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Molecular Systematics of Rhinichthys bowersi and its Taxonomic Status

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by

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ABSTRACT

Molecular Systematics of *Rhinichthys bowersi* and its Taxonomic Status

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Rhinichthys bowersi is a cyprinid fish that is found mostly in tributaries of the Monongahela River. Several investigators have examined the morphology, protein polymorphism, and parasite/host relationships of *R. bowersi* and concluded that it is a valid species of introgressive hybrid origin while others have examined the same data and concluded that it is an F1 hybrid of *R. cataractae* and *Nocomis micropogon*. In this study, mitochondrial DNA was amplified from the 12s gene of *R. bowersi* and compared to that of *R. cataracta, N. micropogon*, and *R. atratulus*, an outgroup. Mitochondrial DNA differences were analyzed by Neighbor Joining (NJ), FITCH, and KITSCH algorithms. Of three individuals of *R. bowersi*, one clustered with *R. cataractae*, one clustered with *N. micropogon*, and another as a cyprinid outgroup. An analysis of 12s DNA sequences of three *R. bowersi* indicated that it was not a valid taxonomic entity, but was the result of multiple, hybrid origins.

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LIST OF SYMBOLS / NOMENCLATURE

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 – Rhinichthy 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*

CHAPTER I

Introduction

From 1890 until 1975, 14 specimens of *Rhinichthys 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 1940a, E.C. Raney collected specimens of the Cheat minnow from the Cheat Bridge area of Shavers Fork and re-described it as a hybrid *Nocomis micropogon* and *Rhinichthys 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).

CHAPTER II

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:

Table 1

	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 indentifying characteristics of *R. bowersi* are between the two intermediate suspected parental species, but do not overlap. *Nocomis 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 Connallsville, 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 Susquehahann 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).

"*Rhinichthys cataractae* is usually found in the rocky bottoms of small to medium size steams with swift waters and high gradients." It is widely distributed throughout North America, including parts of northern Mexico. It is most abundant from the Great Lakes to Appalachian mountains 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. micropon* were 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 1940b, Raney 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 "probably accounts for the majority of known 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).

CHAPTER III

Methods and Materials

The collecting of fish was done by the use of a Smith-Root SR12 Barge with a 7.5 gpp electrofisher (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 (Figure 3). The electrofisher was maneuvered in a zig-zag 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^{o} 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 (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^{o}C for a five-minute cycle; 94^{o}C 45-second denaturation; 55^{o}C one minute annealing; 72^{o}C one minute extension for 30 cycles followed by 72^{o}C extension for three minutes for one cycle and 4^{o}C for infinity. Amplification was checked on 1% gel of Sea Kem GTG agarose (FMC) (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 (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 (Figures 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 non-boostrapped. 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 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

CHAPTER IV

Results

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* (Figure 10). Specimens hyb1 had three variable sites from *R. cataractae* (Rcat2) and one variable site from *R. cataractae* (Rcat1) (Figure 11). Also, specimens hyb2 had 19 variable sites from *C. anomalum* from GenBank (Figure 12), with hyb3 having one variable site from *N. micropogon* (NM1) and two variable sites from *N. micropogon* (NM2) (Figure 13). The distance matrix from each specimen is shown in Table 3. Ten phylogenetic trees (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.

CHAPTER V

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 morphometric 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. *Rhinichthys 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 groups of populations, which are distinguishable on the basis of one or more heritable characters" (Harrison, 1993). Introgression is "the permanent incorporation of genes from one set of differentiated populations into another, i.e. 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 ancestory 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 contrary to what would be expected 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). Also, 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 a 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 later is common in Shavers Fork and known to participate in hybrid reproduction with other cyprinids.

Phylogenetic relationships are represented in 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.

CHAPTER VI

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. cataractatae* 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.

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 .

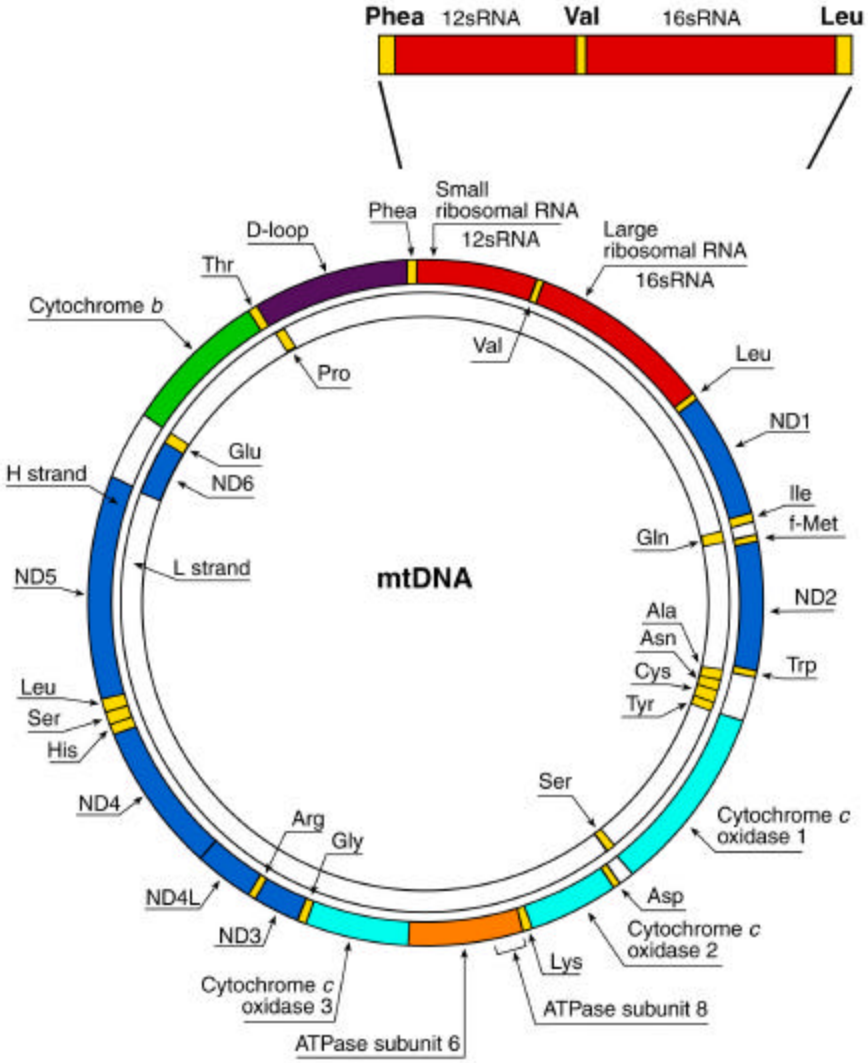

Figure 2. A Smith-Root SR12 Barge was used for collecting *R. bowersi* specimen.

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.

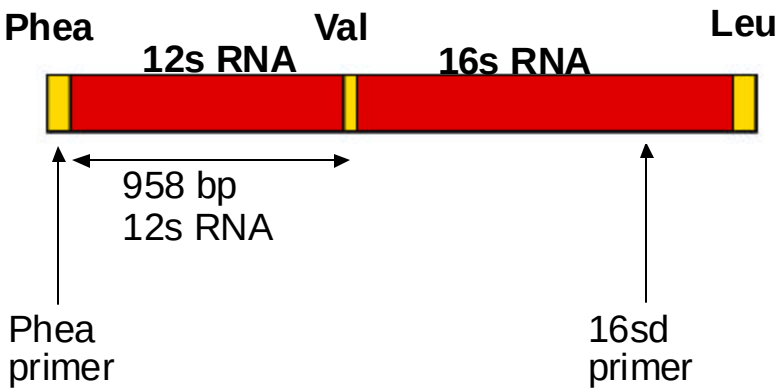

Figure 6. This 1% agarose gel is of all samples that were amplified by PCR. The correct band size was 2,000 bp.

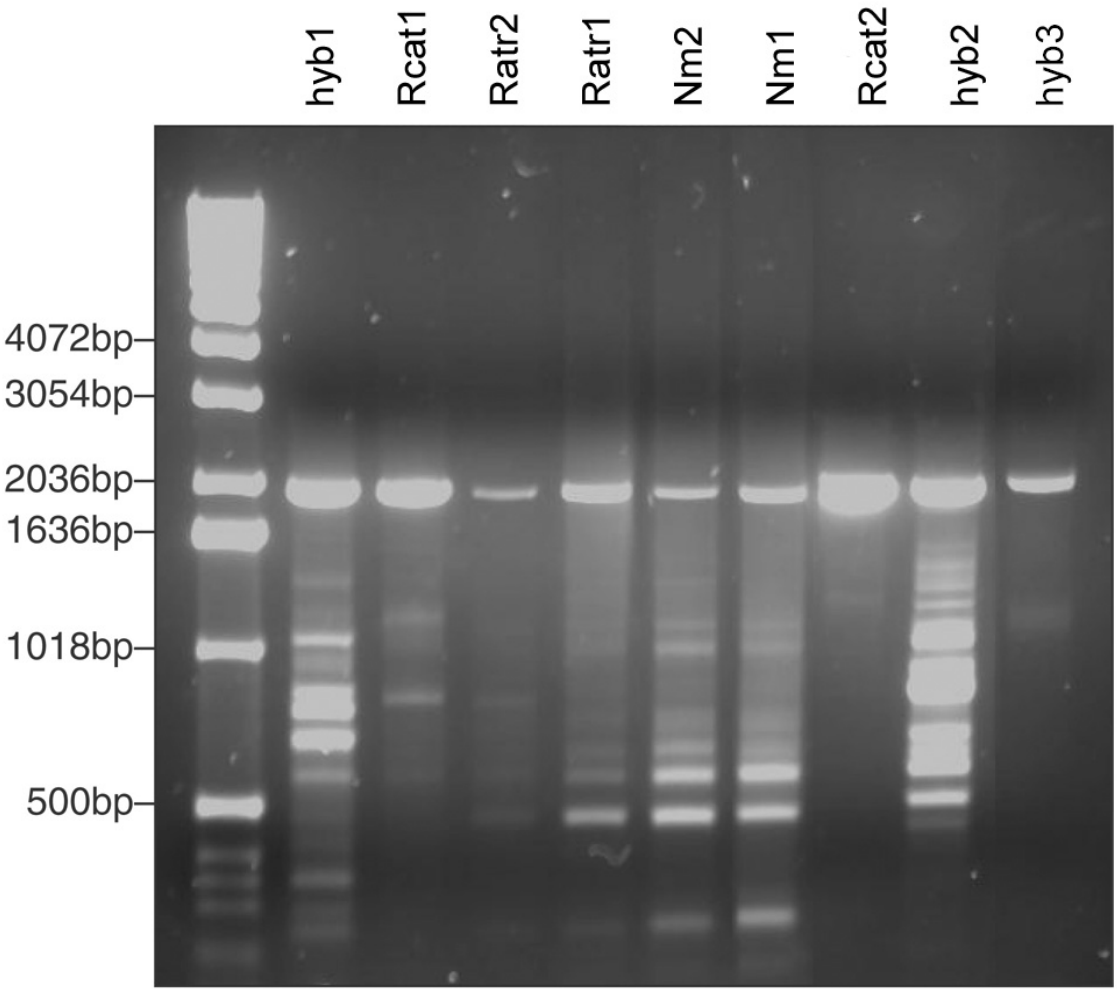

Figure 7. This 1% agarose gel is of a restriction digest using EcoR I on two *F*
bowersi and two *N. micropogon* specimens. The vector size is 3,900 bp, and
insert is 2,000 bp.

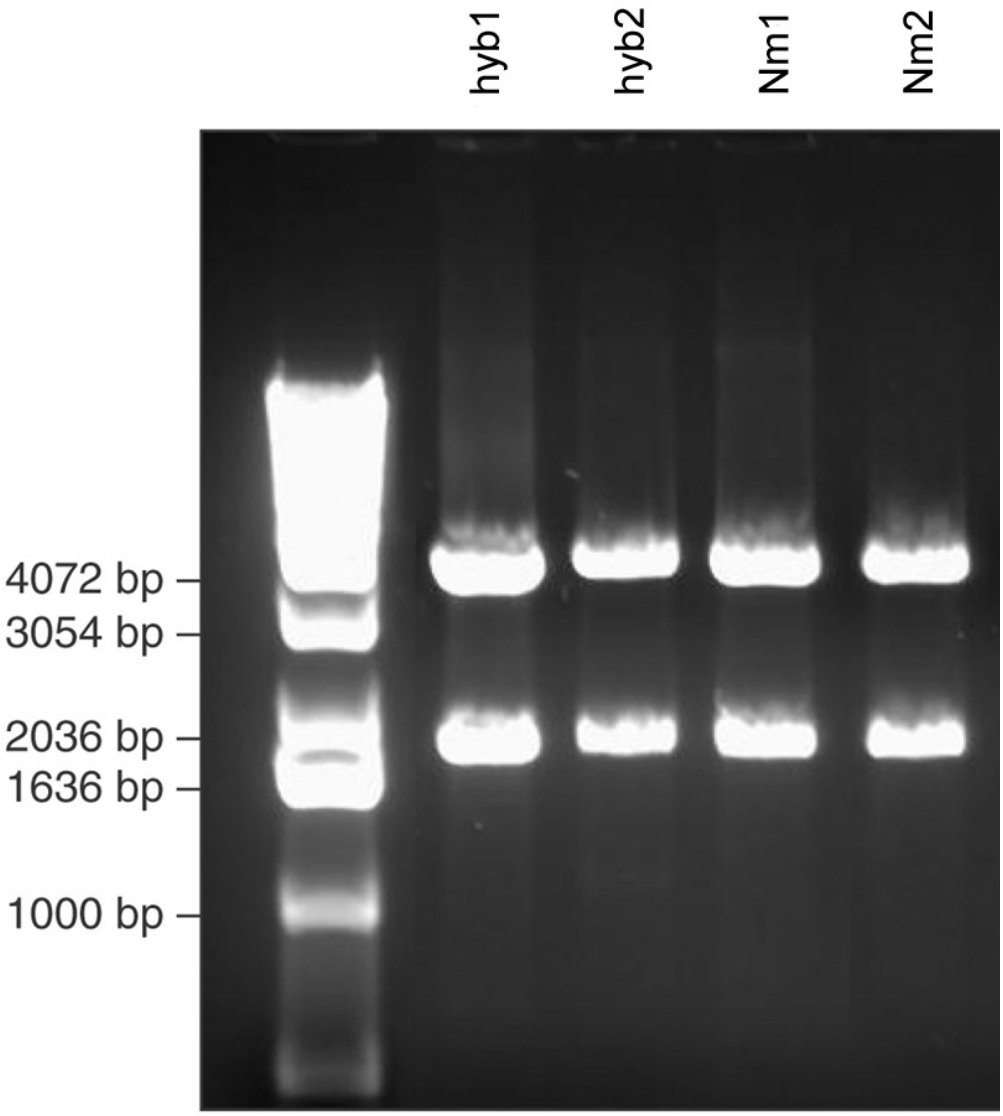

Figure 8. This is a chromatograph of Rhinichthys bowersi. This sample was cloned and sequenced on an ABI 377.

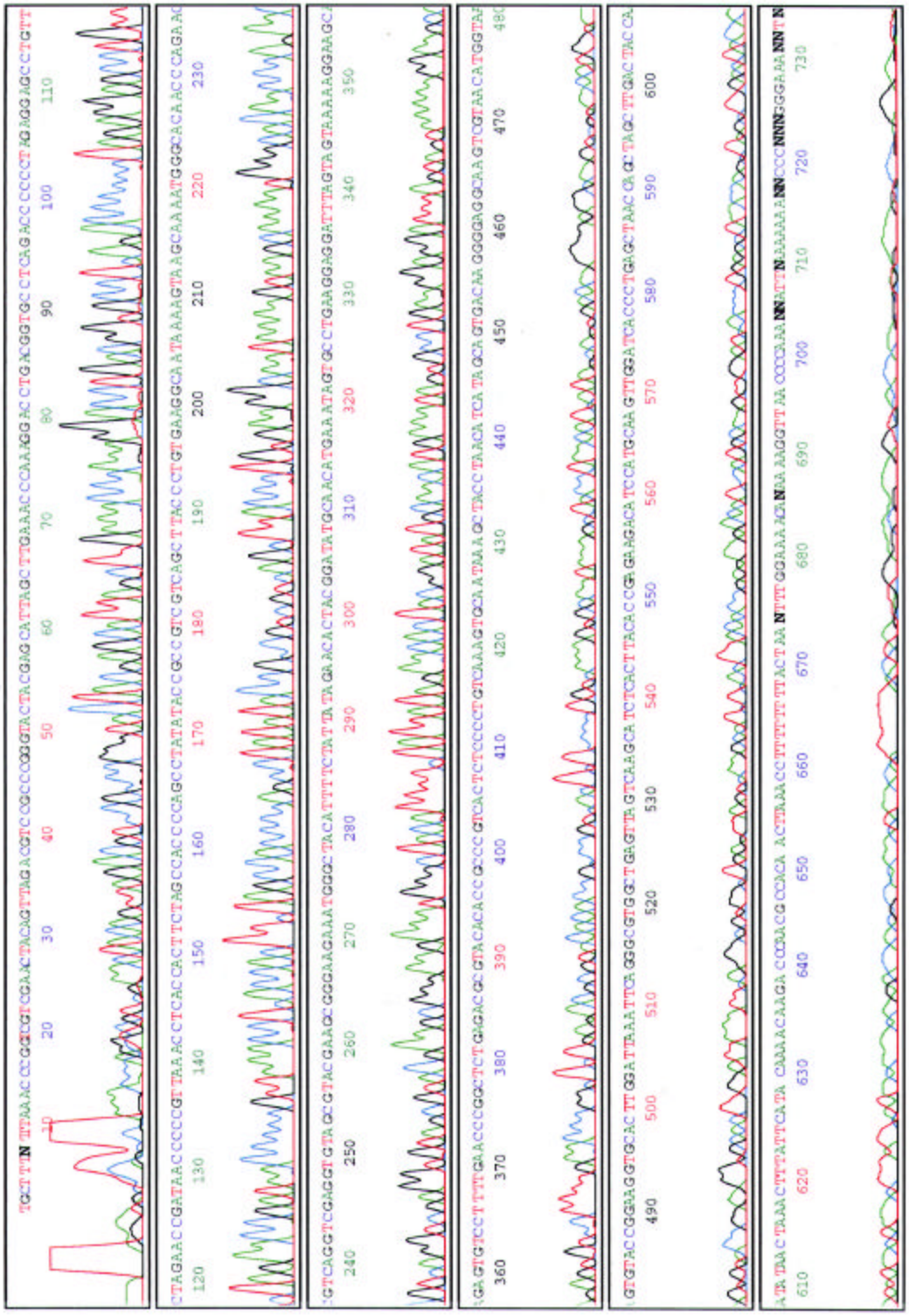

Figure 9. The complete 12s mtDNA for all nine fish, plus two from GenBank (*). The sequences were aligned in Sequencer.

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hyb1          1  CAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Rcat1         1  CAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Rcat2         1  CAAAGGCATGGTCCTGACCTTATTTTTAGCTCTAACCCAACTTACACATG
RA*           1  TAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Ratr1         1  TAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Ratr2         1  TAAAGGCATGGTCCTGACCTTATTATCAGCTCTAGCCCAACTTACACATG
hyp2          1  TAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCTAACTTACACATG
CA*           1  TAAAGGCATGGTCCTGACCTTATTATCAGCTTTAACCTAACTTACACATG
hyp3          1  CAAAGGCATGGTCCCGACCTTATCATCAGCTCTAACCTAACTTACACATG
nm1           1  CAAAGGCATGGTCCCGACCTTATCATTAGCTCTAACCTAACTTACACATG
nm2           1  CAAAGGCATGGTCCCGACCTTATCATCAGCTCTAACCTAACTTACACATG

hyb1         51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
Rcat1        51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
Rcat2        51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
RA*          51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
Ratr1        51  CAAGTCTCCGCAACCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
Ratr2        51  CAAGTCTCCGCAACCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
hyp2         51  CAAGTCTCCGCAATCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
CA*          51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTCAATCCCCTGCCCGGGGACG
hyp3         51  CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
nm1          51  CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
nm2          51  CAAGTCTCCGCACCCCTGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG

hyb1        101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC
Rcat1       101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC
Rcat2       101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC
RA*         101  AGGAGCAGGCATCAGGCACAAACATTTAGCCCAAGACGCCTGGCCTAGCC
Ratr1       101  AGGAGCAGGCATCAGGCACAAACATTTAGCCCAAGACGCCTGGCCTAGCC
Ratr2       101  AGGAGCAGGCATCAGGCACAAACATTTAGCCCAAGACGCCTGGCCTAGCC
hyp2        101  AGGAGCAGGTATCAGGCACGAACCCTTAGCCCAAGACGCCTGGCCTAGCC
CA*         101  AGGAGCAGGTATCAGGCACAAACCCTTAGCCCAAGACGCCTGGCCTAGCC
hyp3        101  AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC
nm1         101  AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC
nm2         101  AGGAGCAGGCATCAGGCACAGATTTCTAGCCCAAGACGCCCAGCCTAGCC

hyb1        151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
Rcat1       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
Rcat2       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATGAGTGAAAA
RA*         151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
Ratr1       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATGAGTGAAAA
Ratr2       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
hyp2        151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
CA*         151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
hyp3        151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
nm1         151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
nm2         151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAG
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hyb1      201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Rcat1     201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Rcat2     201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
RA*       201  CTTGACTCAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Ratr1     201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Ratr2     201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
hyp2      201  CTTGACTCAGTTAAGGCTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
CA*       201  CTTGACTCAGTTAAGGTTAAAAGGGCCGGTAAAACTCGTGCCAGCCACCG
hyp3      201  CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
nm1       201  CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
nm2       201  CTTGACTTAGACAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG

hyb1      251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
Rcat1     251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
Rcat2     251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
RA*       251  CGGTTAAACGAGAGGCCCTAGTTGATAGTACAACGGCGTAAAGGGTGGTT
Ratr1     251  CGGTTAAACGAGAGGCCCTAGTTGATAGTACAACGGCGTAAAGGGTGGTT
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hyp2      251  CGGTTAGACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
CA*       251  CGGTTAGACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
hyp3      251  CGGTTAGACGAGAGGCCCTAGTTAATGATGTAACGGCGCAAAGGGTGGTT
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nm2       251  CGGTTAGACGAGAGGCCCTAGTTAATGATGTAACGGCGTAAAGGGTGGTT

hyb1      301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Rcat1     301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Rcat2     301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
RA*       301  AAGGGTAATAAATTAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Ratr1     301  AAGGGTAGTAAATTAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Ratr2     301  AAGGATACTAAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
hyp2      301  AAGGATACTGAGACAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
CA*       301  AAGGATACTGAGACAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
hyp3      301  AAGGATAGTAAATTAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT
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nm2       301  AAGGATAGTAAATTAATAAAGCCGAATGGCCCTTTGGCTGTCATACGCTT

hyb1      351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT
Rcat1     351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT
Rcat2     351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT
RA*       351  CTAGGAGTCCGAAGCCCAATATACGAAAGTAGCTTTAGGAAAGCCCACCT
Ratr1     351  CTAGGAGTCCGAAGCCCAATATACGAAAGTAGCTTTAGGAAAGCCCACCT
Ratr2     351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGGAAAGCCCACCT
hyp2      351  CTAGGAGTCCGAAGACCAATACACGAAAGTAGCTTTAAAGAAGTTCACCT
CA*       351  CTAGGAGTCCGAAGACCAATATACGAAAGTAGCTTTAAAGGAGTCCACCT
hyp3      351  CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
nm1       351  CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
nm2       351  CTAGGAGTCCGAAGCCCAACATACGAAAATAGCTTTAGAAAAGCCCACCT
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hyb1      401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
Rcat1     401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
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RA*       401  GACCCCACGAAAGCTGAGGAACAAACTGGGATTAGATACCCCACTATGCT
Ratr1     401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
Ratr2     401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
hyp2      401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCC
CA*       401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
hyp3      401  GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
nm1       401  GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
nm2       401  GACCCCACGAAAACTGAGAAACAAACTGGGATTAGATACCCCACTATGCT

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Rcat1     451  CAGCCATAAACCCAGACGTCCAACTACAATTAGACATCCGCCCGGGTACT
Rcat2     451  CAGCCATAAACCCAGACGTCCAACTACAATTAGACATCCGCCCGGGTACT
RA*       451  CAGCCATAAACCCAGGCGTCGAACTACAGTTAGACGTCCGCCCGGGTACT
Ratr1     451  CAGCCATAAACCCAGGCGTCGAACTACAGTTAGACGTCCGCCCGGGTACT
Ratr2     451  CAGCCATAAACCCAGGCGTCGAACTACAGTTAGACGTCCGCCCGGGTACT
hyp2      451  CAGCCGTAAACTTAGACGTCAACCTACAATAAGACGTCCGCCCGGTTACT
CA*       451  CAGCCGTAAACTTAGACGTCAACCTACAATAAGACGTCCGCCCGGGTACT
hyp3      451  CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT
nm1       451  CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT
nm2       451  CAGCCGTAAACTTAGATATTCAATTACAATTAAATATCCGCCCGGGTACT

hyb1      501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
Rcat1     501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
Rcat2     501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
RA*       501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
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Ratr2     501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
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CA*       501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
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nm1       501  ACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTGCCTTAGACCCCCC
nm2       501  ACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTGCCTTAGACCCCCC

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Rcat1     551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
Rcat2     551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
RA*       551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
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CA*       551  TAGAGGAGCCTATTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
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nm1       551  TAGAGGAGCCTGTTCTAGAACCGATAACCCTCGTTAAACCTCACCACTTC
nm2       551  TAGAGGAGCCTGTTCTAGAACCGATAACCCTCGTTAAACCTCACCACTTC
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RA*       601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
Ratr1     601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
Ratr2     601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
hyp2      601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
CA*       601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
hyp3      601  TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA
nm1       601  TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA
nm2       601  TAGCCACTC:AGCCTATATACCGCCGTCGCCAGCTTACCCTGTGAAGGCA

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Rcat1     651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
Rcat2     651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
RA*       651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
Ratr1     651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
Ratr2     651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
hyp2      651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
CA*       651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
hyp3      651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
nm1       651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG
nm2       651  ATAAAAGTAAGCAAAATGGGCACAACCCAGAACGTCAGGTCGAGGTGTAG

hyb1      701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
Rcat1     701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATAATAGAACACTACG
Rcat2     701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
RA*       701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
Ratr1     701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
Ratr2     701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATAATAGAATACTACG
hyp2      701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
CA*       701  CGTACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAACACTACG
hyp3      701  CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG
nm1       701  CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG
nm2       701  CATACGAAGCGGGAAGAAATGGGCTACATTTTCTATTATAGAATATCACG

hyb1      751  GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTAAGTAGTAAAAAGG
Rcat1     751  GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTA:GTAGTAAAAAGG
Rcat2     751  GATATGCAACATGAAATAGTGCTCGAAGGAGGATTTA:GTAGTAAAAAGG
RA*       751  GATGTGCAACATGAAATAGTGCCTGAAGGAGGATTTA:GTAGTAAAAAGG
Ratr1     751  GATATGCAACATGAAATAGTGCCTGAAGGAGGATTTA:GTAGTAAAAAGG
Ratr2     751  GATGTGCAACATGAAATAGTGCCTGAAGGAGGATTTA:GTAGTAAAAAGG
hyp2      751  GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAAAAGG
CA*       751  GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAAGAGG
hyp3      751  GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
nm1       751  GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
nm2       751  GACATGCAACATGAAATAGTGCTTGAAGGAGGATTTA:GTAGTAAGAAGG
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hyb1      801  AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
Rcat1     801  AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
Rcat2     801  AAGCAGAGTGTCCTTTTGAACTCGGCTCTGAGACGCGTACACACCGCCCG
RA*       801  AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGCCCG
Ratr1     801  AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGCCCG
Ratr2     801  AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGCCCG
hyp2      801  AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGGCCG
CA*       801  AAGCAGAGTGTCCTTTTGAACCCGGCTCTGAGACGCGTACACACCGCCCG
hyp3      801  AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG
nm1       801  AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG
nm2       801  AAGCAGCGTGTCCTTCTGAACCCGGCTCTGAGGCGCGTACACACCGCCCG

hyb1      851  TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
Rcat1     851  TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
Rcat2     851  TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACGCCAGAGCGGTG
RA*       851  TCACTCTCCCCTGTCAAAGTGCAATAAAGCTACCTAACATCATAGCAGTG
Ratr1     851  TCACTCTCCCCTGTCAAAGTGCAATAAAGCTACCTAACATCATAGCAGTG
Ratr2     851  TCACTCTCCCCTGTCAAAATGCAGTAAAGCTACCTAACATCATAGCAGCG
hyp2      851  TCACTCTTCCCTGTCAAAATGCAGCAAGACTACCTAATACTAAAGCCATG
CA*       851  TCACTCTCCCCTGTCAAAATGCAACAAGATTACCTAATACTAGAGCCATG
hyp3      851  TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG
nm1       851  TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG
nm2       851  TCACTCTCCCCTGTCAAAATGCAATAAGATTACCTAATGACAAAGCGCCG

hyb1      901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
Rcat1     901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
Rcat2     901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
RA*       901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAG:TGCACTTG
Ratr1     901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
Ratr2     901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAGGGTGCACTTG
hyp2      901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
CA*       901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAG:TGCACTTG
hyp3      901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
nm1       901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG
nm2       901  ACAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCACTTG

hyb1      951  GATTAAAT
Rcat1     951  GATTAAAT
Rcat2     951  GATTAAAT
RA*       951  GATTAAAT
Ratr1     951  GATTAAAT
Ratr2     951  GATTAAAT
hyp2      951  GATAAAAT
CA*       951  GATAAAAT
hyp3      951  GCTTAAAT
nm1       951  GCTTAAAT
nm2       951  GCTTAAAT
```

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

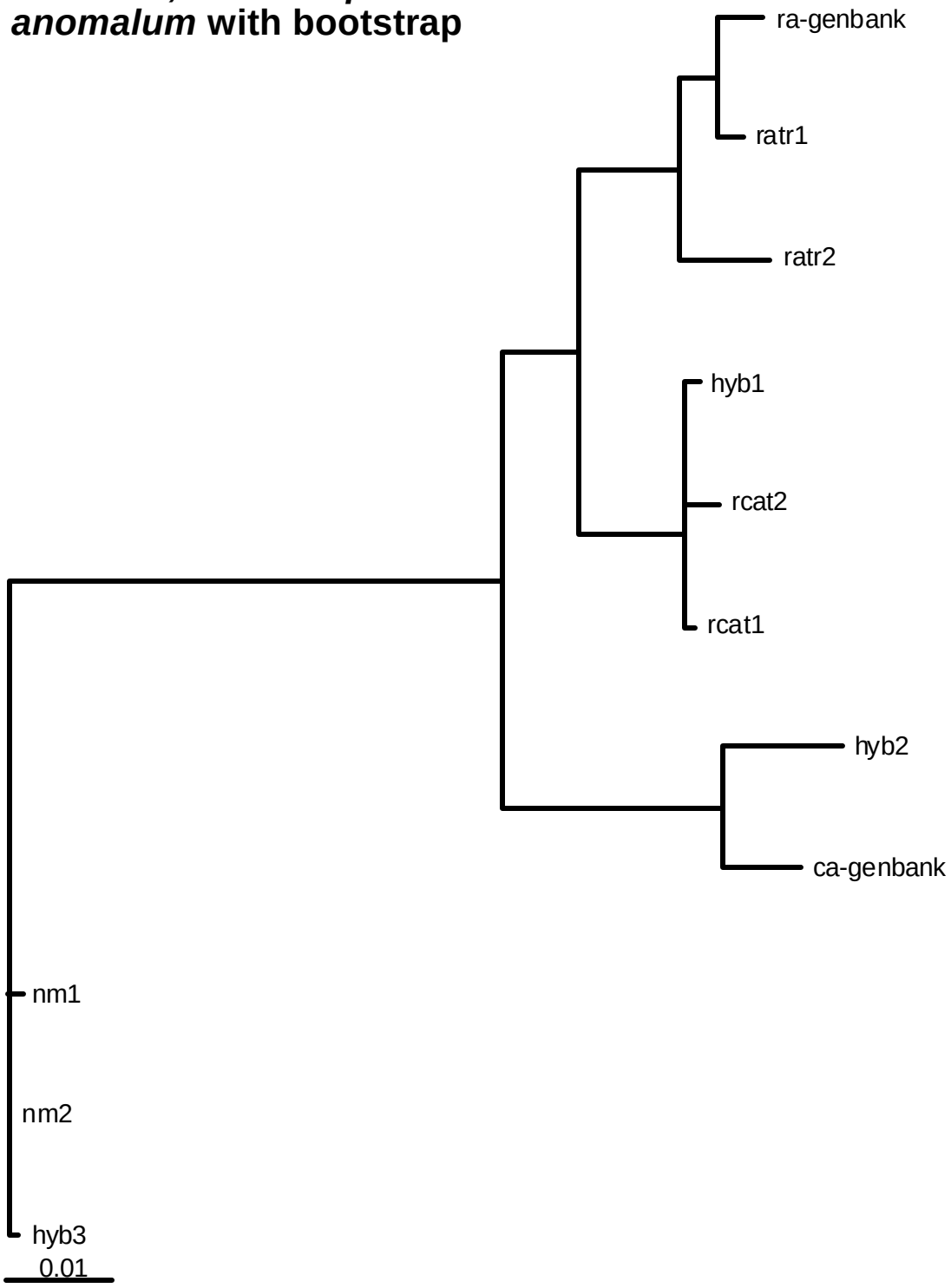

Figure 11. Hyb1 in node with *R. cataractae*.

```
hyb1          1  CAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Rcat1         1  CAAAGGCATGGTCCTGACCTTATTATCAGCTCTAACCCAACTTACACATG
Rcat2         1  CAAAGGCATGGTCCTGACCTTATTTTTAGCTCTAACCCAACTTACACATG

hyb1         51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
Rcat1        51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG
Rcat2        51  CAAGTCTCCGCAGCCCCGTGAGTACGCCCTTAATCCCCTGCCCGGGGACG

hyb1        101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC
Rcat1       101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC
Rcat2       101  AGGAGCAGGCATCAGGCACAAACCTTTAGCCCAAGACGCCTGGCCTAGCC

hyb1        151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
Rcat1       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATAAGTGAAAA
Rcat2       151  ACACCCCCAAGGGAATTCAGCAGTGATAAATATTAAGCCATGAGTGAAAA

hyb1        201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Rcat1       201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG
Rcat2       201  CTTGACTCAGTCAGGGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCG

hyb1        251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
Rcat1       251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT
Rcat2       251  CGGTTAAACGAGAGGCCCTAGTTGATAGTATAACGGCGTAAAGGGTGGTT

hyb1        301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Rcat1       301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT
Rcat2       301  AAGGACAGCGAAATAATAAAGTCGAATGGCCCTTTGGCTGTCATACGCTT

hyb1        351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT
Rcat1       351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT
Rcat2       351  CTAGGAGTCCGAAGCCCGATATACGAAAGTAGCTTTAGAAAAGCCCACCT

hyb1        401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
Rcat1       401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT
Rcat2       401  GACCCCACGAAAGCTGAGAAACAAACTGGGATTAGATACCCCACTATGCT

hyb1        451  CAGCCATAAACCCAGACGTCCAACTACGATTAGACATCCGCCCGGGTACT
Rcat1       451  CAGCCATAAACCCAGACGTCCAACTACAATTAGACATCCGCCCGGGTACT
Rcat2       451  CAGCCATAAACCCAGACGTCCAACTACAATTAGACATCCGCCCGGGTACT
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hyb1      501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
Rcat1     501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC
Rcat2     501  ACGAGCATTAGCTTGAAACCCAAAGGACCTGACGGTGCCTCAGACCCCCC

hyb1      551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
Rcat1     551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC
Rcat2     551  TAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTAAACCTCACCACTTC

hyb1      601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
Rcat1     601  TAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCA
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
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
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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
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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
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.

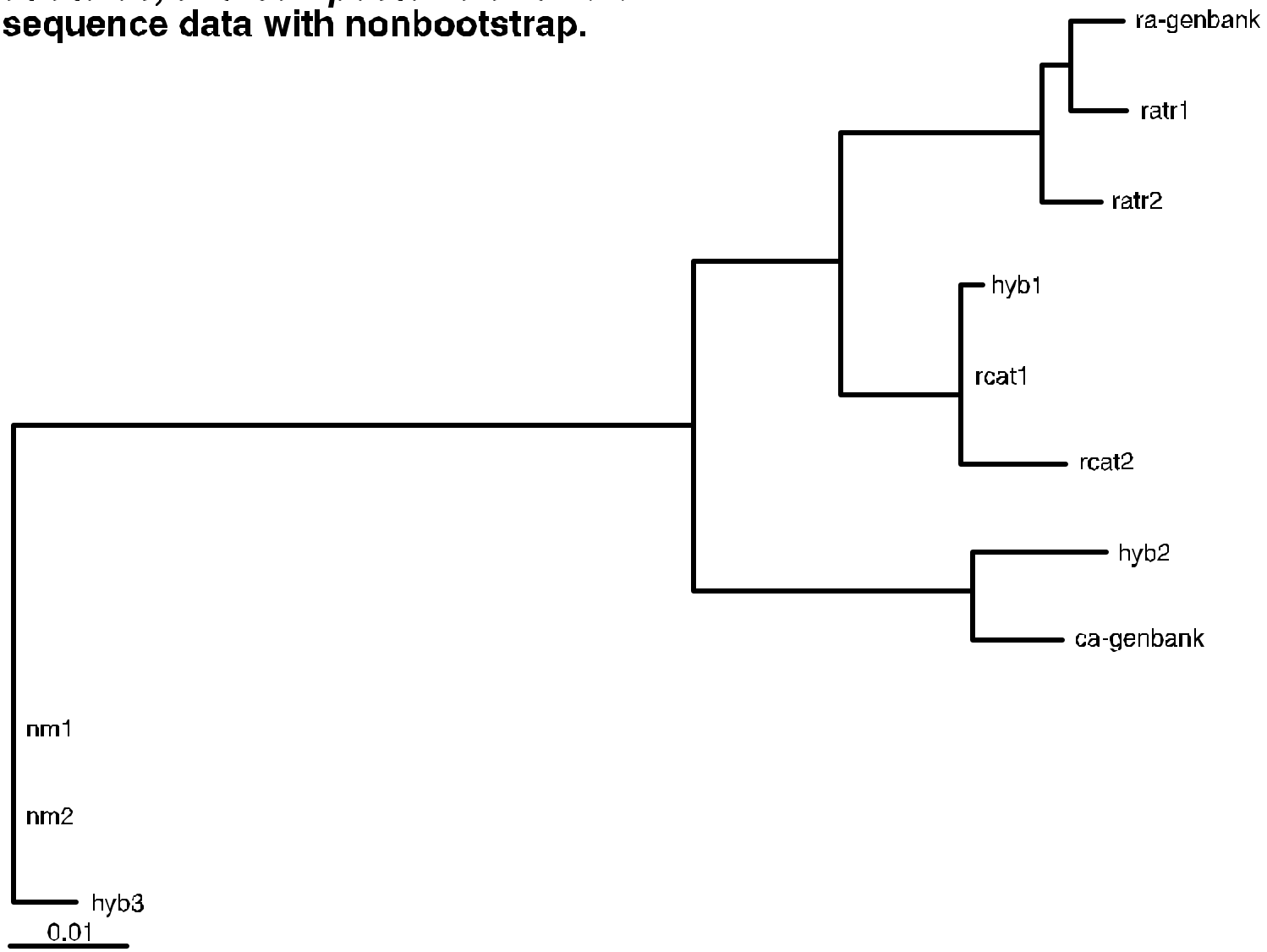


Figure 15. Distance matrix phylogenetic tree using the Fitch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum.* The labels bootstrap values after 100 resamples.

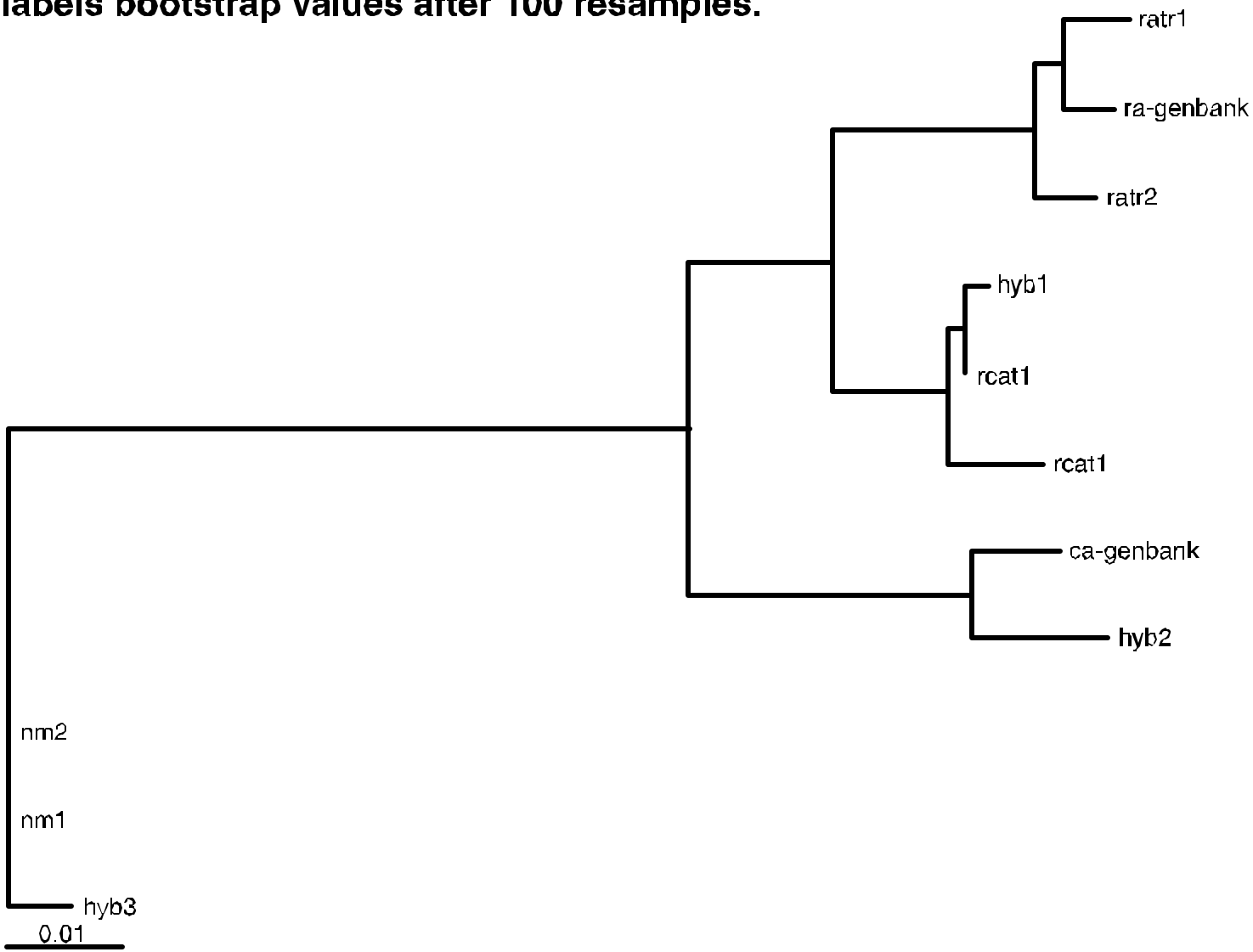


Figure 16. Phylip Neighbor Joining phylogenetic tree using the Fitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with nonbootstrap.

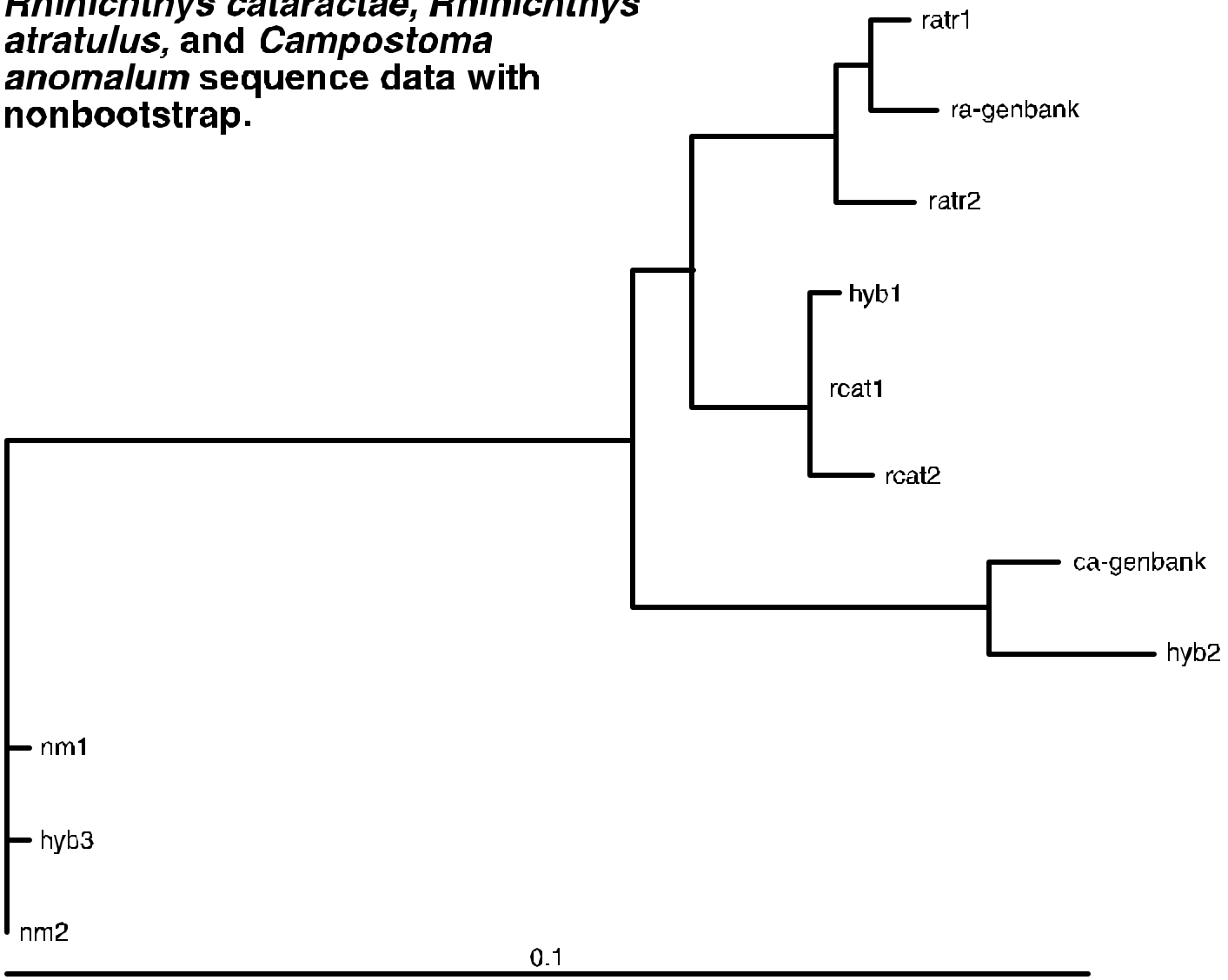


Figure 17. Distance matrix phylogenetic tree using the Fitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with nonbootstrap.

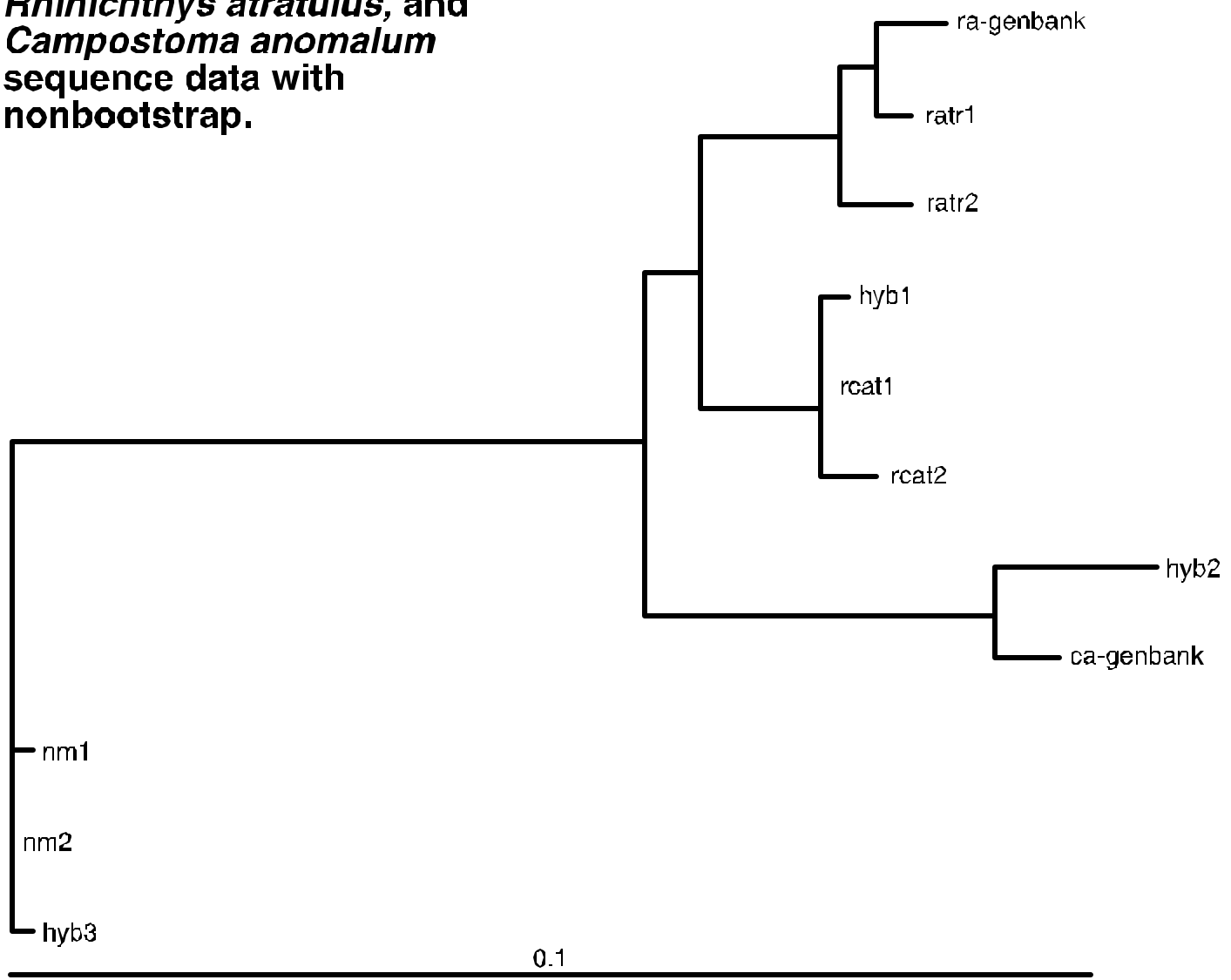


Figure 18. Maximum likelihood non-bootstrapped with jumble (10) option phylogenetic tree of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus*, and *Campostoma anomalum*.

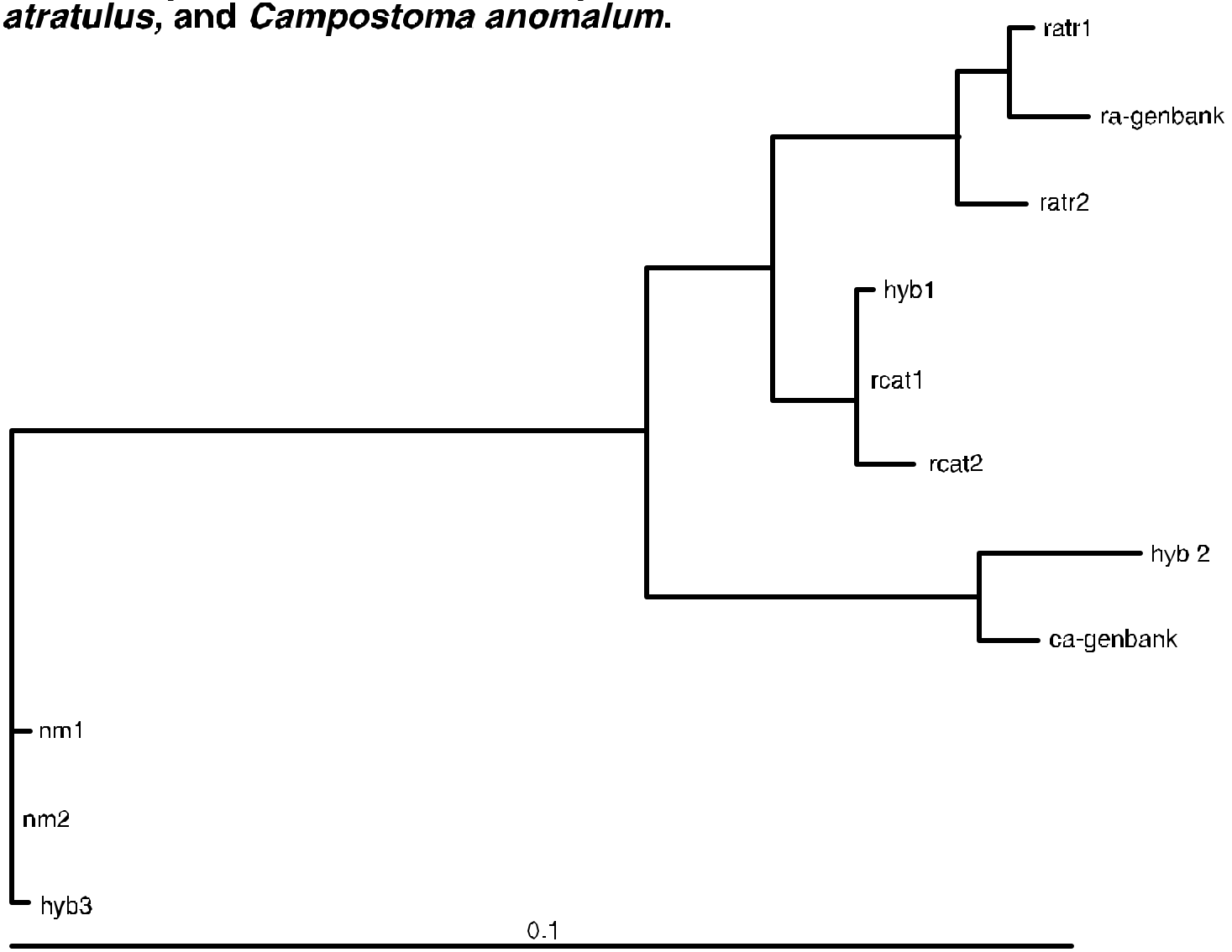


Figure 19. Distance matrix phylogenetic tree using the Kitsch of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data. Internal labels are bootstrap values after 100 resamples.

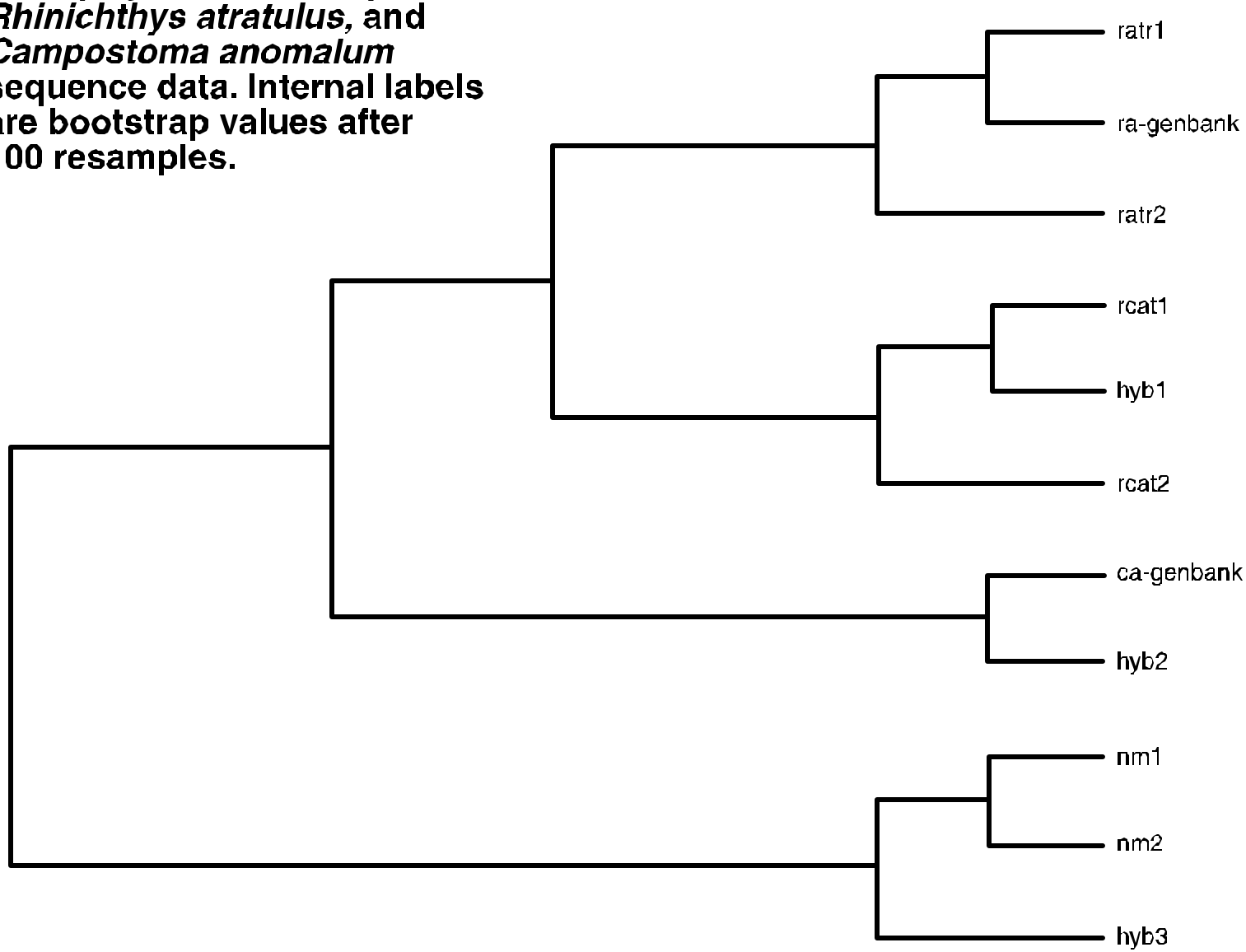


FIgure 20. Distance matrix phylogenetic tree using the Kitsch program of *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* sequence data with non-bootstap.

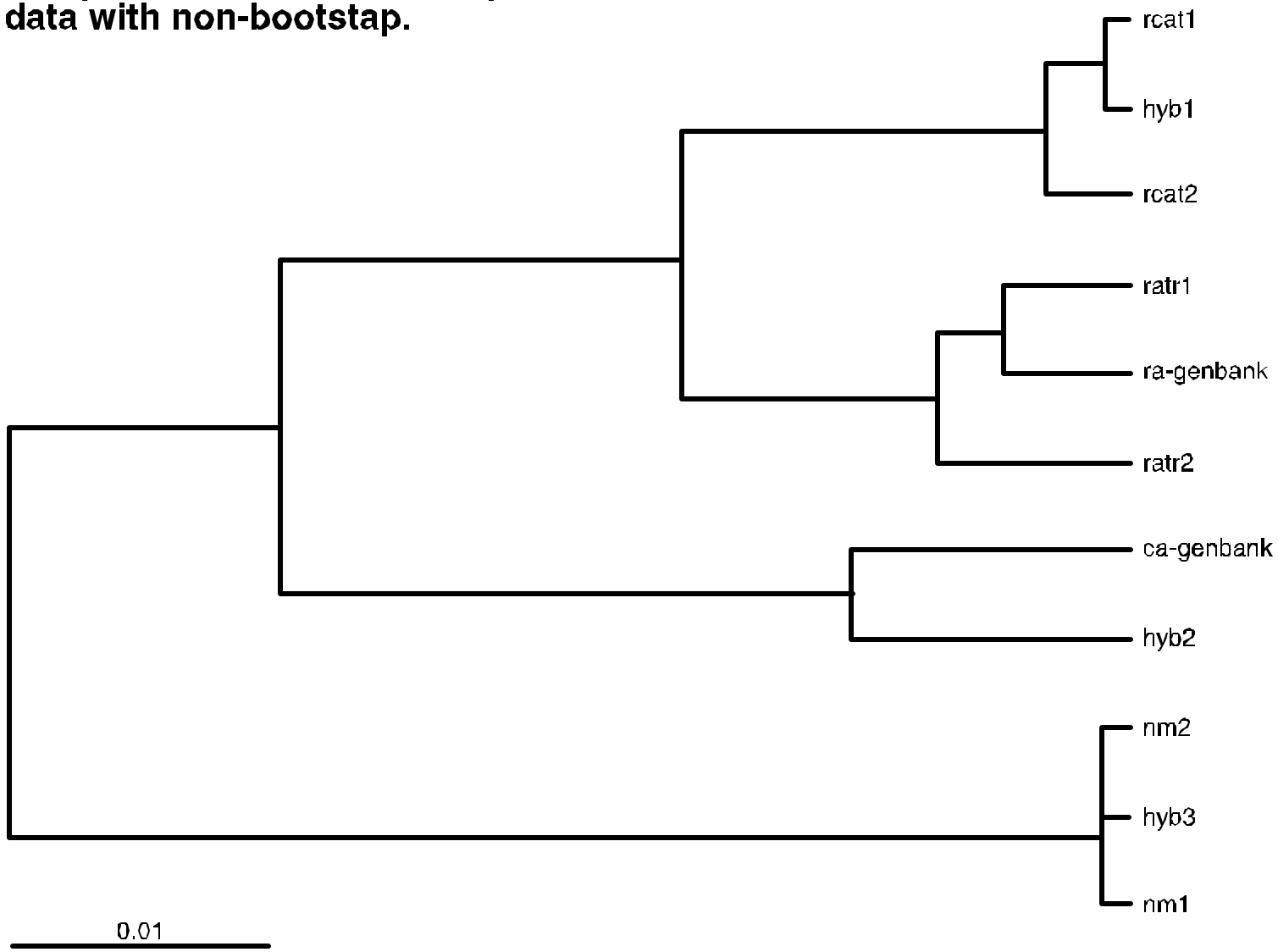


Figure 21. DNA pars phylogenetic tree *Rhinichthys bowersi, Nocomis micropogon, Rhinichthys cataractae, Rhinichthys atratulus,* and *Campostoma anomalum* with 100 jumbles and NM2 as outgroup.

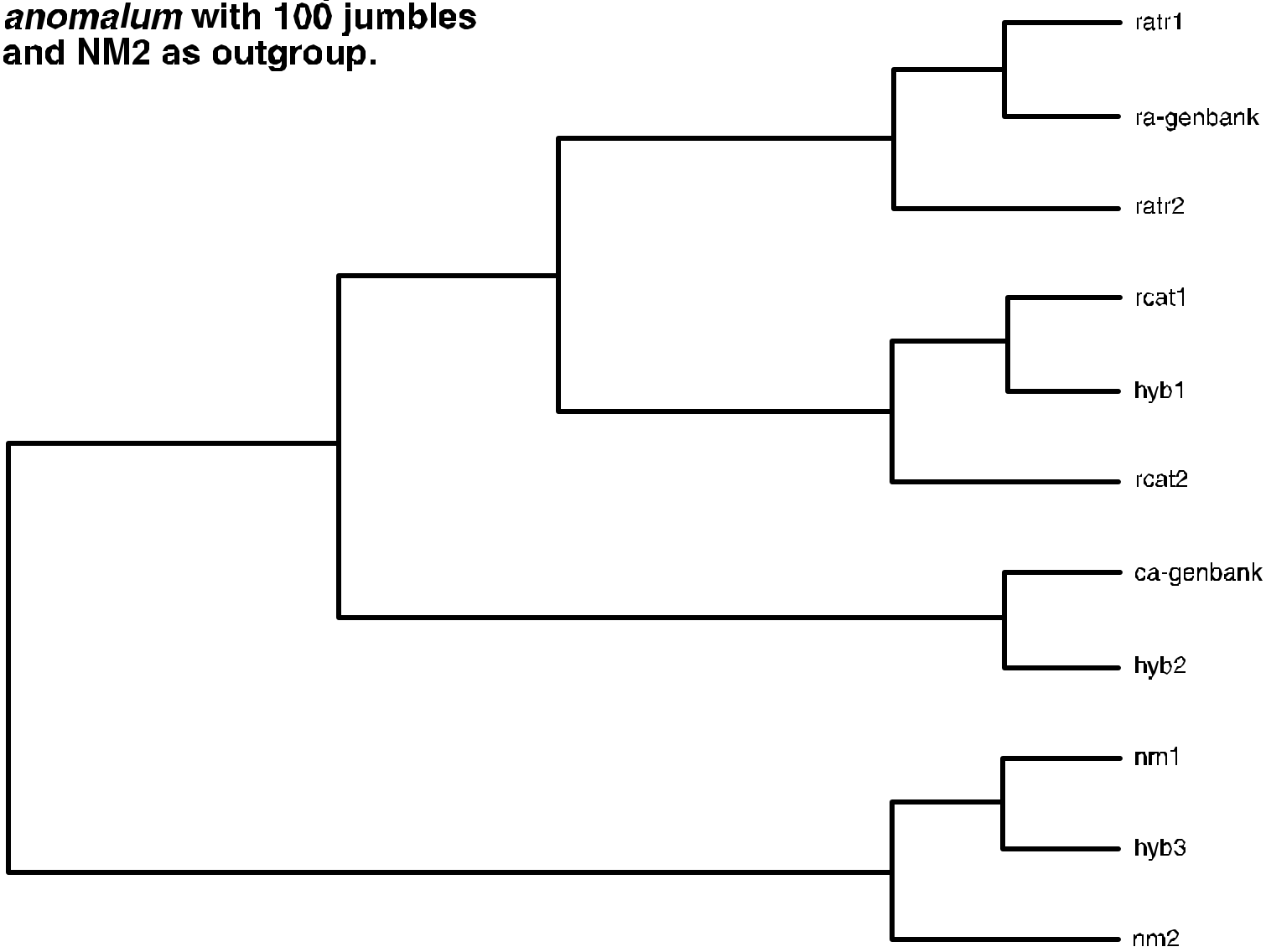


Figure 22. DNA pars of 100 jumbles phylogenetic tree with *Rhinichthys bowersi* as outgroup.

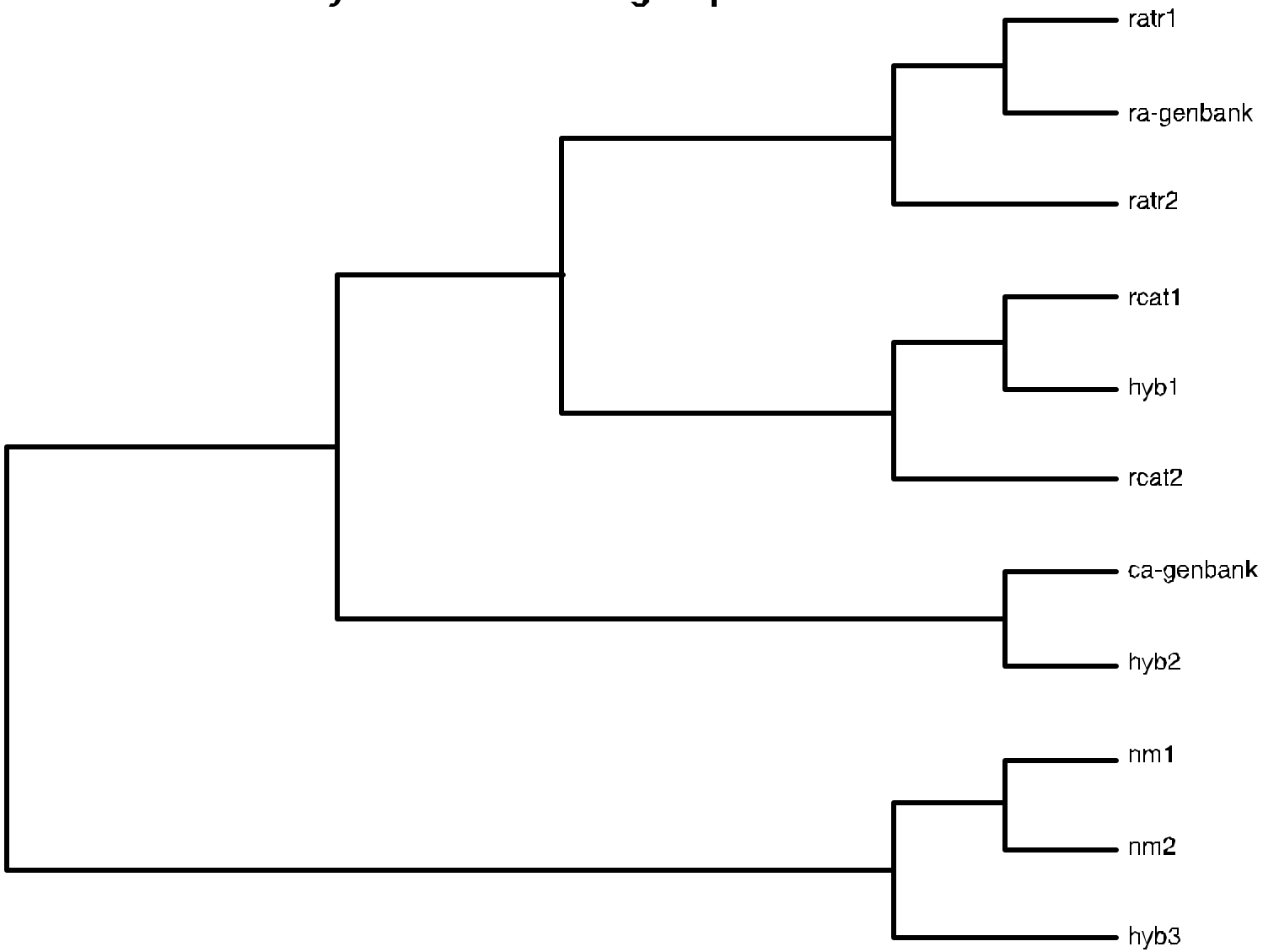


Figure 23. Sites where *Rhinichthys bowersi* were collected on Shavers Fork River near the Cheat Bridge on Route 250.

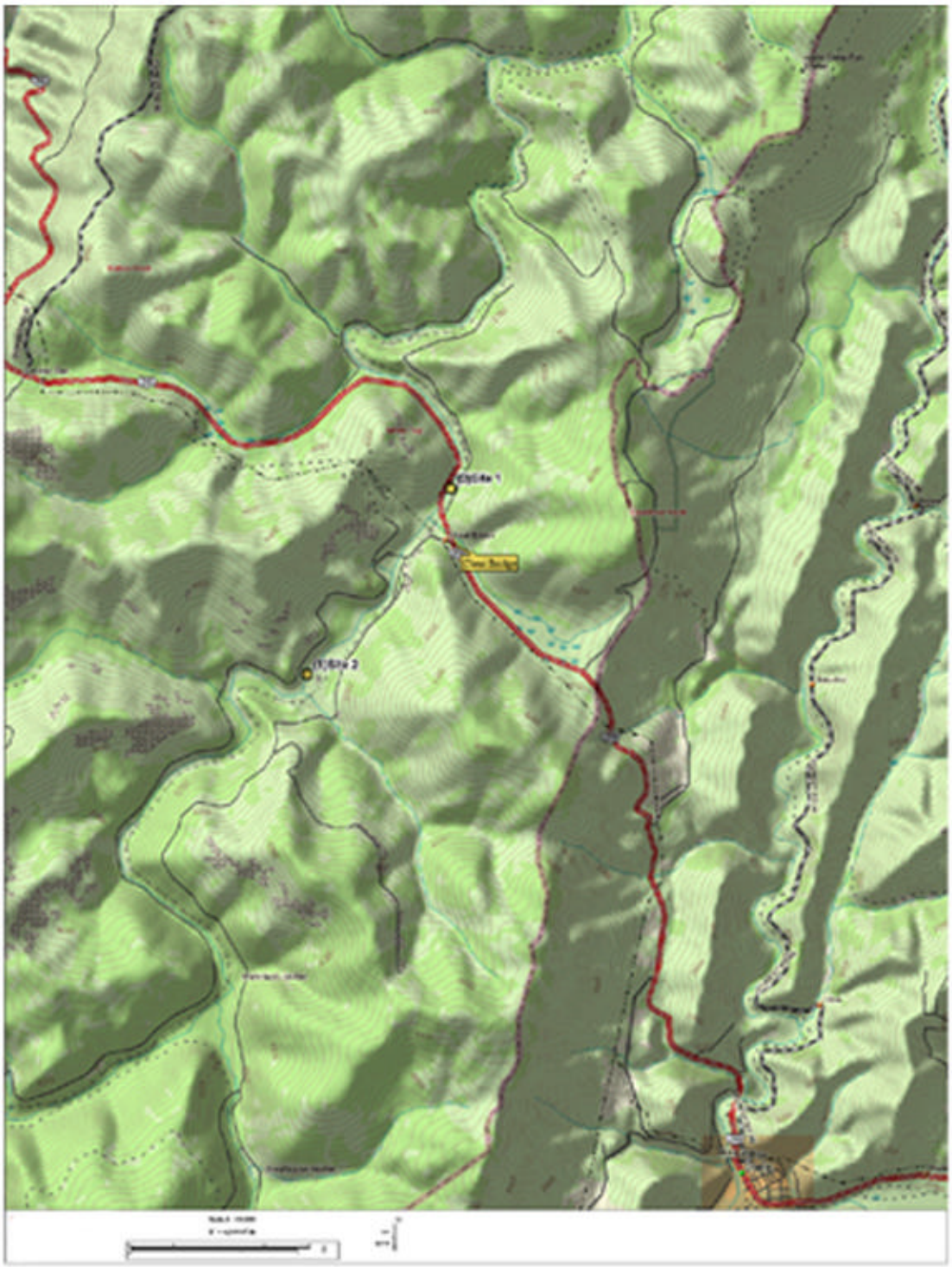
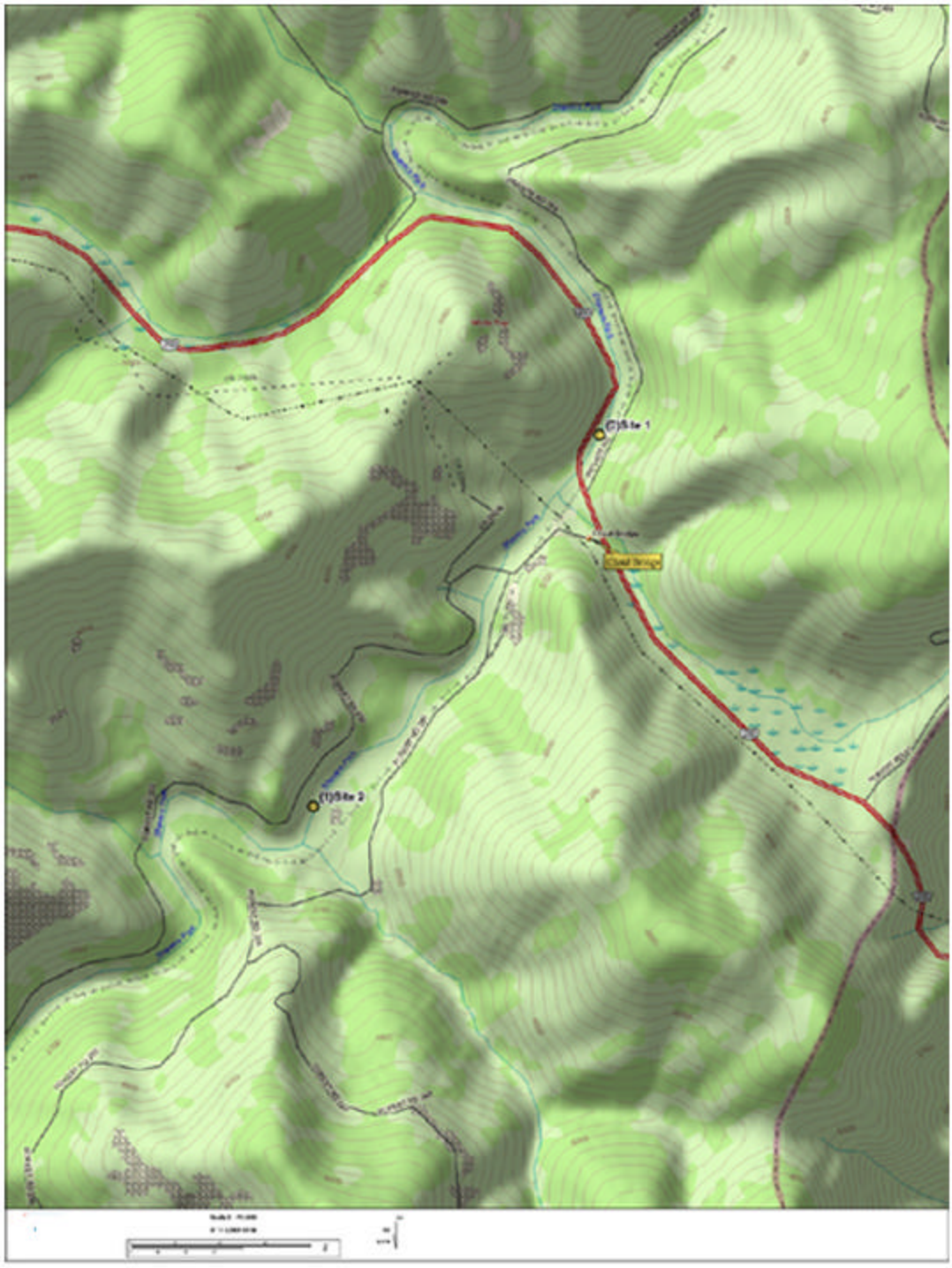


Figure 24. A close-up of the sites where *Rhinichthys bowersi* were collected on Shavers Fork River near the Cheat Bridge on Route 250.



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Appendix

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. Pipet 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 pipet 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:
   $$\frac{\text{x ng PCR product}}{\text{(y bp PCR product) (50 ng pT Adv)}}$$
   (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°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°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. Pipet 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. Pipet 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°C.
6. Heat shock for *exactly* 30 seconds in the 42°C water bath. Do not mix or shake.
7. Remove the tubes from the 42°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°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°C incubator for at least 18 hours.
13. Shift plates to 4°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 $\geq$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 $\geq$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 temperture.

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^{o}C

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
Steriliaze by autoclaving

Antibiotics

Ampicillin 50 mg / ml

Add 50 mg in to 50 ml H_2O
Storage at -20^oC