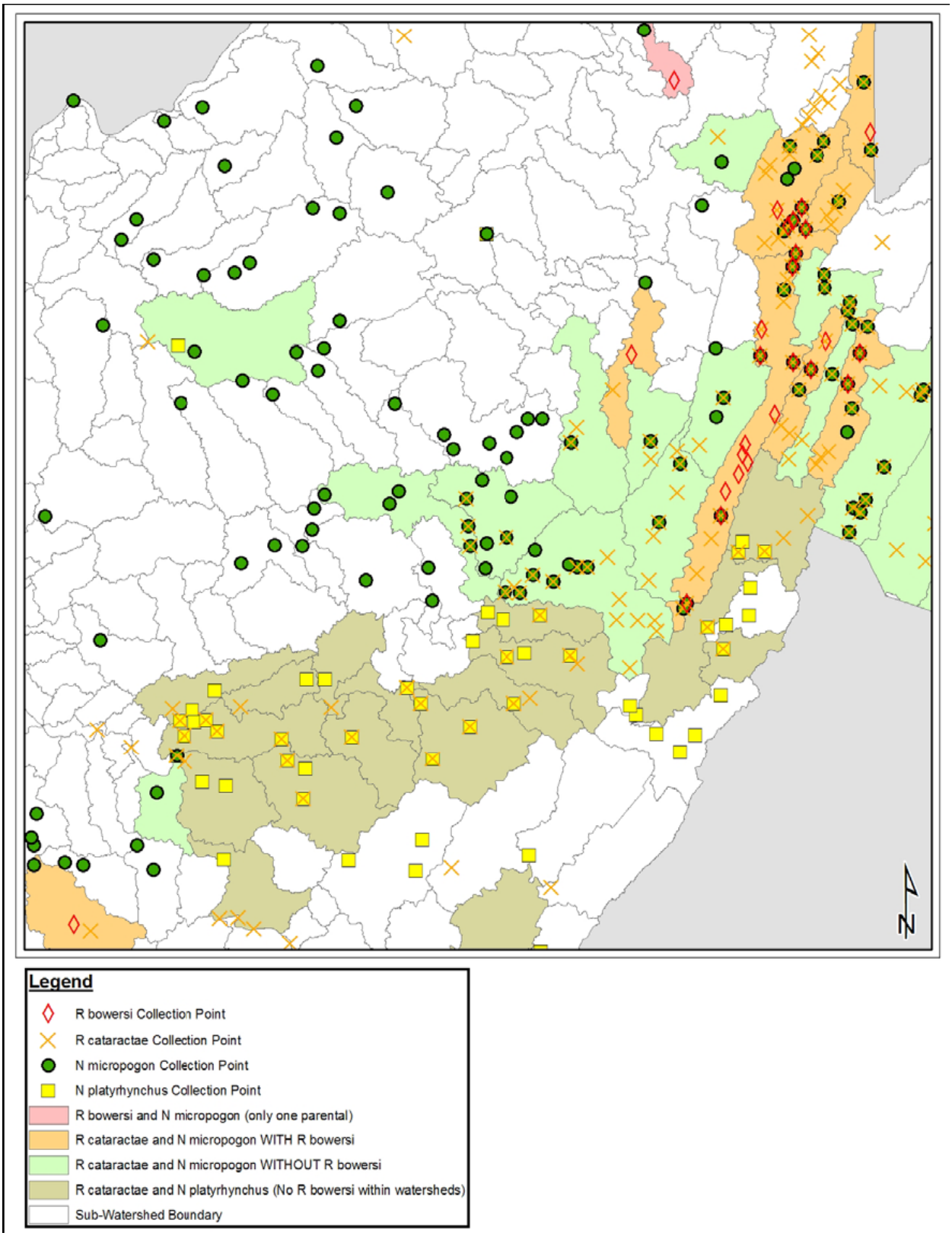
Source: Collection locations from Fishes of West Virginia (Stauffer et al. 1995)

In all other instances within the data model, *R. bowersi* was collected from sub-watersheds yielding both parental species. Fishes of West Virginia records indicate a total of 26 collections of *R. bowersi*, 25 of which occurred in sub-watershed units where both parental species have also been collected, as seen in Table 4 and Figure 10.

Table 4. *R. bowersi* Collections and Parental Species Status within Sub-Watersheds

R. bowersi Collection Location	**County**	**Drainage**	**Stream Name**	***R. cataractae Collected***	***N. micropogon Collected***
South of Mill Run	Randolph	Cheat River	Shavers Fork	YES	YES
South of Saint George	Tucker	Cheat River	Cheat River	YES	YES
South of Oats Run	Randolph	Cheat River	Shavers Fork	YES	YES
North of Suter Run	Randolph	Cheat River	Stalnaker Run	YES	YES
North of Jonathan Run	Tucker	Cheat River	Cheat River	YES	YES
North of Cheat Bridge	Randolph	Cheat River	Shavers Fork	YES	YES
West of Black Fork	Tucker	Cheat River	Shavers Fork	YES	YES
North of Bingham Run	Tucker	Cheat River	Minear Run	YES	YES
North of Johns Run	Randolph	Cheat River	Shavers Fork	YES	YES
East of Hawk Run	Tucker	Cheat River	Shavers Fork	YES	YES
SW of Bonifield Cemetery	Tucker	Cheat River	Horseshoe Run	YES	YES
West of Nichols Lane Run	Randolph	Cheat River	Glady Fork	YES	YES
Dry Fork south of Bethel Church	Randolph	Cheat River	Dry Fork	YES	YES
Dry Fork southwest of HARMAN	Randolph	Cheat River	Dry Fork	YES	YES
West of Sailor Run	Tucker	Cheat River	Minear Run	YES	YES
West of Brushy Creek	Randolph	Cheat River	Glady Fork	YES	YES
North of Cherry Run	Marion-Taylor Line	Monongahela River	Whiteday Creek	**NO**	YES
North of Burnt Bridge	Randolph-Upshur Line	Tygart Valley River	Middle Fork River	YES	YES
Marsh Fork of Big Coal River at Masseyville	Raleigh	Coal	Marsh Fork	YES	YES
North of Wolf Run	Randolph	Cheat River	Shavers Fork	YES	YES
NE of Cheat Junction	Randolph	Cheat River	Shavers Fork	YES	YES
East of Rhine Creek	Preston	Youghiogheny River	Youghiogheny River	YES	YES
South of Mouth of Suter Run	Randolph	Cheat River	Shavers Fork	YES	YES
West of Wildell Cave	Randolph	Cheat River	Shavers Fork	YES	YES
Mouth of Yokum Run	Randolph	Cheat River	Shavers Fork	YES	YES
South of Mouth of Whitmeadow Run	Randolph	Cheat River	Shavers Fork	YES	YES

Figure 10. Distribution of R. bowersi in Fishes of West Virginia Records



Source: Collection locations from Fishes of West Virginia (Stauffer et al. 1995)

RECOMMENDATIONS (INCLUDING SUGGESTIONS FOR ADDITIONAL RESEARCH)

Summary and Conclusion

In this work, DNA evidence is presented for the first time that supports the status of *R. bowersi* as a hybrid developed from a cross of *R. cataractae* and *N. micropogon*. However, these data do not resolve the issue of whether *R. bowersi* is a F1 hybrid or a reproductively isolated species developed through introgressive hybridization. These data do suggest that hybridization involves both male *R. cataractae* with female *N. micropogon* and male *N. micropogon* with female *R. cataractae* hybridizations. Analyses of morphological structure, protein polymorphism, karyotype, and parasite/host relationships have been interpreted to support both species and F1 hybrid status (Stauffer, et. al., 1997; Poly and Sabaj, 1998). *R. bowersi* does have distinguishing numbers of scale counts relative to the presence of a barbel and a frenem that discriminates the form of *R. bowersi* from other cyprinids. However, these morphological characteristics fail to identify *R. bowersi* as a species as opposed to a hybrid formed from introgressive hybridization of two well-defined species.

From field experiences during this work, *R. bowersi*, as expected, was found to be rare within its range and more common in some streams than others. *R. bowersi* was collected from North Fork of Snowy Creek, Glady Fork, and Shavers Fork of the Cheat, but was not found in streams from which it had previously been reported, Laurel Fork of the Cheat, Horseshoe Run of the Cheat, and Middle Fork of the Monongahela. Many streams inhabited by *R. bowersi* were heavily impacted by sediment deposition and channelization. The decline in habitat quality for *R.* bowersi described by Goldsborough and Clark nearly 100 years ago persists to a lesser degree to this date. Thus, the restricted distribution of *R. bowersi* primarily to the Monongahela drainage may result from higher rates of hybridization in stressed environments and not to genetic or reproductive isolation.

Analyses of morphological structure, protein polymorphism, karyotype, and parasite/host relationships have been interpreted to support both species and F1 hybrid status for *R. bowersi* (Stauffer et al., 1997; Poly and Sabaj, 1998). In this work, DNA evidence is presented for the first time that supports the status of *R. bowersi* as a hybrid developed from a cross involving *R. cataractae, N. micropogon,* and another cyprinid species.

However, these data do not resolve the issue of whether *R. bowersi* is a F1 hybrid or a reproductively isolated species developed through introgressive hybridization.

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APPENDICES

Figure 1. A diagram of the entire mitochondrial DNA consists of about 16,659 bp, with 22 transfer RNA genes, 2 ribosomal RNA genes, and 13 protein coating regions. Within the mitochondrial DNA I used primers at phea and 16s RNA, which produced a 2,000 bp product .

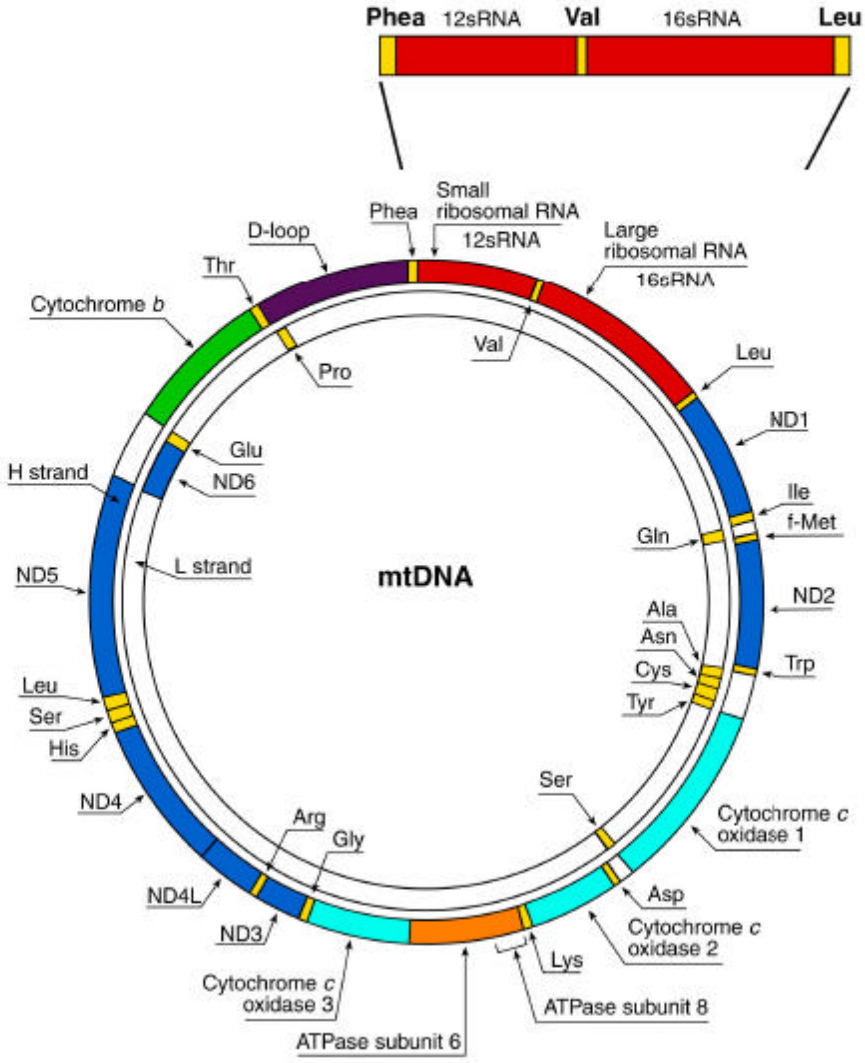

Figure 2. A Smith-Root SR12 Barge was used for collecting *R. bowersi* specimen.

Figure 3. A Honda generator was used for collecting *R. bowersi*.

Figure 4. Three minnows were caught in Shavers Fork River. The fish at the top is *Nocomis micropogon*, middle is *Rhinichthys bowersi*, and bottom is *Rhinichthys cataratae*.

FIgure 5. Illustration of the amplified area of PHEa and 16sd primers.

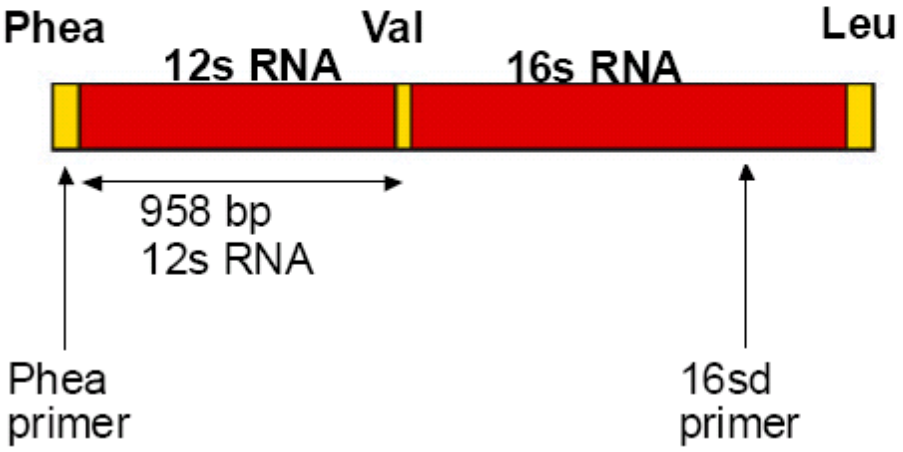

Figure 6. This 1% agarose gel is of all samples that were amplified by PCR. The correct band size was 2,000 bp.

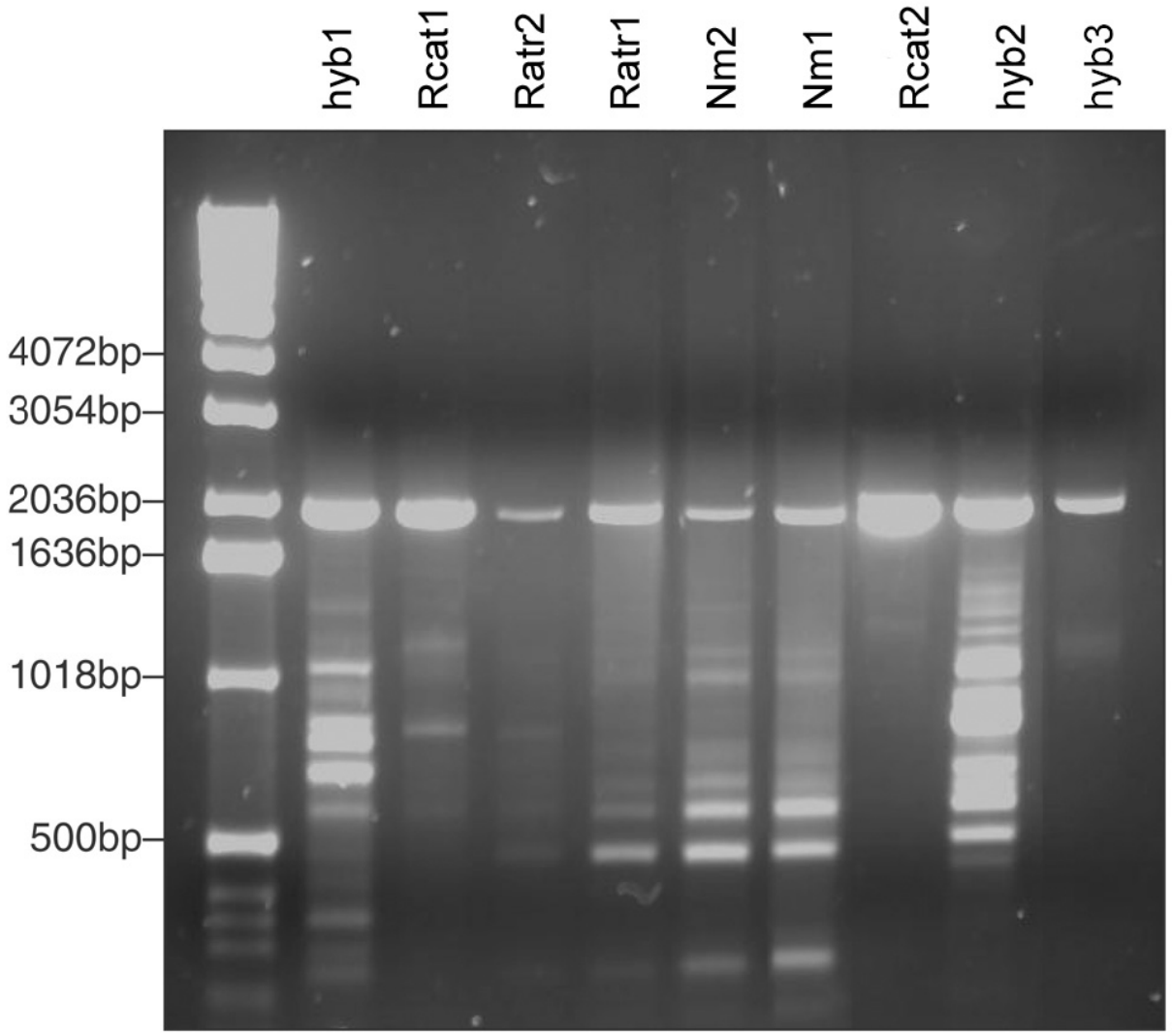

Figure 7. This 1% agarose gel is of a restriction digest using EcoR I on two *R. bowersi* and two *N. micropogon* specimens. The vector size is 3,900 bp, and the insert is 2,000 bp.

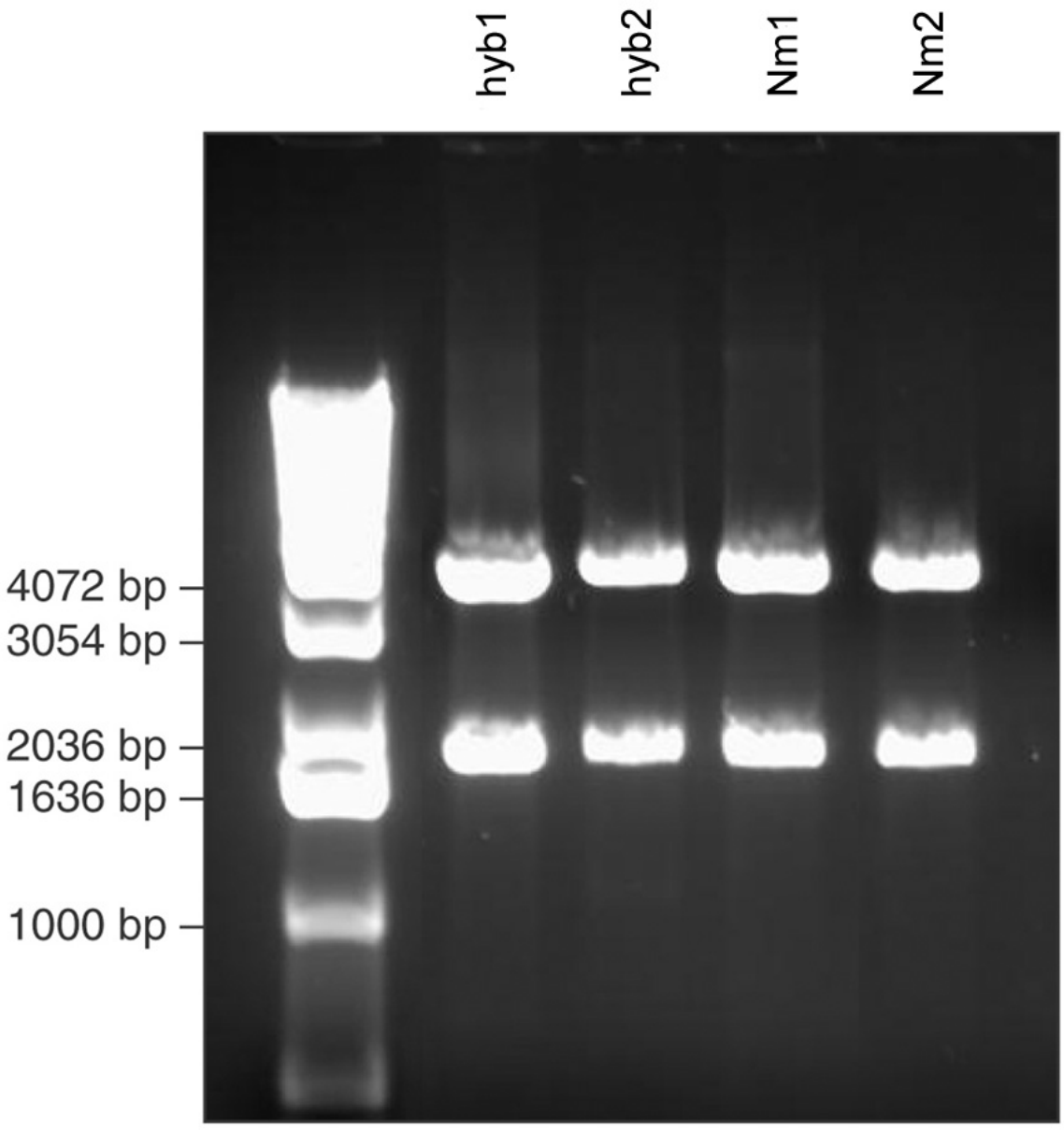

Figure 8. This is a chromatograph of Rhinichthys bowersi. This sample was cloned and sequenced on an ABI 377.

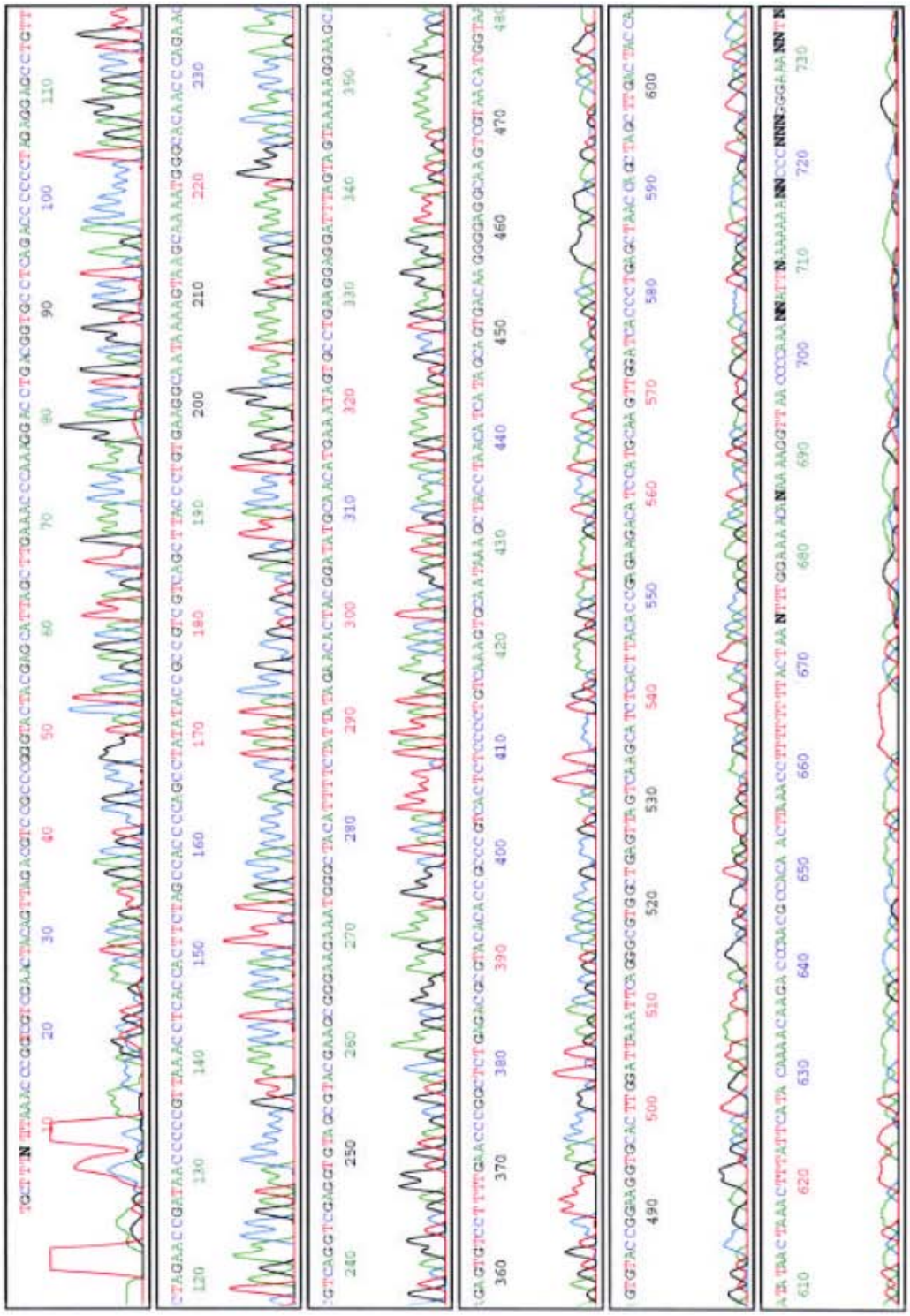

Figure 9. The complete 12s mtDNA for all nine fish, plus two from GenBank (*). The sequences were aligned in Sequencer.

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hyp3 951 GCTTAAAT
nm1 951 GCTTAAAT
nm2 951 GCTTAAAT
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Figure 10. Neighbor joining cluster phylogenetic tree of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* with bootstrap

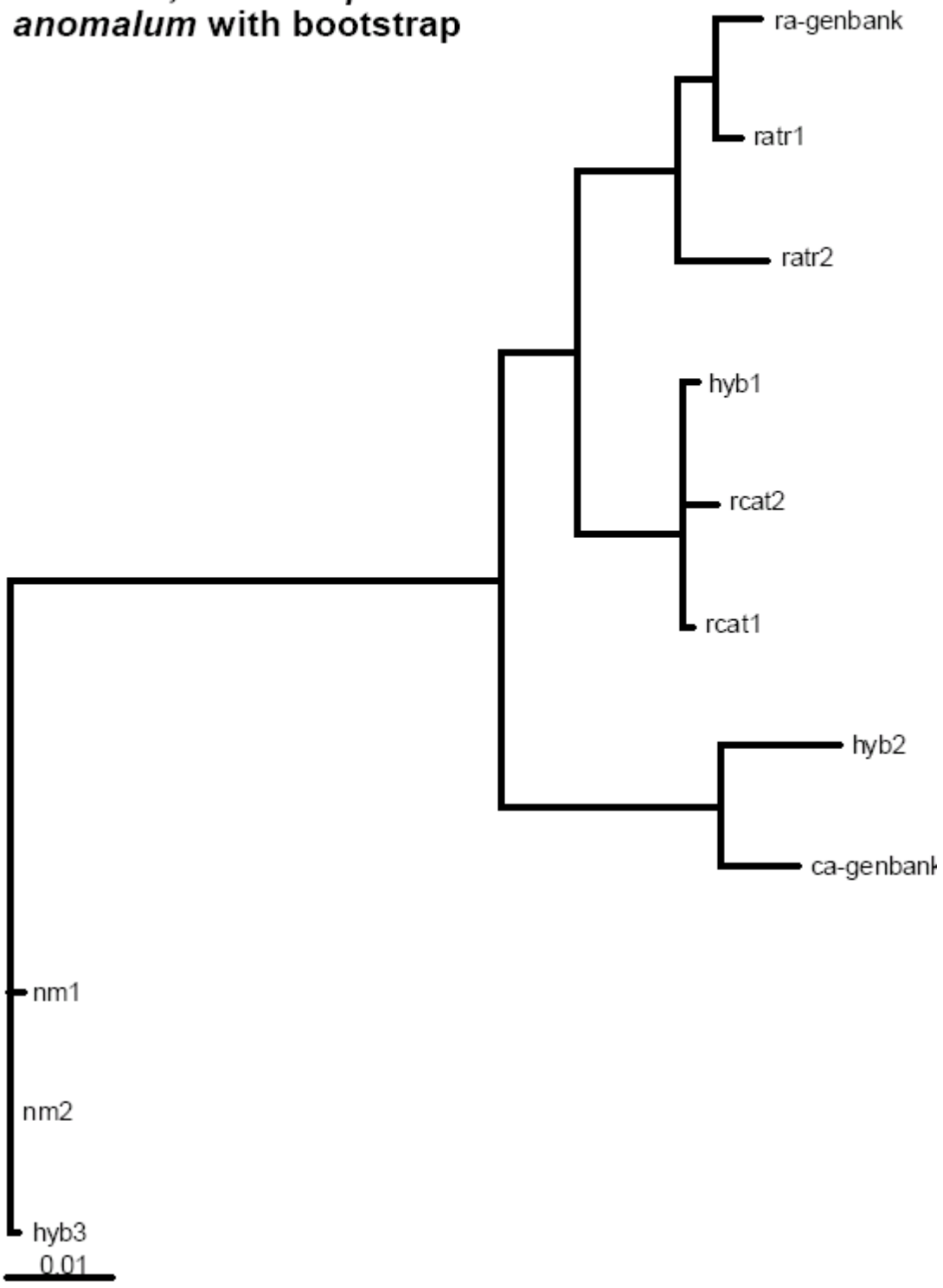

Figure 11. Hyb1 in node with *R. cataractae*.

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Rcat2 601 TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
hyb1 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
Rcat1 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
Rcat2 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
hyb1 701 CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
Rcat1 701 CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATAATAGAACACTACG
Rcat2 701 CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
hyb1 751 GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTAAGTAGTAAAAAGG
Rcat1 751 GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTA:GTAGTAAAAAGG
Rcat2 751 GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTA:GTAGTAAAAAGG
hyb1 801 AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
Rcat1 801 AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
Rcat2 801 AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
hyb1 851 TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
Rcat1 851 TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
Rcat2 851 TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
hyb1 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
```

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Rcat1 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
Rcat2 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
hyb1 951 GATTAAAT
Rcat1 951 GATTAAAT
Rcat2 951 GATTAAAT
```

Figure 12. Hyb2 in node with *C. anomalum.*

```
hyp2 1 TAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCTAACTTACACATG
CA* 1 TAAAGGCATGGTCCTGACCTTATTATCAGCTTTAACCTAACTTACACATG
hyp2 51 CAAGTCTCCGCAATCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
CA* 51 CAAGTCTCCGCAGCCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
hyp2 101 AGGAGCAGGTATCAGGCACGAACCCTTAGCCCAAGACGCCTGGCCTAGCC
CA* 101 AGGAGCAGGTATCAGGCACAAACCCTTAGCCCAAGACGCCTGGCCTAGCC
hyp2 151 ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
CA* 151 ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
hyp2 201 CTTGACTCAGTTAAGGCTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
CA* 201 CTTGACTCAGTTAAGGTTAAAAGGGCCGGTAAAACTCGTGCCAGCCACCG
hyp2 251 CGGTTAGACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
CA* 251 CGGTTAGACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
hyp2 301 AAGGATACTGAGACAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
CA* 301 AAGGATACTGAGACAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
hyp2 351 CTAGGAGTCCGAAGACCAATACACGAAAGTAGCTTTAAAGAAGTTCACCT
CA* 351 CTAGGAGTCCGAAGACCAATATACGAAAGTAGCTTTAAAGGAGTCCACCT
hyp2 401 GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCC
CA* 401 GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
hyp2 451 CAGCCGTAAACTTAGACGTCAACCTACAATAAGACGTCCGCCCGGTTACT
CA* 451 CAGCCGTAAACTTAGACGTCAACCTACAATAAGACGTCCGCCCGGGTACT
hyp2 501 ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
CA* 501 ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
hyp2 551 TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
CA* 551 TAGAGGAGCCTATTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
hyp2 601 TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
CA* 601 TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
hyp2 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
CA* 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
hyp2 701 CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
CA* 701 CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
hyp2 751 GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAAAAGG
CA* 751 GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAAGAGG
hyp2 801 AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGGCCG
CA* 801 AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGCCCG
hyp2 851 TCACTCTTCCCTGTCAAAATGCAGCAAGACTACCTAATACTAAAGCCATG
CA* 851 TCACTCTCCCCTGTCAAAATGCAACAAGATTACCTAATACTAGAGCCATG
hyp2 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
CA* 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAG:TGCACTTG
hyp2 951 GATAAAAT
CA* 951 GATAAAAT
```

Figure 13. Hyb3 in node with *N. micropogon.*

```
hyp3 1 CAAAGGCATGGTCCCGACCTTATCATCAGCTCTAACCTAACTTACACATG
nm1 1 CAAAGGCATGGTCCCGACCTTATCATTAGCTCTAACCTAACTTACACATG
nm2 1 CAAAGGCATGGTCCCGACCTTATCATCAGCTCTAACCTAACTTACACATG
hyp3 51 CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
nm1 51 CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
nm2 51 CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
hyp3 101 AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC
nm1 101 AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC
nm2 101 AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC
hyp3 151 ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
nm1 151 ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
nm2 151 ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
hyp3 201 CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
nm1 201 CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
nm2 201 CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
hyp3 251 CGGTTAGACGAGAGGCCCTAGTTAATGATGTAACGGCGCAAAGGGTGGTT
nm1 251 CGGTTAGACGAGAGGCCCTAGTTAATGATGTAACGGCGTAAAGGGTGGTT
nm2 251 CGGTTAGACGAGAGGCCCTAGTTAATGATGTAACGGCGTAAAGGGTGGTT
hyp3 301 AAGGATAGTAAATTAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
nm1 301 AAGGATAGTAAATTAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
nm2 301 AAGGATAGTAAATTAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
hyp3 351 CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
nm1 351 CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
nm2 351 CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
hyp3 401 GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
nm1 401 GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
nm2 401 GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
hyp3 451 CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT
nm1 451 CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT
nm2 451 CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT
hyp3 501 ACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTGCCTTAGACCCCCC
nm1 501 ACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTGCCTTAGACCCCCC
nm2 501 ACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTGCCTTAGACCCCCC
hyp3 551 TAGAGGAGCCTGTTCTAGAACCGATAACCCTCGTTAAACCTCACCACTTC
nm1 551 TAGAGGAGCCTGTTCTAGAACCGATAACCCTCGTTAAACCTCACCACTTC
nm2 551 TAGAGGAGCCTGTTCTAGAACCGATAACCCTCGTTAAACCTCACCACTTC
hyp3 601 TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA
nm1 601 TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA
nm2 601 TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA
hyp3 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
nm1 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
nm2 651 ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
hyp3 701 CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG
nm1 701 CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG
nm2 701 CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG
hyp3 751 GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
nm1 751 GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
nm2 751 GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
hyp3 801 AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG
nm1 801 AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG
nm2 801 AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG
hyp3 851 TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG
nm1 851 TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG
nm2 851 TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG
hyp3 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
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nm1 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
nm2 901 ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
hyp3 951 GCTTAAAT
nm1 951 GCTTAAAT
nm2 951 GCTTAAAT
```

Table 3. Distance matrix for all nine fish, plus two from GenBank (*).

	Hyb1	**Ract2**	**Ract1**	**RA***	**Ratr1**	**Ratr2**	**Hyb2**	**CA***	**Hyb3**	**Nm2**	**Nm1**
Hyb1	0.0000	0.0075	0.0019	0.0362	0.0342	0.0323	0.0679	0.0579	0.0778	0.0757	0.0778
Rcat2	0.0075	0.0000	0.0056	0.0401	0.0342	0.0362	0.0721	0.0619	0.0819	0.0799	0.0779
Rcat1	0.0019	0.0056	0.0000	0.0342	0.0323	0.0304	0.0659	0.0559	0.0757	0.0737	0.0757
RA*	0.0362	0.0401	0.0342	0.000	0.0094	0.0170	0.0742	0.0640	0.0859	0.0839	0.0859
Ratr1	0.0342	0.0342	0.0323	0.0094	0.0000	0.0131	0.0700	0.0639	0.0860	0.0840	0.0860
Ratr2	0.0323	0.0362	0.0304	0.0170	0.0131	0.0000	0.0679	0.0618	0.0925	0.0905	0.0925
Hyb2	0.0679	0.0721	0.0659	0.0742	0.0700	0.0679	0.000	0.0207	0.1074	0.1053	0.1074
CA*	0.0579	0.0619	0.0559	0.0640	0.0639	0.0618	0.0207	0.0000	0.0989	0.0968	0.0989
Hyb3	0.0778	0.0819	0.0757	0.0859	0.0860	0.0925	0.1074	0.0989	0.0000	0.0019	0.0037
Nm2	0.0757	0.0799	0.0737	0.0839	0.0840	0.0905	0.1053	0.0968	0.0019	0.0000	0.0019
Nm1	0.0778	0.0779	0.0757	0.0859	0.0860	0.0925	0.1074	0.0989	0.0037	0.0019	0.0000

Figure 14. Distance matrix phylogenetic tree using the Fitch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with nonbootstrap.

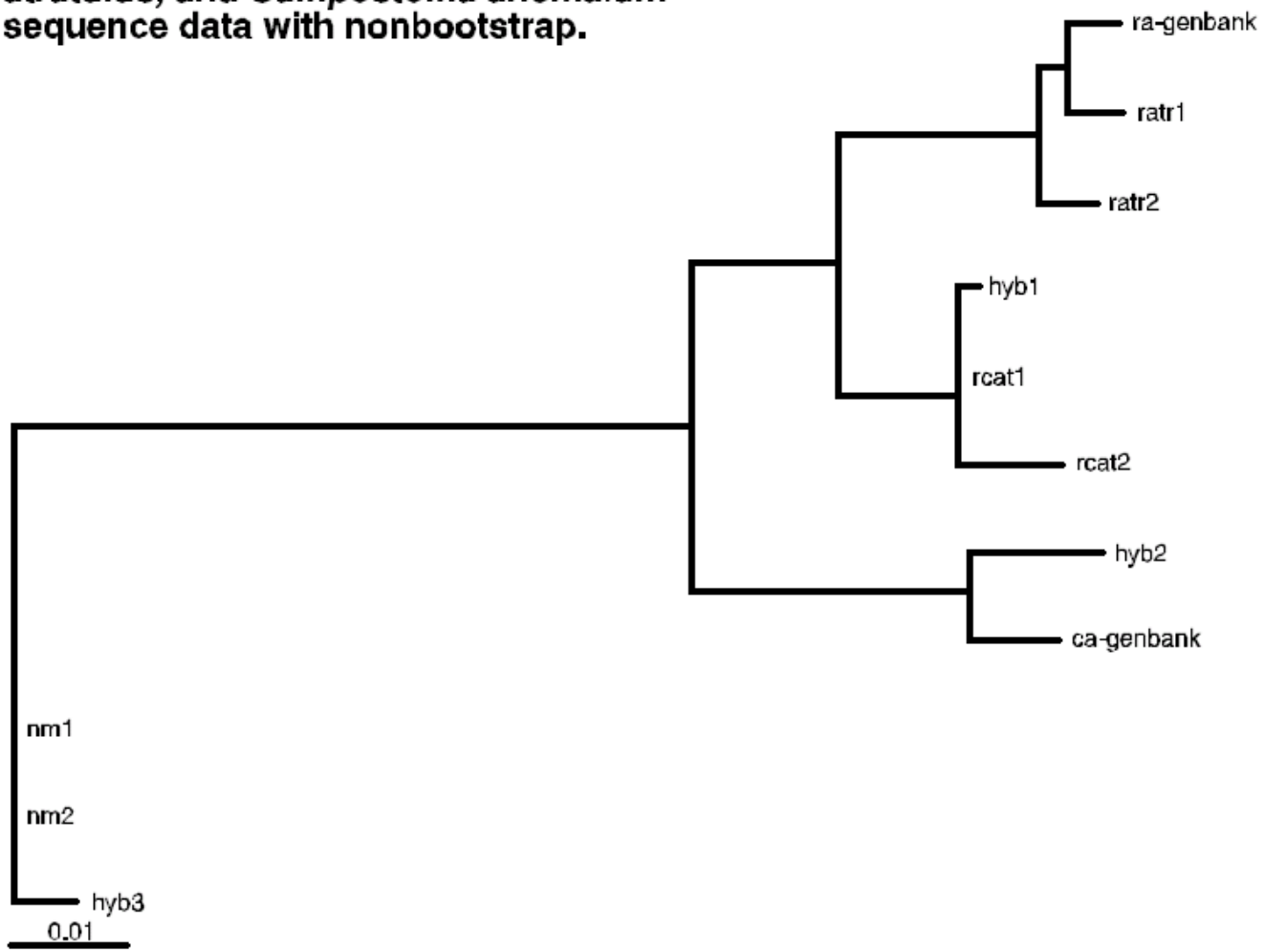


Figure 15. Distance matrix phylogenetic tree using the Fitch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum.* The labels bootstrap values after 100 resamples.

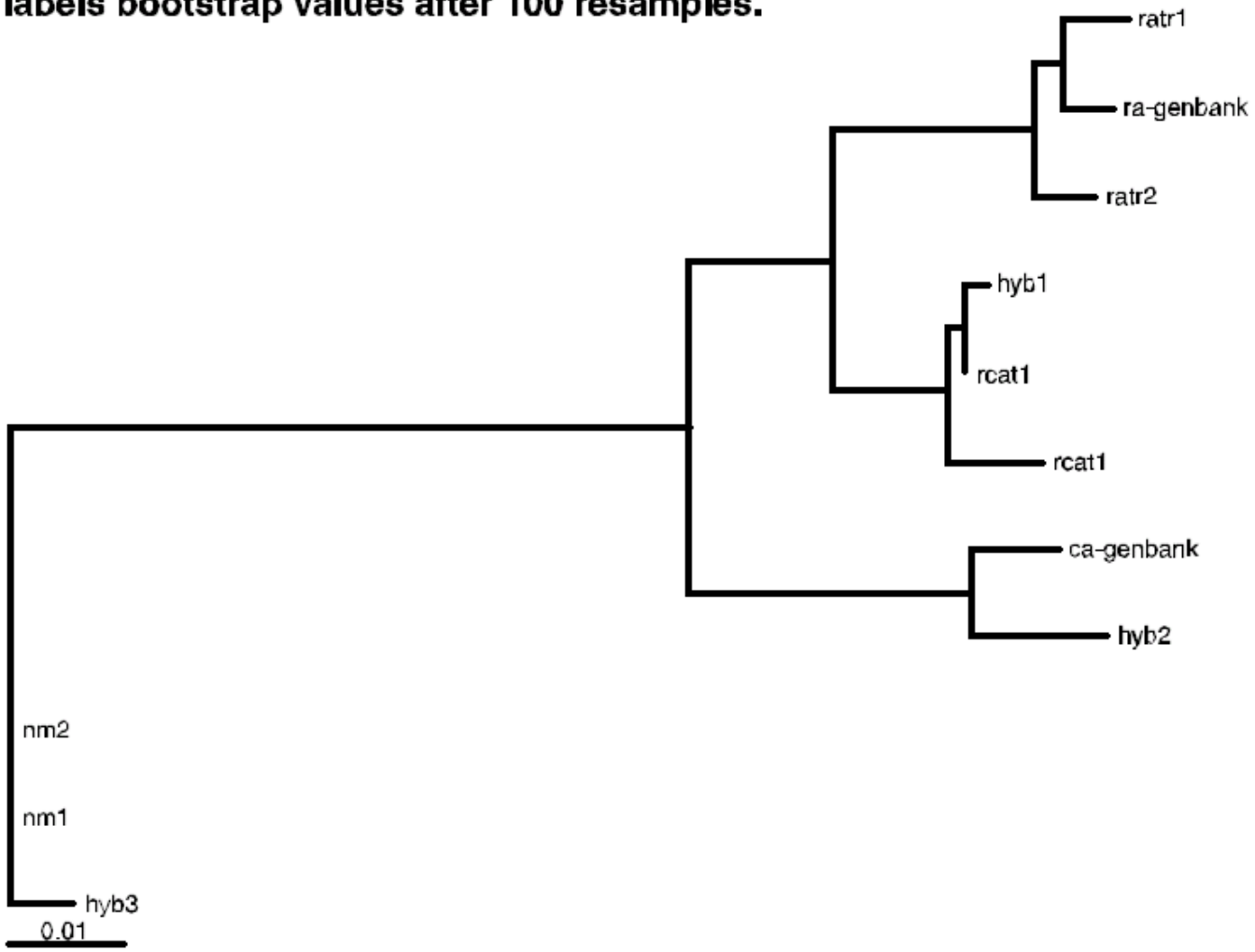


Figure 16. Phylip Neighbor Joining phylogenetic tree using the Fitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with nonbootstrap.

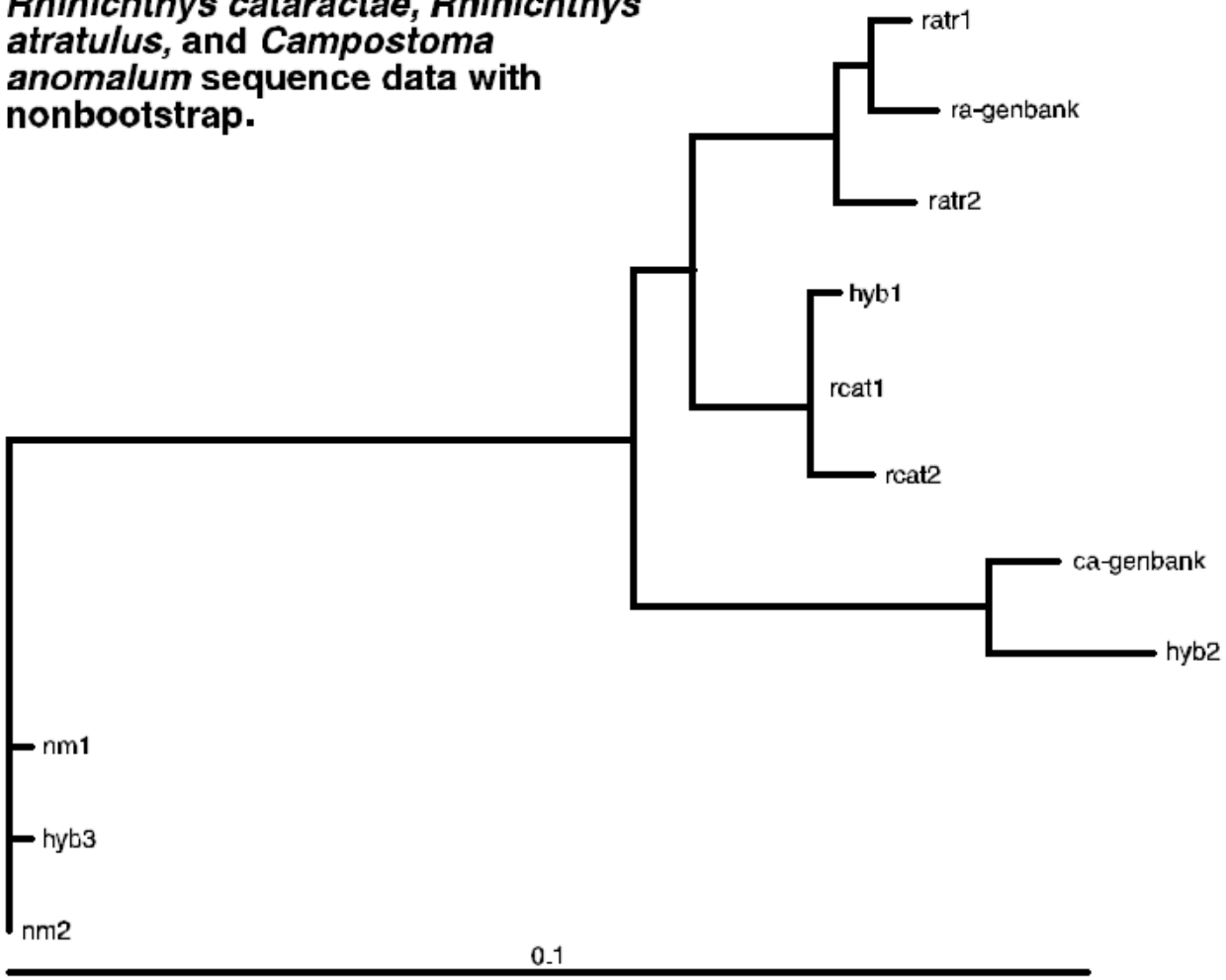


Figure 17. Distance matrix phylogenetic tree using the Fitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with nonbootstrap.

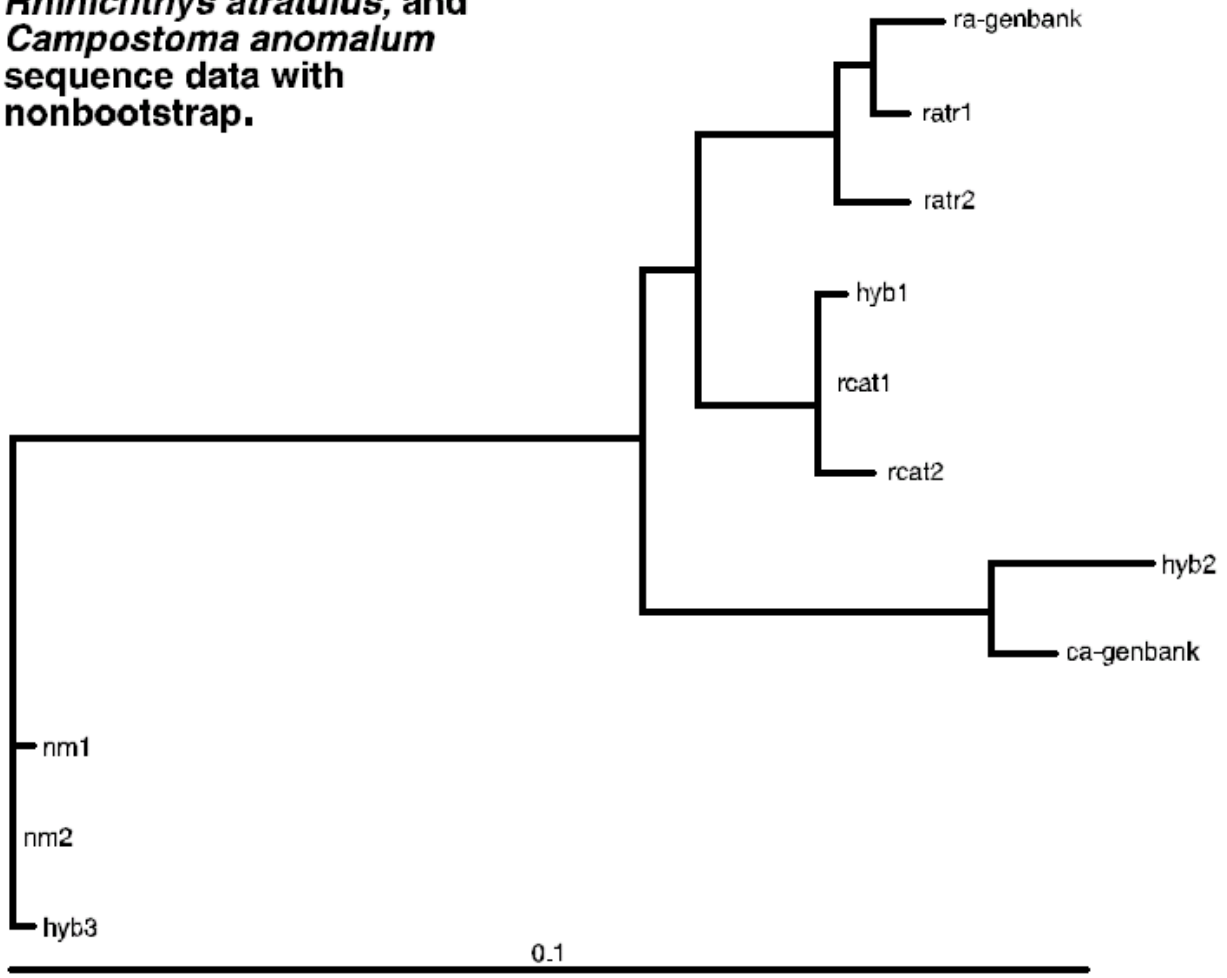


Figure 18. Maximum likelihood non-bootstrapped with jumble (10) option phylogenetic tree of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum*.

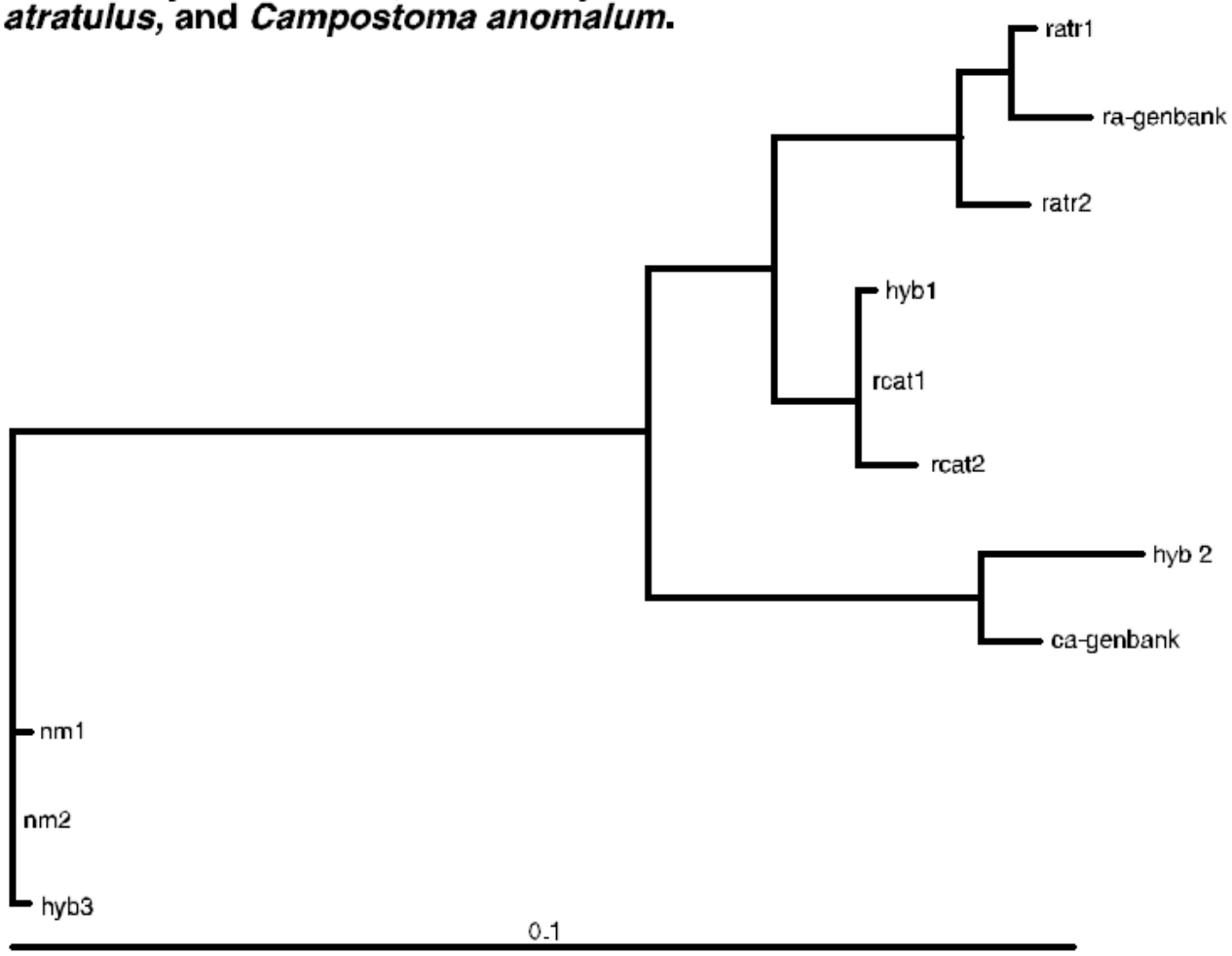


Figure 19. Distance matrix phylogenetic tree using the Kitsch of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data. Internal labels are bootstrap values after 100 resamples.

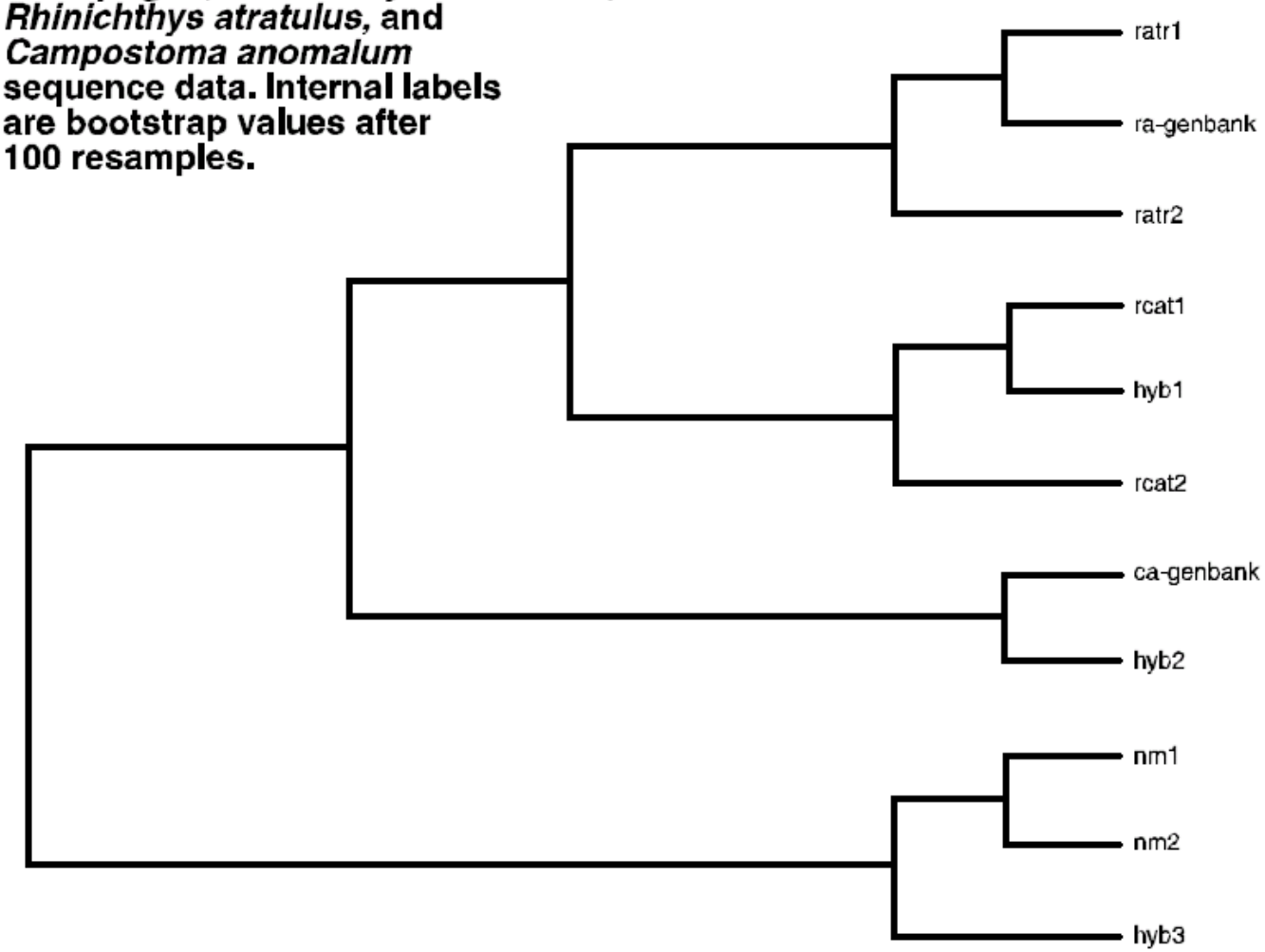


FIgure 20. Distance matrix phylogenetic tree using the Kitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with non-bootstap.

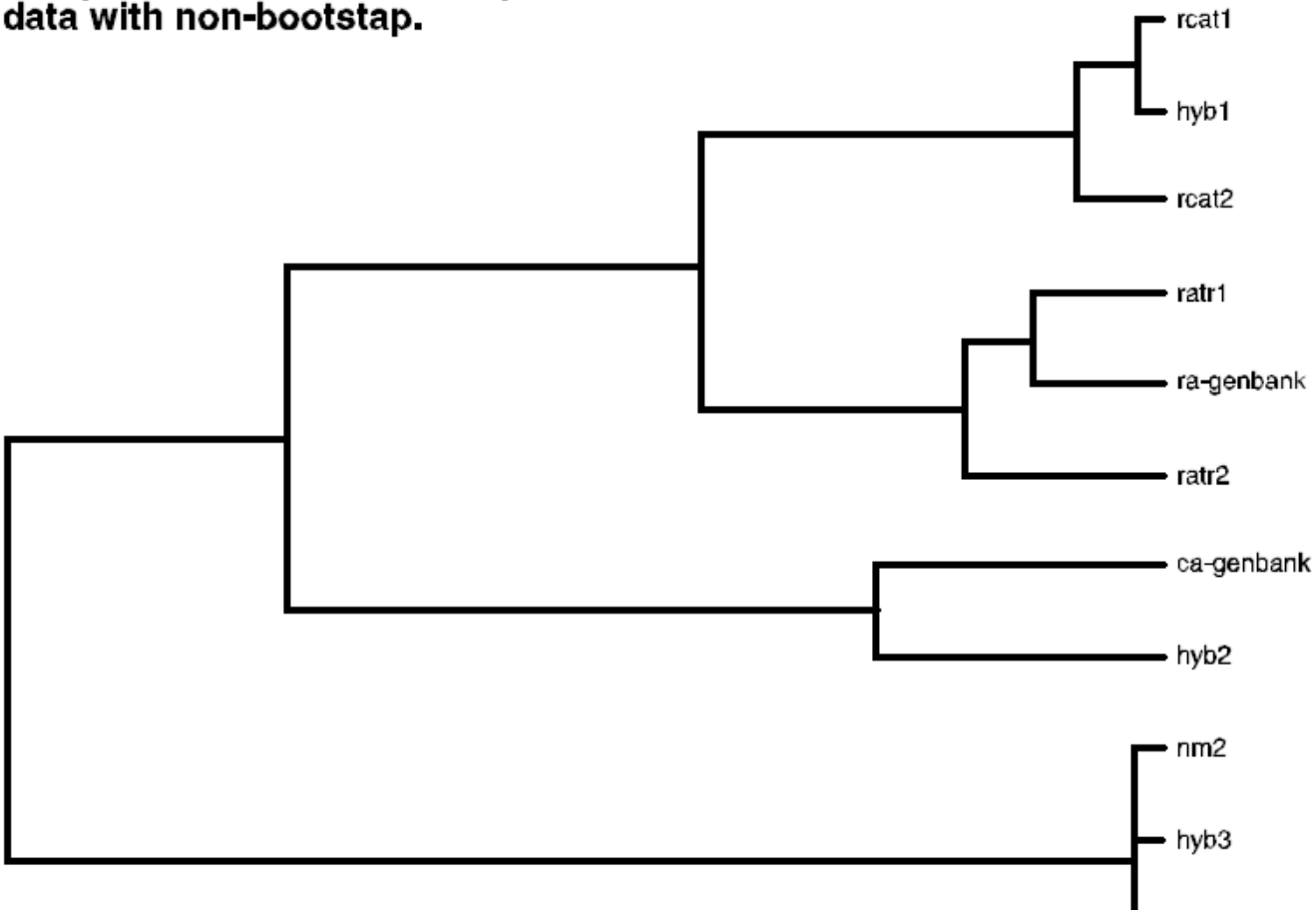


Figure 21. DNA pars phylogenetic tree *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* with 100 jumbles and NM2 as outgroup.

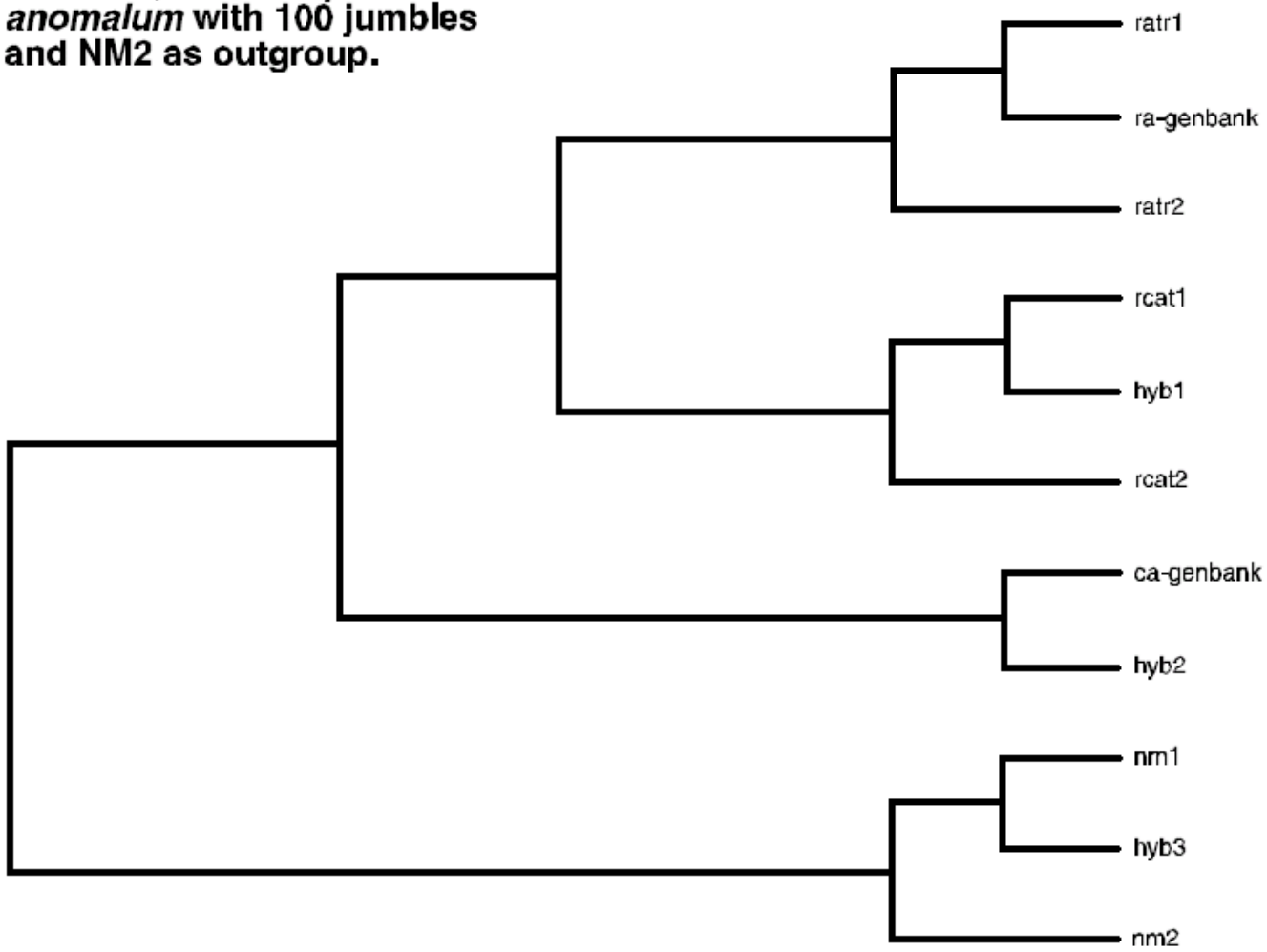


Figure 22. DNA pars of 100 jumbles phylogenetic tree with *Rhinichthys bowersi* as outgroup.

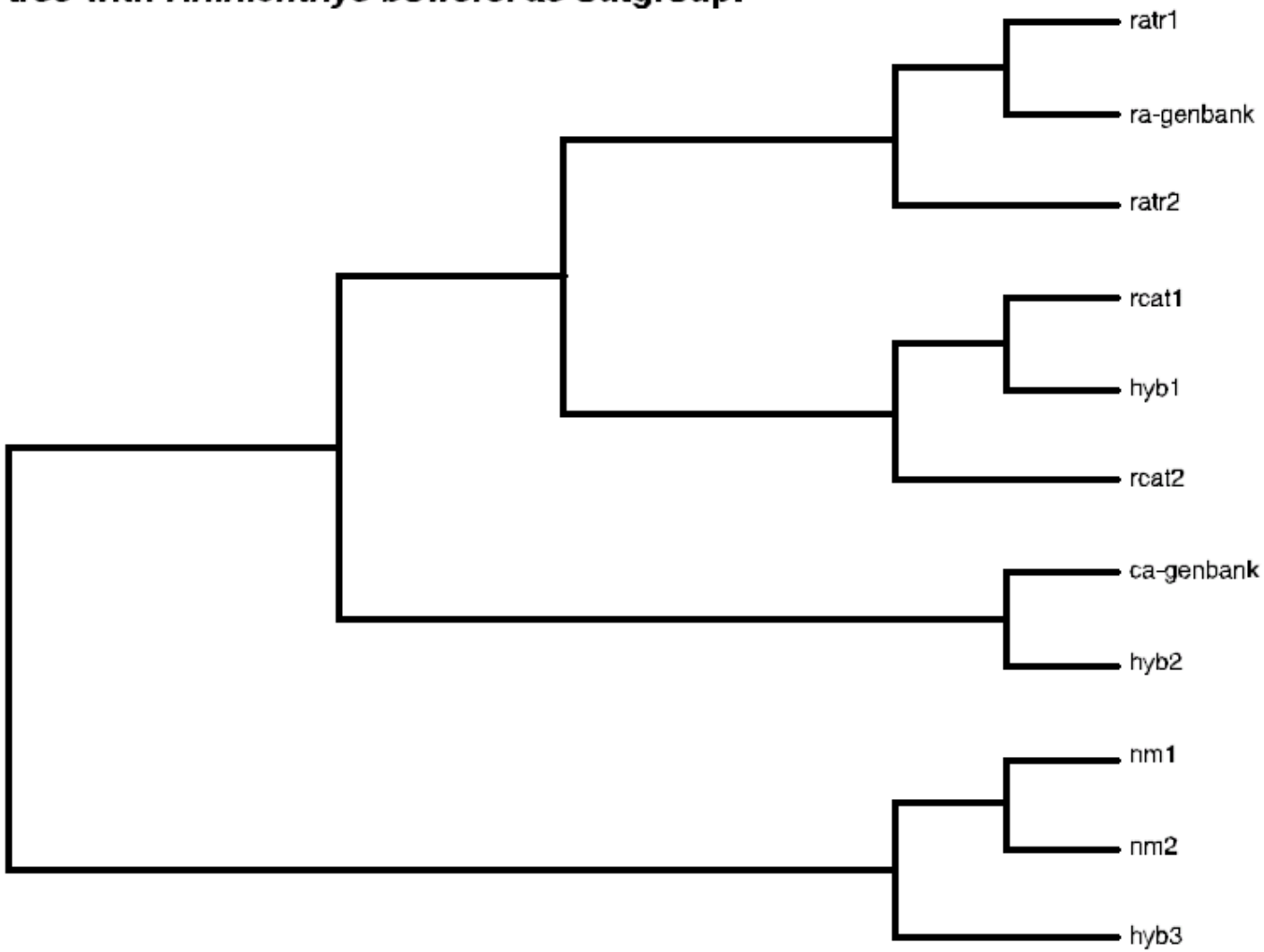


Figure 23. Sites where *Rhinichthys bowersi* were collected on Shavers Fork River near the Cheat Bridge on Route 250.

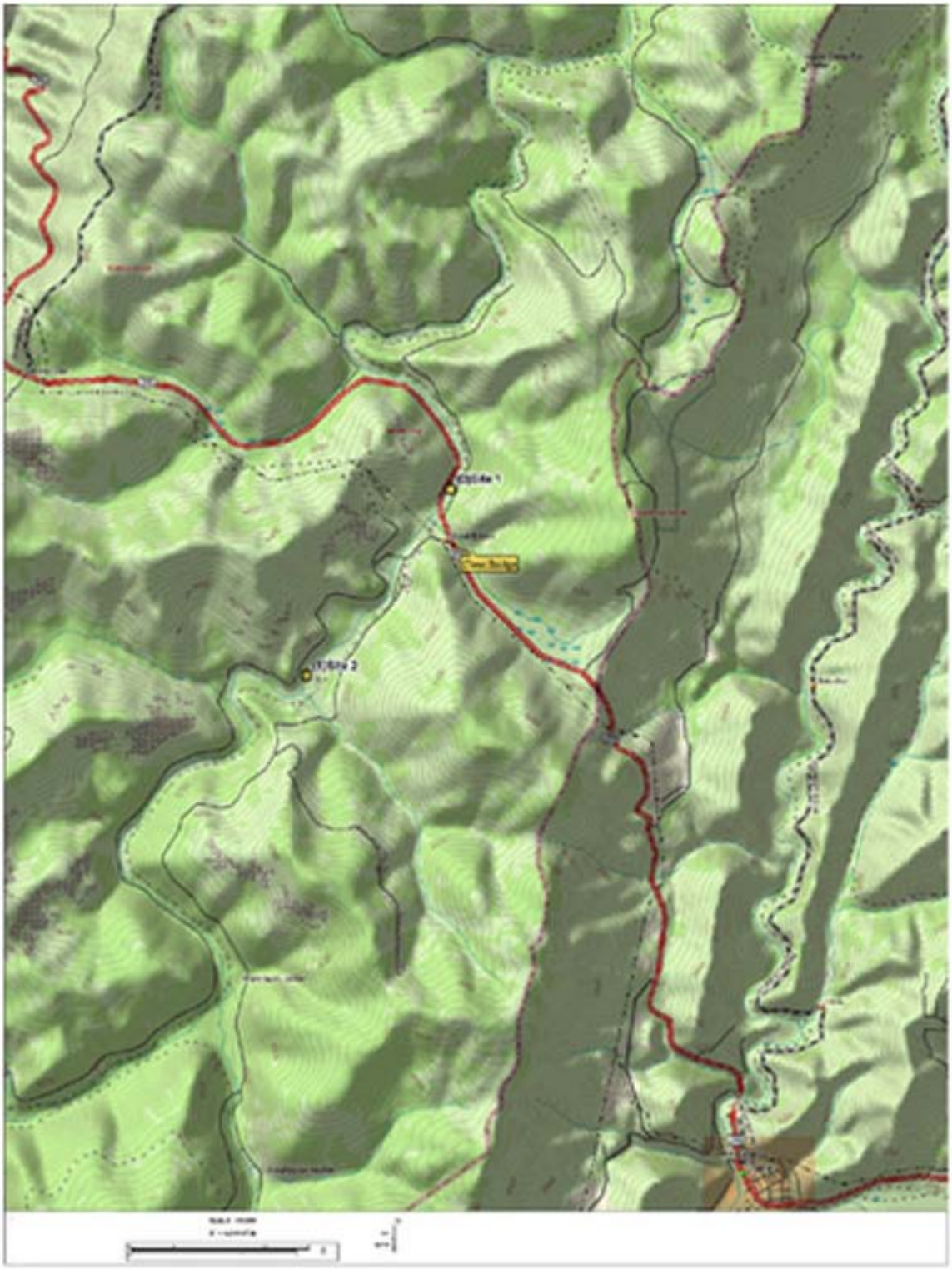
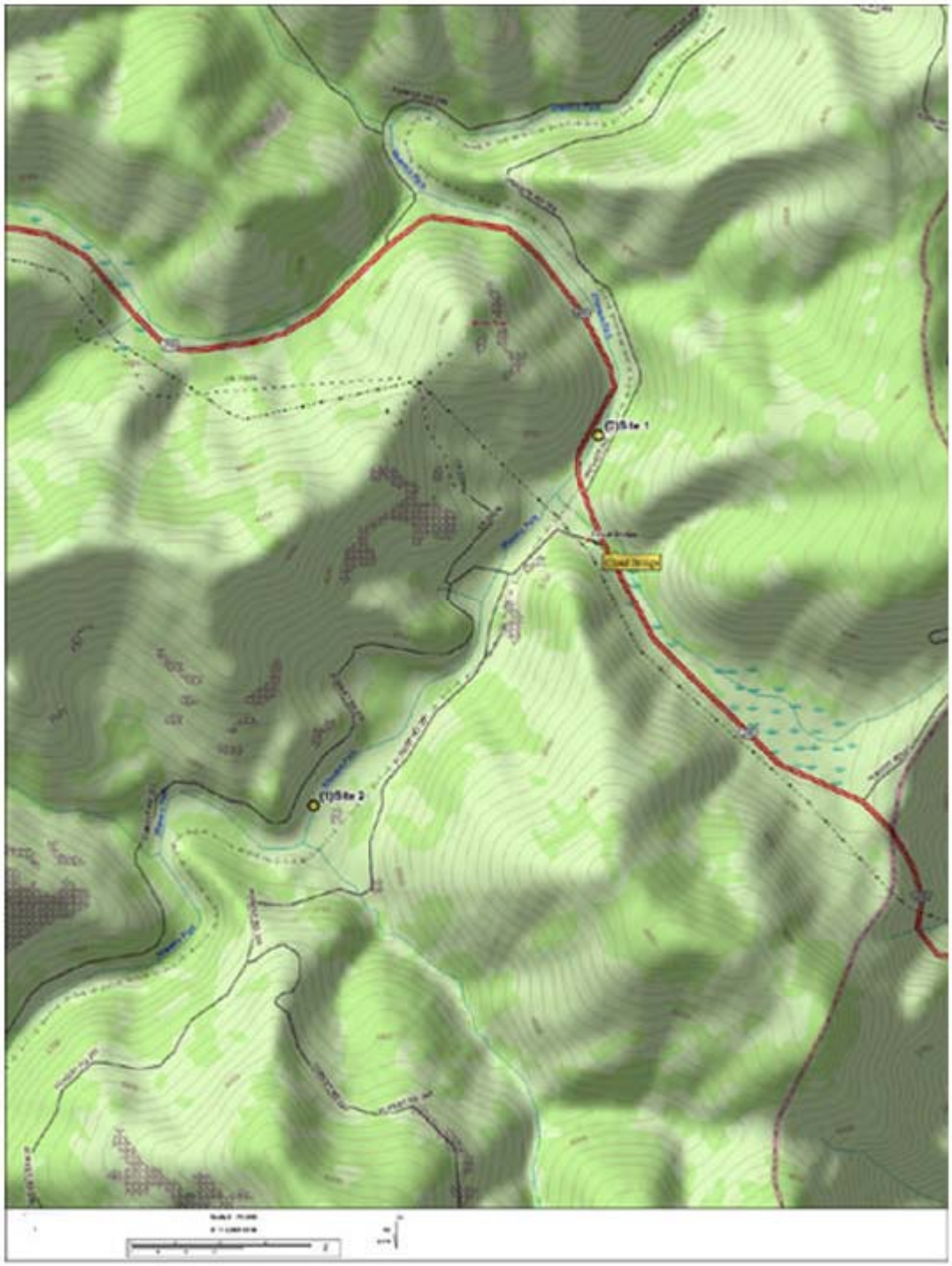


Figure 24. A close-up of the sites where *Rhinichthys bowersi* were collected on Shavers Fork River near the Cheat Bridge on Route 250.



PROTOCOLS

Qiagen DNeasy Protocol for Animal Tissues

1. Cut up to 25-50 mg tissue (up to 10 mg spleen) into small pieces, place in a 1.5-ml microcentrifuge tube, and add 180 µl Buffer ATL.
2. Add 20 µl Proteinase K, mix by vortexing, and inclubate at 55^{o}C until the tissue is completely lyed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.
3. Vortex for 15 seconds. Add 200 µl buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70^{o}C for 10 minutes.
4. Add 200 µl ethanol (100%) to the sample, and mix thoroughly by vortexing.
5. Pipette the mixture from step 4 into the DNeasy mini column sitting in a 2-ml collection tube. Centrifuge at greater than or equal to 6000 x g (8000 rpm) for 1 minute. Discard flow-through and collection tube.
6. Place the DNeasy mini column in a new 2- ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 minute at greater than or equal to 6000 x g (8000 rpm). Discard flow-through and collection tube.
7. Place the DNeasy mini column in a 2- ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 minutes at full speed to dry the DNeasy membrane. Discard flow-through and collection tube.
8. Place the DNeasy mini column in a clean 1.5-ml or 2-ml microcentrifuge tube (not provided), and pipette 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute, and then centrifuge for 1 minute at greater than or equal to 6000 x g (8000rpm) t elute.
9. Repeat elution once as described in step 8.

Source: Qiagen Manual

Qiagen Protocol for Cleanup of Dye-Terminator Sequencing Reactions
Using DyeEx Spin Kits

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn.
3. Snap off the bottom closure of the spin column, and place the spin column in a 2-ml collection tube (provided).
4. Centrifuge for 3 minutes at 3000 rpm for Eppendorf Centrifuge 5415C.
5. Carefully transfer the spin column to a clean microfuge tube. Slowly apply the sequencing reaction (10 µl – 20 µl) to the gel bed.
6. Centrifuge for 3 minutes at the calculated speed.
7. Remove the spin column from the microfuge tube.
8. Dry the sample in a vacuum centrifuge and proceed according to the instructions provided with the DNA sequencer.

Source: Qiagen Manual

Clontech AdvanTage™ PCR Cloning Kit

Cloning Procedure

1. Briefly centrifuge one tube of pT-Adv to collect all the liquid in the bottom.
2. Mark the date of first use on the tube. If there is any vector remaining after the experiment, store at –20^{o}C or –70^{o}C.

3. Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmol) of pT-Adv: x ng PCR product = (y bp PCR product) (50 ng pT Adv) (size of pT-Adv: ~3,900 bp)
4. Calculate the volume of PCR product needed for x ng (determined in step 3). Dilute your PCR sample with sterile H_2O if necessary.
5. Set up the ligation reaction as follows;
PCR product (<1 day old) x µl
10X ligation buffer 1 µl
pT-Adv Vector (25 ng/µl) 2 µl
Sterile H_2O x µl
T4 DNA ligase (4.0 Weiss units) 1 µl
Total volume 10 µl
6. Incubate the ligation reaction at 14^{o}C for a minimum of 4 hours (preferably overnight). Higher of lower temperatures may reduce ligation efficiency.
7. Proceed to Transormation. If you cannot transform immediately, store your ligation reaction at –20^{o}C until you are ready.
Source: Clonetech Manual

Transformation
1. Briefly centrifuge tubes containing the ligation reactions and place them on ice.
2. On ice, thaw the tube of 0.5 M β–mercaptoethanol (β-ME), along with one 50-µl tube of frozen TOP10F$_1$ *E. coli* competent cells for each ligatin/transformation.
3. Pipette 2 µl of 0.5 M β-ME into each tube of competent cells and mix by stirring gently with the pipette tip. *Do not mix by pipetting up and down.*
4. Pipette 2 µl of each ligation reaction directly into the mixture from Step 3 and mix by stirring gently with the pipette tip.
5. Incubate the tubes on ice for 30 minutes. Store the remaining ligation mixtures at –20^{o}C.
6. Heat shock for *exactly* 30 seconds in the 42^{o}C water bath. Do not mix or shake.
7. Remove the tubes from the 42^{o}C water bath and place on ice for 2 minutes.
8. Add 250 µl of SOC medium (at room temperature) to each tube.
9. Shake the tubes horizontally at 37^{o}C for 1 hour at 225 rpm in a rotary shaking incubator.
10. Place the tubes containing the transformed cells on ice.
11. Spread 50 µl and 200 µl from each transformation on separate, labeled LB/Amp/X-Gal/IPTG plates containing 50 µg/ml of either kanamycin or ampicillin.
12. Make sure the liquid is absorbed, then invert the plates and place them in a 37^{o}C incubator for at least 18 hours.
13. Shift plates to 4^{o}C for 2-3 hours to allow proper color development.
Source: CloneTech Manual

Qiagen QIAprep Spin Miniprep Kit Protocol
1. Resuspend pelleted bacterial cells in 250 µl of Buffer P1 and transfer to a microfuge tube.
2. Add 250 µl of Buffer P2 and invert the tube gently 4-6 times to mix.
3. Add 350 µl of Buffer N3 and invert the tube immediately but gently 4-6 times.
4. Centrifuge for 10 minutes. During centrifugation, prepare the vacuum manifold and QIAprep columns: QIAvac 24.

5. Apply the supernatant from step 4 to the QIAprep column by decanting or pipetting.
6. Switch on vacuum source to draw the solution through the QIAprep columns, and then switch off vacuum source.
7. (Optional): Wash QIAprep column by adding 0.5 ml of Buffer PB.
Switch on vacuum source. After the solution has moved through the column, switch off vacuum source.
8. Wash QIAprep columns to a microfuge tube. Centrifuge for 1 minute.
9. Transfer the QIAprep columns to a microfuge tube. Centrifuge for 1 minute.
10. Place QIAprep column in a clean 1.5-ml microfuge tube. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O to the center of the QIAprep column, let stand for 1 minute, and centrifuge for 1 minute.
Source: Qiagen Manual

Analysis of the Transformations
1. Pick 10 white colonies for plasmid isolation and restriction analysis.
2. Grow colonies in 6 ml of LB broth containing 100 µ g/ml of ampicillin.
3. Isolate plasmid and analyze by restriction digestion. Do digestion for 1 hour.
2µl DNA
2µl 10x buffer
1 µl enzyme
15 µl water
TOTAL 20µl
Source: CloneTech Manual
Qiagen QIAquick PCR Purification Kit Protocol
1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and
mix. It is not necessary to remove mineral oil or kerosene.
2. Prepare the vacuum manifold and QIAquick columns.
3. To bind DNA, load the samples into the QIAquick columns by decanting or pipetting, and apply vacuum. After the samples have passed through the column, switch off the vacuum source.
4. To wash, add 0.75 ml of Buffer PE to each QIAquick column and apply vacuum.
5. Transfer each QIAquick column to a microfuge tube or the provided 2-ml collection tubes. Centrifuge tubes. Centrifuge for 1 minute at greater than or equal to 10,000 x g (~13,000 rpm).
6. Place each QIAquick column into a clean 1.5-ml microfuge tube.
7. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or
H_2O) to the center of each QIAquick column, and centrifuge for 1 min at greater than or equal to 10,000 x g (~13,000 rpm).
Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of each QIAquick column, let stand for 1 min, and then centrifuge.
Source: Qiagen Manual

PCR and Conditions
1. 10X Buffer for KlenTaq LA 5µl
2. Deoxynucleotide mix (dNTP) 1µl
3. Primer 1 (10pmol/µl) 1µl
4. Primer 2 (10pmol/µl) 1µl
5. DNA Template (500ng-1µg) ? µl

6. Betaine (5M) 10µl
7. KlenTaq LA Polymerase 0.5µl
8. Sterile water ? µl
Total 50µl
94 ^{o}C 5 min denaturation 1 cycle
94 ^{o}C 1.0 min denaturation
55 ^{o}C 1.0 min annealing 30 cycles
72 ^{o}C 1.5 min extension
72 ^{o}C 3.0 min extension 1 cycle
Source: Sigma Manual
QIAquick Gel Extraction Kit Protocol
1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50^{o}C for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 minutes during the incubation.
4. After the gel slice has dissolved completely, check that the color of mixture is yellow (similar to buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. To bind DNA, pipet the sample onto the QIAquick column and apply vacuum. After the sample has passed through the column, switch off vacuum source.
7. (Optional) Add 0.5 ml of Buffer QG to QIAquick column and apply vacuum.
8. To wash, add 0.75 ml of Buffer PE to QIAquick column and apply vacuum.
9. Transfer QIAquick column to a clean 1.5-ml microfuge tube or to a provided 2-ml collection tube. Centrifuge for 1 minute at
>10,000 x g (~13,000 rpm).
10. Place QIAquick column in a clean 1.5-ml microfuge tube.
11. To elute DNA, add 50 ul of Buffer EB (10 mM Tris-Cl, pH
8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 minute at >10,000 x g (~13,000 rpm). Alternatively, for increased DNA concentration, add 30 ul elution buffer, let stand for 1 minute, and then centrifuge for 1 minute.
Source: Qiagen Manual

Stock Solution
0.5M (pH 8) EDTA
Dissolve 186.1 g 800ml of H_2O use a magnetic stir
Add 20 g of NaOH to adjust the pH to 8
Allow to cool then bring volume to 1 liter and autoclaving
Ethidium Bromide (10mg/ml)
Add 1 g of ethidium bromide to 100ml of H_2O stir on magnetic stir for several hours
Store in dark container and room temperature.
3M Sodium Acetate
Dissolve 408.3 g of sodium acetate in 800ml of H_2O
Adjust pH 5.2 with glacial acetic acid
Adjust the volume to 1 liter with H_2O autoclaving
1M Tris-Cl

Dissolve 121.1 g of Tris base in 800ml of H_2O.
Add 42 ml of concentrated HCL
Bring volume to 1 liter and autoclaving

Buffers Solution

10x TE

100mM Tris-cl (pH 8)
10mM EDTA (pH 8)
autoclaving

50x TAE

Dissolve 242g of Tris base in 700ml of H_2O.
Add 57.1ml of glacial acetic acid
Add 100ml of 0.5M EDTA
Bring volume to 1 liter and autoclaving

6x Gel-loading Buffer type II

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
15% (w/v) Ficoll (type 400; Pharmacia) in H_2O

Enzyme Stocks

Proteinase K (20mg/ml)

Dissolve lyophilized powder at concentration of 20mg/ml in sterile 50mM Tris (pH 8), with 1.5mM calcium acetate. Store at -20^oC

Pancreatic Rnase 10mg/ml

Dissolve 10 mg in 10 ml of TE

Media

LB Medium Broth

To 950 ml of H_2O
Add 10 g tryptone
Add 5 g yeast extract
Add 10 g NaCl
Adjust volume to 1 liter
Sterilize by autoclaving
When cool add 100 μg/ml ampicillin

LB Medium Plates

To 950 ml of H_2O
Add 10 g tryptone
Add 5 g yeast extract
Add 10 g NaCl
Add bacto agar 15 g per liter
Adjust volume to 1 liter
Sterilize by autoclaving
When cool add 50 μg/ml ampicillin, X-Gal and IPTG

SOB Medium

To 950 ml of H_2O
Add 20 g tryptone
Add 5 g yeast extract
Add 0.5 g NaCl

Add 10 ml of 250mM solution KCl (1.86 g of KCl in 100 ml of H_2O)
Adjust pH 7 with 5 N NaOH
Adjust volume to 1 liter
Sterilize by autoclaving