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PHYLOGENETIC ANALYSIS OF BLACKNOSE DACE (*RHINICHTHYS*) IN WEST VIRGINIA STREAMS

A Thesis submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science

Physical Science

by
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Approved by

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Marshall University
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Abstract

PHYLOGENETIC ANALYSIS OF BLACKNOSE DACE (*RHINICHTHYS*) IN WEST VIRGINIA STREAMS

Samantha Taylor

Blacknose dace (*Rhinichthys*) are one of the most common cyprinid fishes in eastern North America. They also have been a topic of debate for over 30 years because morphology-based systematics has failed to clearly define their taxa. Taxonomists classify the complex into two species and one subspecies: the eastern form, *R. atratulus atratulus*; and the western form *R. obtusus obtusus*, and southern form *R. obtusus meleagris*. This research uses the mitochondrial cytochrome *b* gene and genomic RAG 2 gene in a phylogenetic analysis to help clarify species relations according to differences between each current species. Maps have been created to give a visual representation of how these fish may have evolved from one another in their respective stream locations in West Virginia, both by morphological and genetic characteristics. Current results with the mitochondrial cytochrome *b* gene indicates that there is a distinct separation between *R. atratulus atratulus* and *R. obtusus obtusus,R.atratlus atratulus* having 51-58 site changes compared to the *R. obtusus obtusus* reference. The difference between *R. obtusus obtusus* and *R. obtusus meleagris* is not as direct. In the collection of *R. obtusus obtusus* two groupings were recorded. The first group had 0–5 differences; the second grouping had 10–19 base pair changes. The *R. obtusus meleagris* species group had 19–21 changes. The RAG2 genetic tree was composed of two branches, one mixed with *R. obtusus obtusus* and *R.obtusus meleagris* and a second composed of *R. atratulus atratulus.* Even though there are strong indications that these fish have been separated long enough to begin to look different through there mitochondrial lineage, there is evidence that the groups are interbreeding.

Chapter 1: Introduction

Classification of species has become an important aspect of biology. Knowing what species are in what geographic regions is important to the conservation of regions and species. Although taxonomists are expert in their fields, there are still species that baffle scientists as to their classification. One such species is the blacknose dace genus (*Rhinichthys*). This group of fish has caused debate among ichthyologists for almost 30 years (Smith, 2007). Some studies have suggested ways to separate the species from one another (Smith, 1985). Others have found no conclusive way to distinguish between the species according to physical characteristics (Matthews *et al,.* 1982; Fraser *et al*., 2005).

The problem that arises is that the connections and separations of stream systems can result in genetic differentiation over time (Haponski *et al.*, 2009). Matthews *et al.* (1982) suggest that it is possible that mating coloration can be used as a characteristic of classification. Therefore, it is hypothesized that the genus is two separate species, *Rhinichthys atratulus* and *Rhinichthys obtusus* with *R. obtusus* containing a subspecies *R. obtusus meleagris*. A second hypothesis is the possibility that *R. obtusus meleagris* is a hybrid form created by breeding between the other two species. Determining the genotype of mitochondrial and genomic markers could help in the debate of how these fish should be classified according to their genetic make-up and allow for better classification of these species.

Speciation

The concept of speciation is an important biological problem. There has been debate concerning the appropriate definition of a species:

> A commonly held view is the evolutionary species concept, according to which as species is "a single lineage of ancestral-descendant populations which maintains its identity from other such lineages and which has its

> own evolutionary tendencies and historical fate" (Wiley, 1978). For sympatric, sexually reproducing species, this reduces to the biological species concept (Mayer, 1969), according to which a species consists of a group of individuals capable of exchanging genetic material with each other but which are reproductively isolated from all other groups. (Hillis *et al.,* 1996)

New species are thought to develop from geographically isolated populations that develop independently of one another in allopatric divergences (Dieckmann and Doebeli, 1999). If a species is separated for a long period of time and allowed to reproduce, the gene pools will begin to evolve differently for each separated group based on selective advantages for the organism. Vellend and Geber (2005) note that even within a sympatric area, individuals may represent different species in a community or even different genetic variation within a species. They suggest that species diversity and genetic diversity need to be used together in determining classification of species.

Being able to separate organisms according to their species seems like it would be a simple concept. If it looks significantly different, then it is its own species. This is not the case for all animals, though. The physical differences make it easy to identify a lion and tiger as different species. Dogs, on the other hand, separated into many different breeds rather than species based on their size and shape, are all considered to be in the same species, *Canis familiaris.* A simple explanation by Mayr is that a group of animals that can interbreed with one another must be the same species because they share the same gene pool (Schilthuizen, 2001).

> Mayr's biological species concept seems like a nice, simple definition for what species are. No matter how different two organisms look, if they can interbreed, they share a common gene pool, and, by definition, they belong to one and the same species. But the apparent clarity and practicality of the [biological species concept] are deceptive, because nature is not as simple as Mayr's definition. (Schilthuizen, 2001).

This explanation becomes questionable when looking at hybridization between separate species.

Hybridization

A hybrid is formed when different species are able to interbreed, allowing the mixing of genetic material that had been isolated from one another (Schwenk, 2008). When hybrids backcross to one or both parent populations, introgression is observed. When the gene flow of introgression is seen through numerous generations of crosses of both parent populations as well as mating among hybrids themselves, a hybrid swarm is formed. For a long time, hybridization between animal species was thought to be rare and held no value for the animal for adaptation or in an evolutionary way (Cunha *et al.*, 2004). Hybrids are often thought to be sterile, such as the mule, and stop the evolutionary process of their species. A closer look into the genetics of some animals has shown that this preconceived notion is not fully true. When studying the genetics of species and the possibilities of hybrids, many scientists are more concerned with the loss of gene pools than viewing hybrids as a creative force, leading into debates and concerns about species conservation and protection (Moyl and Cech, 2003; Nerras and Spruell, 2001; Rhymer and Simberloff, 1996).

According to Fishes: An Introduction to Ichthyology (Moyl and Cech, 2003), hybridization is quite common among fish, and its involvement in the evolution of fishes is often debated. The mixtures of genes can cause a complex system of mating. There are examples of hybridization that incorporates multiple sets of chromosomes to maintain the species. A minnow exists in the river basins of Portugal and Spain that is known to be only males, and has a numeral set of paired chromosomes, and is not hermaphroditic (Alves *et al.*, 2002). This minnow is part of *Squalius alburnoides*. It is a hybrid of two species that are either triploid, tetraploid, or

diploid. The ancestral form, now extinct as a "normal" species, maintained themselves by mating with triploid hybrid females that would produce haploid eggs with one set of chromosomes that is identical to the males. Those matings only produce males, so the female genome has to be maintained though crosses with other forms. Another complication is the replacement of a phenotype. The cutthroat trout has become extinct in its native regions because of the introduction of rainbow trout. The two fish species are able to breed and hybridize with one another, and after several generations of breeding the phenotype of the rainbow trout dominate those of the cutthroat trout (Moyl, 2003), causing a decline in their numbers.

Genetic mixing in some ecological zones can result from human activities (Nerras and Spruell, 2001; Rhymer and Simberloff, 1996). Humans modify, fragment, and introduce exotic species to previously balanced habitats. The development of dams has had a great impact on the habitat of fish in rivers, since the construction of dams causes fragmentation of the river habitats. Fish that were once able to move freely up and down a river system, now, have a blockage that prevents their movement. This can have a large impact on the life cycle of many fish. Fish often have migratory life histories. They live as adults in the large rivers and travel to small inland streams for their spawning events. The Bull trout (*Salvelinus confluentus*) is listed on the U.S. Endangered Species Act. A large factor in their decline is the fragmentation of their habitat due to hydroelectric impoundments (Nerras and Spruell, 2001). In such situations, fish are able to travel downstream with the flow of the river though the dams. This forces a genetic population downstream and into other species habitat and increases the chance of hybridization among populations.

Humans also have introduced new, exotic species to a habitat for sport fishing, as a biological control, or accidently as bait species while on fishing trips. Some instances, like the

introduction of the smallmouth bass (*Micropterus dolomieui*) into the Guadeloupe River, has become a threat to native bass species because of the interbreeding, hybridization, and backcrossing of offspring (Rhymer and Simberloff, 1996).

Diversity has been lost in the western US watersheds because of introgression of rainbow trout (*Oncorhynchus mykiss*) as well. The rainbow trout hybridize with the Apache trout (*O. apache*), a native and threatened species in the west. Of the Apache trout populations, 65% of the individuals have rainbow trout alleles, and one native population is now completely rainbow trout (Rhymer and Simberloff, 1996). So, it is seen that the rainbow genes are moving into the Apache populations, but the reverse introgression has not been seen (Rhymer and Simberloff, 1996). This could highly affect the future populations of these fish, where eventually the Apache populations will be wiped out completely due to the lack of genetic material being transferred throughout the two populations. There are many other instances of once native populations being genetically over run by introduced invasive species.

Hybrids that are born can either be non-fertile or fertile. It is common for hybrids to develop to adulthood but be sterile when it comes to breeding. One example is the bluegill-green sunfish cross. These occur when, it is assumed, male green sunfish release sperm in a bluegill nest when bluegills are spawning. The resulting hybrid offspring are all male and grow faster than either parent species. These fish will build a nest and defend them, dominating non-hybrid fish during spawning events, even though they have no chance of producing future offspring. Hybrid offspring that are fertile often have lower survival rates and poor reproductive success. It is thought that the two parent species have an advantage over the hybrid, and the hybrid is only likely to be successful when the stream has been modified and conditions give them an

advantage, as noted in other examples. It is more common that these mating are accidental when two species spawn in the same area at the same time (Moyl, 2003).

Some hybrid offspring have physical characteristics of each of their parental species. The saugeye, a hybrid of the sauger and walleye, has a physical appearance that is in between the two parent species. It is intermediate in size between the sauger and walleye, and the saugeye may have the white tip of the lower caudal fin lobe that is a trait of the walleye parent. Another example is the hybrid white bass. This fish is the offspring of a striped and white bass. It so closely resembles both parents that it makes physical identification difficult. It has distinct stripes along its body and has hyoid teeth that lie in two parallel patches, both traits of the striped bass. But, its deep body shape and a second anal spine two-thirds or more the length of the third spine makes it appearance similar to the white bass (Steve Foster, personal contact).

As studies on hybrid populations increase, so do the concerns of species protection and conservation. Even though a new genotype maybe better suited for survival of a species, some view it as a tragedy, and something that needs to be corrected. There seems to be more evidence that hybrids are less able to adapt than their parental populations. The topic of sterile hybrids has already been touched upon, but a sterile hybrid's inability to breed and produce offspring is a strong indication of the lack of fitness of the genetic makeup of the hybrid. Hybrids are also seen to die more often in their natural habitats because they are often more susceptible to diseases and illnesses to which the parental species are resistant (Rhymer and Simberloff, 1996). Many ecological changes do not fully result from the breeding of hybrids since it is the introduced species that causes difficulties for the native populations. The mating process is interrupted by both the introduced species and any adult hybrids. It is the males who fight for the rights to breed with the females, whether it is of their own species or the other. Competition

in mating will cause a great decrease in future populations, especially if the female is unable to produce offspring because of the poor match in mating partners, or if a hybrid mate is sterile. It can be difficult to distinguish hybrids by physical characteristics alone, especially if several generations have backcrossed in their breeding (Rhymer and Simberloff, 1996). A revolution in the studies of hybridization started when the polymerase chain reaction was perfected. PCR allowed the amplification of DNA fragments. These fragments could be sequenced and analysis of the DNA could be done. Researchers could now focus on genetic analyses of questioned species rather than physical differences. Even further development of measurements of genetic variation at the nuclear and mitochondrial level allowed the focus of gene flow among species to become a main focus (Schwenk *et al.*, 2008). In depth studies can not only determine the extent of hybridization and introgression, but also the gender of hybridizing individuals in the populations (Rhymer and Simberloff, 1996).

Use of Mitochondrial and Genomic DNA to follow gene flow

Mitochondrial DNA is maternally inherited. Female lineages are isolated from one another, even within the species, through the transfer of mitochondrial DNA (Avise *et al.*, 1983). The evolution of one species to another can be seen though the changes in mitochondrial DNA of closely related species. The use of mitochondrial and genomic DNA has been a popular method to create an idea of how closely related species are to one another. Even recently it has been used to look at the relations between species that live in closely to one another and possibly form hybrid species. It helps in determining if hybridization is occurring between the males of one species and the females of another, or vice versa (Rhymer and Simberloff, 1996). It has become important to look at the genetics of isolated populations, which may have low genetic variability

within them, and the populations as a whole that have possible high genetic divergences (Natacha *et al.,* 2001).

Many studies use similar methods for extracting these DNAs from their subject species. DNA is extracted from each sample specimen from tissue collected, either a DNA extraction kit is used or a protocol of incubation with SDS and proteinase K digestion, followed by phenol-chloroform extraction. Each study used primers that were specific to their species in isolating the mitochondrial cytochrome *b* gene or the genomic RAG2 gene though PCR (Smith, 2007; Haponski *et al.,* 2009; Cunha *et al.,* 2004; Natacha *et al.,* 2001; Dowling *et al.,* 2002; Lovejoy and Collette, 2001; Pfrender *et al.,* 2004; Ray *et al.,* 2006; Wang *et al.,* 2007). Sequences were obtained after PCR and alignments into phylogram trees to use as a visual of the relationship between samples.

A study was done in Oregon (Pfrender, 2003) studying the distribution of mitochondrial cytochrome *b* of *Rhinichthys osculus*. This study recorded haplotype ranges of 0.15% to 16.38% between the divergences of the *Rhinichthys osculus* group. Their locations were grouped within their tree formed. Only one area separated into two groupings, Klamath, and they observed four distinct clades within that. Those clades contained several different haplotypes.

Ray *et al.* (2006) considered that the mitochondrial cytochrome *b* distribution in the rainbow darter (*Etheostoma caeruleum*) according to their isolation from other drainages, (drainages to the Mississippi River, Great Lakes, Potomac River and the Hudson Bay). Their results were able to distinguish four separate grouping of the species though out these sites relative to their location. The clades (White-Little red river clade, Mississippi River clade, Northern Ozarks-upper midwest clade, and the Eastern clade) were each separated from one another, and within each clade the river systems were separated from one another. Each of these

groups had unique haplotypes, and many were not shared across drainages. It was noted in their paper that an agreement of gene trees and systematic information suggests that genetic trees are accurately separate by location. They record that their findings reflect dispersal and vicariant events that occurred in that area. Their overall conclusion was that the gene revealed a great deal of isolation and getic divergence among the different watersheds, but within the watershed there was a high level of genetic variation.

In a similar study of rainbow darter, Haponski *et al*. (2009) used other sites within the Lake Erie catchment and the Ohio River, and used eight nuclear DNA markers, the mitochondrial cytochrome *b* gene, and a control region. Their study only resulted in two different clades within their sites. However, when the sequences from Ray's study were added, four clades were then formed. Such a different result such as this could be related to the number of locations samples were collected from for the study. The study done by Ray *et al.* (2006) contained a large number of sites in their own collection in multiple different streams. Haponski *et al.* (2009) only has five points of reference for their collection of fish. Having a large distribution of specimens showed greater amount of variation based on location of the samples.

Nuclear markers of genes can also be used in the separation of species. Lovejoy and Collette (2001) used RAG2, the encoding components in recombination of the immunoglobin and T-cell receptor genes, to compare a new clade of fish with previously studied clades in accordance with the water type (freshwater or saltwater) they were found in. Their resulting tree separates the fish into two distinct groups. Group A consisted of *Pseudotylosurus* from freshwater areas in South America and *Strongylura* from the western and eastern Atlantic. In group B *Potamorrhaphis* and *Belonion* were grouped with *Strongylura* from marine and freshwater areas of the eastern Pacific and western Atlantic. The grouping in B allowed Lovejoy

and Collette (2001) to draw the conclusion that there had been a movement from the marine waters around Central and South America into freshwater streams. This study gave some clarity to the not only the classification of these fish, but also a historical lineage of evolution from marine species to freshwater species.

A study in China also used the RAG2 gene to organize many species of Cyprinidae within their freshwater streams. Based on morphological data, Cyprininae could be divided into four subfamilies, Barbinae, Cyprininae, Labeoninae, and Schizothoracinae. According to this study's RAG2 sequences only Labeoninae could clearly be defined (Wang, 2007). There was a second branch to their tree that they classified as Cyprininae, but stated that it was a weakly supported family because it also contained the families of Barbinae and Schizothoracinae.

Mitochondrial and genomic DNA are now commonly used to determine how closely related species are in sympatric areas. Fish are a popular organism for these studies since they can easily be moved around by people and often have impacts on the other species in an ecosystem. The blacknose dace is a fish species that has attracted the attention of ichthyologists for many years in their relation to one another and their proper classification according to physical characteristics. In West Virginia there is an area of sympatric contact of the blacknose dace complex. The use of mitochondrial and genomic DNA markers could help resolve the debate of classification for this species.

Challenges with the Blacknose Dace

Blacknose dace are an abundant cyprinid minnow distributed widely in West Virginia and throughout North America. For more than 30 years the taxonomy of the blacknose dace species complex (*Rhinichthys*) has been an area of disagreement (Smith, 2007; Fraser *et al.*, 2005), and recently had a reclassification in 2004 (Smith, 2007; Fraser *et al.*, 2005), from three

subspecies of *Rhinichthys atratulus* into two separate species and a subspecies, *Rhinichthys atratulus* and *Rhinichthys obtusus* with subspecies *Rhinichthys obtusus meleagris,* based on Smith's publication of the New York's fish population (Smith, 1985).

Smith concedes that the eastern and the western blacknose dace are very difficult to tell apart. He states that the significant difference is in the males. Eastern forms have fine speckling on the back and sides, but the western has larger blotches of dark scales. He also observes that the western males have a broad band of rust coloring along the mid-lateral strip and is present all year long, while the eastern form loses the reddish color after breeding season. His final morphological difference is that the western blacknose dace is more humpbacked than the eastern.

Multiple studies have been done since the reclassification involving the physical appearance and measurements of each of the members of the complex. There has not been an agreement yet on how to classify each of the species to an individual level and both Matthews and Fraser demonstrate that variations among individuals, in both the James River in Virginia and Lake Ontario studies, are not great enough to propose a classification of different species (Matthews *et al.*, 1982; Fraser *et al.*, 2005). The only trait that was agreed upon that may be possible to use, is the coloration of mating males. The mating colors, however, are not a good reference trait due to the fact that the colors are only seen for about three months of the year.

All three of the subspecies can be found in the state of West Virginia. The eastern form, *Rhinichthys atratulus,* can be found in the streams of the eastern panhandle that flow into the Atlantic Ocean. The southern form, *Rhinichthys obtusus obtusus,* is located in the streams in the southern part of the state that flow into the lower portion of the Ohio River. The western or central form, *Rhinichthys obtusus meleagris*, is in the upper area of the state that drains into the

upper section of the Ohio River (Smith, 2007; Matthews *et al.*, 1982). An area in central West Virginia has known to have streams that have more than one of the complex members in its waters (Smith, 2007; M. Little, personal contact), making it an area of interest for collection and analysis.

Throughout most of the year, it is difficult to distinguish the three species from one another, or distinguish males from females. During their mating season (mid-April to late July) the males develop unique coloration that has been used to distinguish these three taxa of the fish. For most fish identifications there are multiple characteristics that are used. Morphometric measurements, melanophore coloration, nuptial coloration, breeding patterns, and locations are often used to distinguish fish from within species complexes. An extensive review of these identifiers for the original *Rhinichthys* complex was completed by Matthews *et al.* (1982) and is used as the basis for this section. Any information not cited in this section can be credited to the work of Matthews *et al*.

When working in the field, collecting specimens from streams and rivers, physical characteristics are used to determine species. Currently there is not a definitive way to differentiate between the different forms of *Rhinichthys*; however, there are studies that suggest ways that maybe used to distinguish. Studies stated that it is possible to distinguish *R. a. atratulus* and *R. o. obtusus*. Hubbs recorded that *R. a. atratulus* can be distinguished from *R. o. meleagris* by its more slender caudal peduncle (1958) and larger eye (1928). In their Canadian study, Scott and Crossman (1973) suggested a difference in number of pectoral fin rays between *R. a. atratulus* and *R. o. meleagris*. *R. o. obtusus* was considered to differ from *R. o. meleagris* by head shape and by having a larger mouth (Trautman, 1957), and it had a different number of lateral line scales according to Bollman (1889). Alternatively, Matthews (1982) does not have

confidence in these distinctions because the authors did not adequately describe the characters. In their own research Matthews *et al.* (1982) suggest that *R. o. obtusus* might have higher scale counts, separating them from *R. a. atratulus*. Conversely, they do suggest that their measurement may not be fully accurate because the samples they compared were from different elevations, and the scale count could be affected by the different temperatures or altitudes. In *R. a. atratulus* the lower side of the fish is often has lighter speckling or is absent. In *R. o. obtusus* and *R. o. meleagris* the lateral stripe is broken up in irregular blotches. The lower side of the fish often has moderate development of distributed dark scales (Matthews, 1982). The Matthews study found variation in this characteristic, however, making them doubt the accuracy of the character for identification.

Most often the easiest way to distinguish between the species is with nuptial coloration. In *R. a. atratulus* the pectoral fin pad will become bright orange to red. The lateral line of *R. a. atratulus* remains a dark brown or black, but is highlighted with an orange shade. Sometimes the under belly will turn a slight shade of yellow to green (Figure 1). In *R. o. obtusus* the main coloration that is noted is that the dark lateral line is replaced with a bright shade of orange (Figure 2). *R. o. meleagris* also loses the dark lateral line for a bright orange one. It becomes distinctive from *R. o. obtusus* by the orange coloration that develops on its underbelly and into its cheeks (Figure 3). Nuptial coloration is not a good identifier characteristic for field work. The coloration is only seen during three months of the year. The rest of the year the coloration is lost and only the dark lateral line exists. It is also difficult to obtain good photographic images of the nuptial coloration because the fish looses the coloration when stress levels increase during capture (M. Little personal contact).

Figure 1: ***Rhinichthys atratulus atratulus*****. Lateral stripe remains during mating and is overlapped with color pigmentation. Fin pad coloration.**

Figure 2: ***Rhinichthys obtusus obtusus*****. Lateral stripe vanishes and is replaced with matting pigmentation. No coloration in cheeks or fin pad.**

Figure 3: ***Rhinichthys obtusus meleagris*****. Lateral stripe vanishes and is replaced with matting pigmentation. Pigmentation also appears on the under belly and into cheeks.**

Geobiophysical Modeling

Physical maps are made to give a visual representation of where a species is located within an area by color coding. Geobiophysical modeling allows scientists to update and design maps that incorporate their findings of biological features or speciation.

A previous study (Fraser *et al.*, 2005) of *R. atratulus* and *R. obtusus* published a map that simply showed the distribution of the two species from sites though out Canada. Each site was marked with a circle. The legend contained three types of circles: 1) a white circle representing sites that contained only *R. atratulus*. 2) A black circle for locations with only *R. obtusus*. 3) A circle split in two with black and white sections, these sites are zones of sympathy for both species.

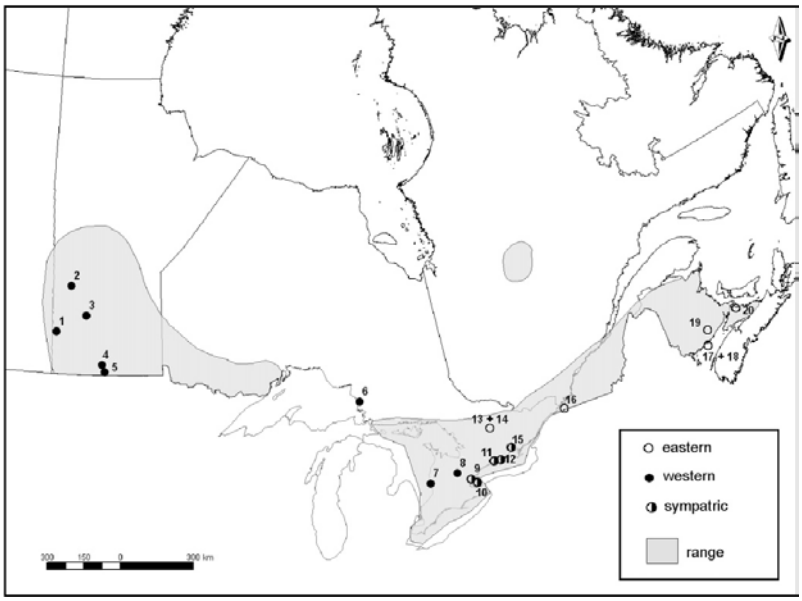


Figure 4: Population collection map from Fraser *et al.*, 2005.

Scientists can color code data results, such as phylogenic trees, and incorporate those areas into a map. Fujita and Papenfuss (2011) made this type of map with their Afro-Arabian gecko species complex. Each species branch was given a specific color. The specimen from each branch was then plotted on a map of North Africa. This created points that were either

solid with one constant species, or points that contained specimen from two or more branches of their genetic tree, giving their readers an understanding of where exactly their species co-exist with one another.

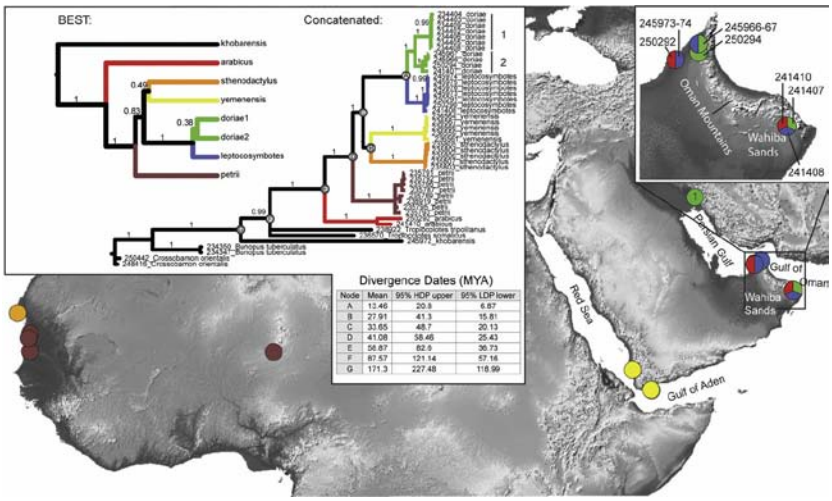


Figure 5: Tree information and plotted co-occurrence of Fujita and Papenfuss' gecko species. The pie coloration agrees with the colors assigned to the tree branches.

A similar map was published in a genetic study of the Danubian rheophilic barb *Barbus petenyi* (Kotlik and Berrebi, 2002) in order to show that there is a distinct difference within a classified species. Again, each grouping within the phylogenic tree formed was given a different color representation. They had a site that had two of the three species within one site. They also had ellipses that grouped sites together according to partitioning within their fish lineages. Their resulting map gave a strong example of species that is divided amongst itself into three specific species and should be revaluated for classification.

The Scope of this Thesis

Often it is difficult to identify hybrids with only physical characteristic (Rhymer and Simberloff, 1996). The different colorations during the blacknose dace mating may suggest possible hybridization within the species, or it can mean that there is a separation between

species. This thesis uses specimens collected from blacknose dace sympatric areas of West Virginia, to examine these fish on a molecular level. I will analyze cytochrome *b* mitochondrial genes and Rag2 genomic genes that are found in the fish and look for indications of introgression that can distinguish between separate species or possible mixing of gene pools indicating hybrids. Outlying areas of the state that have no mixing of species can be used to further define differences between the species because of their geographic separation. Making geographical maps of the sites within West Virginia can help further explain the relationships between each species and streams. In an analysis of the zones of syntopy and allopatry in West Virginia it can be seen that these species have been able to hybridize as a result of mixing habitats. If that is not the case, it is expected that different genetic variations will be seen within the various species. Being able to record these genes it is expected to add valuable evidence to a debate that has plagued the ichthyologist for almost 30 years, and assist in resolving the classification of the blacknose dace.

Chapter 2: Methods

Collection

Initial fish samples were collect by backpack electrofishing by Geoffrey Smith using a Coffelt Manufacturing Mark 10 Backpack Electrofisher. At that time only male specimen were collected that were in nuptial coloration. Areas of collection were streams that were in the zone of contact known to home *Rhinichthys* species. Because the males mate within cover objects, the electrofishing protocol was modified to include flipping and disturbing of cover objects to collect adequate numbers of samples (Smith, 2007). After capture, Smith labeled the fish with a random numeric code (MU_BND_XXX). Each individual was digitally photographed on site to ensure maximum coloration expression (Appendix A). Afterwards, samples were put into Mylar foil pouches (Sorbent Systems, Los Angeles, CA, #16MFS24ZTE) and preserved in dry ice to maintain the integrity of DNA. The Mylar pouches were then stored in a -80˚C freezer in the laboratory. Mylar pouches were used instead of plastic baggies as they tend to hold up better than plastic at -80°C.

A second collection was done in a similar fashion, with the help of Dr. Tom Jones, Tyler Hern and Paul Huggs. This trip allowed the collecting of any sample, male or female, with or without nuptial coloration, that was found. Collection areas in this sampling were done in streams that had shown potential results of hybridization within the zone of contact and areas that were known to hold only one species with no zones of contact. A control group of fish was collected from Swan creek in Maryland by Tyler Hern. These fish were controls for the *R. atratulus atratulus* species.

Morphological Data Collection

Smith identified each specimen in the field to subspecies level using characteristics that had been identified as the most predictive characters for taxonomy of the members of the species complex (Smith, 2007; Matthews *et al*, 1982; Jenkins and Burkhead, 1994). The purpose of this section was to blindly group the specimen only on character combinations and to determine which of these characters best predicted the identity of the species.

All fish were cataloged into a Microsoft Access Datatable. The sites collected had the stream conditions and the North and East coordinates recorded (Table 1). In a second table (see results), each specimen collected was recorded as well as the physical characteristics of the fish and the species classification that were reflected by the physical characteristics.

Table 1: Collection site information

StreamCode	StreamName	LocationDescription	MajorDrainage	GPSMethod	GPSDatum	Zone	East	North
Laurel	Laurel Fork Cheat River	in Laurel Fork Camping Area	Laurel Fork	Garmin GPS	NAD83	17N	613650	4288760
Pheasant	Pheasant Run	upstream of village of Pleasant Run	Shavers Fork	Garmin GPS	NAD83	17N	609423	4322255
UYR	UNT to Youghiogheny River	near confluence with Youghiogheny River	Youghiogheny River	Garmin GPS	NAD83	17N	630228	4346931
Cove	Cove Run	near culvert under FR44	West Fork Greenbrier River	Garmin GPS	NAD83	17N	603981	4276264
Files	Files Creek	upstream of Beverly	Tygart Valley River	Garmin GPS	NAD83	17N	600242	4297917
Horseshoe	Horseshoe Run	near Horseshoe Camping Area	Cheat River	Garmin GPS	NAD83	17N	620958	4337996
DryFork	Dry Fork	near town of Job	Black Fork River	Garmin GPS	NAD83	17N	625041	4302957
WFGR	West Fork Greenbrier River	near FR35 bridge	West Fork Greenbrier River	Garmin GPS	NAD83	17N	606096	4285431
UWFGR	UNT to West Fork Greenbrier River	downstream of FR44 near May	West Fork Greenbrier River	Garmin GPS	NAD83	17N	605641	4280692
Johnny	Johns Run/ Johnnys Run	between Durbin and Bartow	East Fork Greenbrier River	Garmin GPS	NAD83	17N	604725	4267130
OLR	Otter Lick Run	near Big Otter, WV	Elk River	Garmin GPS	NAD83	17N	498621	4267192
Abernathy	Abernathy Run	near Millesons Mill, WV	South Branch Potomac	Map Interpolation	NAD83	17N	699980	4368103
BIRCH2	Birch River	near Cowen, WV	Upper Elk River	Garmin GPS	NAD83	17N	536306	4253769
BIRCH1	Back Fork Birch River	near Cowen, WV (Rt 82)	Upper Elk River	Garmin GPS	NAD83	17N	536442	4253258
Brushy	Brushy Fork	NA	NA	NA	NAD83	17N	599678	4339426
Pheasant2	Pheasant Run	NA	NA	NA	NAD83	17N	607553	4321559
Swan	Swan Creek	Mary Land	NA	NA	NAS83	18N	402073	4375021

Molecular Analysis

This study was designed to look at the genetic variations among this species complex, as well as any relation that there may be with the physical characteristics and site locations. Therefore, all individuals collected were used in the molecular analysis of this study.

DNA Extraction

DNA was extracted from samples using the following two methods. The first method used 100mg of manually cut fish tissue that was cut from the fish, the first cut included the tail fin structure. This piece was minced to help break down the tissue. The minced tissue was then placed in a 2 mL screw top microfuge tube with 700 µL of sterile Sodium Chloride homogenizing buffer (Aljanabi and Martinez, 1997) and 500 µL of 0.1 mm Zicronium beads and beat at 3300 rpm for 1 minute to continue the breakdown of tissue. The samples were iced for 2 minutes and beat again for 1 minute at 3300 rpm. After the second beating session, the liquid was allowed to settle and 500 µL of tissue/liquid mixture was removed and transferred to a new tube. 50 µL of 20% SDS and 20µL of proteinase K were added to the tube. Samples were mixed by inverting the tubes and were incubated at 55˚C overnight to complete digestion. Following digestion, 100 µL of 6M NaCl was added and the tube was centrifuged at 10,000 x g for 30 minutes at 4˚C to precipitate out proteins. After centrifugation, 750 µL of supernatant was transferred to a new 1.5 mL tube and an equal volume of 100% isopropanol was added and the sample was placed in -20˚C freezer for one hour. After chilling, the sample was centrifuged at 10,000 x g for 20 minutes at 4˚C precipitate DNA. Supernatant was removed and the isopropanol was allowed to evaporate off. The DNA pellet was then resuspended in 100 µL sterile deionized water.

Mitochondrial DNA Extraction

Mitochondrial (mtDNA) was extracted though a phenol chloroform clean-up procedure. 5Prime Phase Lock Gels (#2302840) were used. These were premade tubes that had soft wax centrifuged to the bottom. 100 µL of Phenol-Chloroform was added above the wax. 100 µL of resuspended DNA was added to the tube and centrifuged at 14,000 x g for 5 minutes. 100 µL of chloroform was added to the tube and centrifuged again at the same settings. The liquid layer above the wax was removed with a pipette and placed into a new 1.5 mL tube and 200 µL of 100% ethanol per 100 µL of DNA was added. The ethanol and DNA mixture was spun at 10,000 x g for 20 minutes at 4˚C, precipitating DNA from the solution. Supernatant was discarded and 500 µL of 70% ethanol was added. The mixture was spun at 14,000 x g for 30 minutes. Supernatant was removed again and the DNA pellet was left to dry overnight. Once all ethanol had evaporated off the pellet 100 µL of sterile deionized water. The presence of DNA was verified by running 10 µL of resuspended DNA on a 1% agarose gel and yield was checked with the Nanodrop 1000. DNA samples were stored in -20˚C freezer.

Second protocol for DNA extraction

The QIAGEN-DNeasy Blood and Tissue kit (#69504) was also used following the protocol from the kit. A 25mg section of fish tissue was cut from fish samples and that piece was cut into smaller pieces to help with the lysis process. The tissue was placed in a 1.5 mL tube and 180 µL of Buffer ATL was added. Then, 20 µL proteinase K was added. The tube was mixed by vortexing, and incubated at 56°C until the tissue was completely lysed. The samples were vortexed occasionally during incubation to disperse the samples during the lysing process. When the tissue was completely lysed it was vortexed again for 15 seconds. 200 µL of Buffer AL was added to the sample, and mixed thoroughly by vortexing. 200 µL of ethanol (100%) was added

and mixed again by vortexing. The mixture was pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 minute. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed into a new 2 mL collection tube, 500 µL of Buffer AW1 was added, and the tube was centrifuged for 1 minute at ≥6000 x g. The flow-through and collection tube were discarded. The Mini spin column was placed into a new 2 mL collection tube, 500 µL of Buffer AW2 was added, and the tube was centrifuged for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. The membrane of the spin column must be dry, since any residual ethanol may interfere with the rest of the reactions. The spin column was carefully removed from the collection tube so that the column does not come in contact with the flow-through. The spin column was placed into a clean 1.5 mL or 2 mL microcentrifuge tube and 200 µL of Buffer AE was pipet directly onto the DNeasy membrane. This was incubated at room temperature for 1 minute, and was then centrifuged for 1 minute at ≥6000 x g (8000 rpm) to elute DNA. Again, resulting DNA was checked on a 1% agarose gel and nanodropped.

Measuring DNA yield using Nanodrop 1000

The concentration of DNA yielded from the extraction was measured using a Nanodrop 1000 Spectophotometer (Fisher Scientific, #J046). The nanodrop was attached to a computer and the ND-1000 V3.6.0 software was installed to it. Once that program was opened the button for nucleic acids was selected. A pop-up screen informed the user that water needed to be used to initiate the nanodrop device. The arm of the nanodrop was moved to the up position and 2 µL of dH_2O was pipetted onto the eye of the lower measurement pedestal (Figure 6). The arm was carefully lowered and the OK button was clicked on the pop-up screen.

Figure 6: How to load a sample onto the Nanodrop 3300

When the pop-up screen was gone and the machine had stop initiating, the upper arm was raised and the upper and lower measuring pedestals were wiped with a Kimewipe to remove all water from the measuring pedestals. Next, a blank measurement was made. 2 µL was pipetted onto the lower pedestal and the upper arm was lowered. On the computer, the Blank button was clicked. This calibrates the machine to 0ng/µL for accurate readings of nucleic acids. When the machine was done blanking, again the upper arm was raised and both measuring pedestals were wiped with a Kimewipe to remove the water. The ID of the sample was typed into the text box on the right side of the screen. 2 µL of sample was pipetted onto the lower measuring pedestal and the upper arm was lowered. The 'Measure' button was clicked to signal the machine to measure the sample. When the machine was finished a screen image such as in Figure 7 was created. If the curve was not smooth it meant that other material was still in the sample. A second measurement was done to check that the first test was not correctly, if the second

measurement still showed poor results the DNA was extracted and cleaned again. The measuring process was repeated with all samples, cleaning the upper and lower measuring pedestals after each measurement with a Kimewipe.

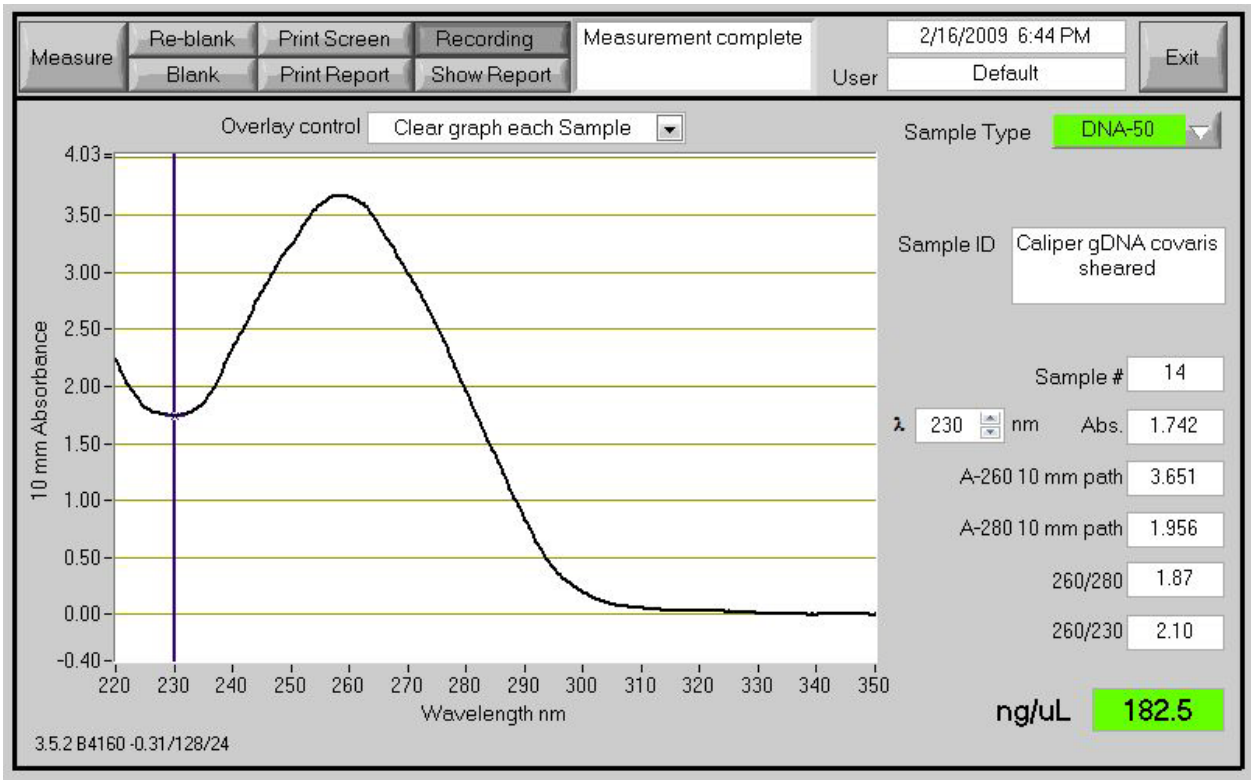


Figure 7: Final Nanodrop result screen of DNA yield.

Preparing and running a 2% Agarose gels using TBE buffer

To check DNA purification and PCR amplifications 2% agarose gels were ran. To prepare a gel the electrophoresis box had to be set up first. The gel dock was placed into the gel box so that the rubber grips to the sides of the box, it was important to have a seal around the gel dock to keep the agarose from leaking out. A gel comb was placed in the top notch in the gel dock. Often, one litter of 1X TBE was needed to be made to run a gel (one liter will make and run about 2 gels). To make 1X TBE, 100 mL of 10X TBE (Roskams and Rodgers, 2002) was combined with 900 mL of distilled H_2O, and inverted to mix into a homogeneous mixture. To

prepare the 2% agarose gel, 2.0 grams of agarose was measured out and placed into a 250 mL bottle. 100 mL of 1X TBE was added to the bottle. The bottle was placed into the microwave for 2 minutes. The mixture was watched carefully while in the microwave for boiling. When the solution began to boil the microwave was stopped and using a heat resistant glove the solution was swirled to disperse the heated material. The bottle was placed back into the microwave and started again. The microwave was stopped each time the solution began to boil and was swirled to mix until all the bubbles were gone and the liquid was clear. When the agarose was completely dissolved 0.35 µL of Ethidium Bromide (EthBr) was added to the solution and mixed in by swirling. Once, the EthBr was mixed throughout the solution the gel was slowly poured into the gel dock until there was about 2mm of agrose in the dock. It took about 20 minutes for the gel to completely set.

While the gel was setting up, 2 µL bubbles of loading dye for each sample was placed on a piece of parafilm. Each sample was thawed and vortexed for 15-30 seconds. Once the gel had set the gel dock was removed and rotated to the left by 90°. This placed the wells towards the black electrode. 1X TBE buffer was added to the gel box until no portion of the gel was exposed. 10 µL of sample was removed from its tube using a 0.5-10 µL pipet and was dispensed into one of the bubbles of loading dye. The bubble was then pipetted up and down until the sample and dye were well mixed. Using the pipette 10 µL of this mixture was collected and dispensed into an empty well of the gel, each sample location was recorded while loading the gel. After each sample the pipette tip used was disposed before moving to a new sample. This avoided any cross contamination between samples. One end well of the gel was left empty of sample, this well was used for a DNA ladder which helped in determining if the DNA was clean enough, or if the PCR amplicon was the correct size. 2 µL of DNA ladder was pipetted into that

well. When all samples were loaded the lid was placed onto the box and the electrodes were plugged into the power outlet. The gel was then ran at 70 volts for 60 minutes. Turn off the power supply when the gel has completed its run.

When the gel was finished running, the loading dye had traveled about half way down the gel, the gel was removed from the gel box and checked using the Gel Doc camera (Biorad, #75S 02285). The lower drawer of the machine was opened and checked for cleanliness. If the UV screen was not clean it was cleaned with 70% ethanol. The gel was then placed onto the screen and the drawer was closed. The Quality One program on the computer was opened. Gel Dock was selected under the File tab. When the program began working the Trans UV button was pushed on the machine. Manual Expose was selected, exposing the gel to UV light and giving us an image. The amount of light could be controlled by increasing or decreasing using the up or down arrows under the Manual Expose button. The image was improved by using the Gamma slider near the bottom of the screen. When the image was clear it was saved to the DACE file on that computer and printed from the video printer by clicking the Video Print button above the save button.

PCR Amplification of cytochrome b

After extraction all samples were quantified using the labs Nanodrop. They were then diluted to 100ng/µL from their original concentration. Initial work for the mitochondrial cytochrome *b* gene was adopted from Pfrender *et al.*(2003) work with speckled dace (*Rhinichthys osculus*). Pfrenders *et al.* primers were used to amplify a 670-bp segment of mitochondrial DNA that contained a portion of the cytochrome *b* gene, which has been used frequently to trace the evolutionary history of cyprinids. (Pfrender *et al*, 2003; Kotlik and Berrebi, 2001) Initially the full protocol for amplification was followed using Pfrender's setup.

PCR amplification was set up in 50 µL reactions in 50 µL tubes. A master cocktail was made for each set up in the PCR hood. The amount of materials used depended on how many samples were being set up. Three reactions were set up for each DNA sample. An Excel table was set up in the following manner to help with calculations of the amount of materials needed (Table 2). The number within the yellow box was changed depending on the number of reactions that were set up. The Excel table was set up that each value in the "amount in µl" column was multiplied by the yellow box value and the product was inserted into the "amount for X samples" column.

Table 2: Calculation table for PCR reaction materials.

	amount in µl	amount for X samples in µl	X=	3	
GoTaq	12.5	37.5			
MgCl2	3	9			
Forward Pirmer	1	3			
Reverse Primer	1	3			
dH20	31.5	94.5			
total	49	147			

The "amount for X samples" amounts for each material was pipetted into a 2 mL tube, a larger tube was used if a large amount of reactions was set up. This tube was vortexed for 15 seconds to make sure the materials mixed into a homogeneous solution. 49 µl of the cocktail solution was pipette into one of the 50 µl tubes. The tubes were labeled for each reaction being set up, using the fish number of the fish DNA being used, 1 µl of the corresponding DNA was pipette into each of the 50 µl tubes labeled and the tubes were capped and the sample centrifuged down for a few seconds to make sure all materials were mixing together. The PCR amplification followed the thermocycler settings of an initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of 92˚C for 45 seconds, 48˚C for 60 seconds, and 72˚C for 90 seconds, that Pfrender *et al.*(2003) used. PCR samples were stored in the -20°C freezer until they could be run on a 2% gel to check for amplicons.

PCR Amplification of RAG2

Following Lovejoy's *et al.* (2001), primer combinations were used of the following: F1/R4, F1/R6, F2/R4, and F2/R6. PCR reactions were set up in the same manner as they were for the mitochondrial cytochrome *b* reaction was. Amplification was done with the following settings: denaturing at 95°C for 30 seconds, a decreasing annealing temperature gradient was done every two cycles decreasing 2°C every cycle from 58°C to 52°C for 60 seconds, then 72°C for 90 seconds. A total of 8 cycles were run. Then, in the same program, 95°C for 30 seconds, 50°C for 60 seconds, and 72°C for 90 seconds for 27 cycles. Those that worked went through clean-up and sequencing. Primer-3 was used to help design new primers from the recent sequencing. Once the new primers arrived a test experiment was set up mixing Lovejoy's forward primer with our reverse and his reverse with our forward. The combination that used Lovejoy's forward and our reverse primers with the published thermocycler programming produce a significantly higher amount of product, and were used for the completion of this study.

PCR Clean-up

Clean-up of both cytochrome *b* and RAG2 amplicon was done using the Amicon Ultra-0.5 mL Centrifugal Filters for DNA Purification and Concentration (Millipore, # UFC503024) A reservoir was placed into a 1.5 mL tube, the reservoir would collect the amplicon product and any extra material would flow through to the tube. To the reservoir 150 µL of PCR product was added, as well as 350 µL of dH_2O. The tube was spun at 1,000 x g for 15 minutes. The reservoir was moved to a new tube and 20 µL distilled water was added. The reservoir was then place upside down into the new tube and spun at 1,000 x g for 2 minutes. A small amount of product will have spun out of the reservoir. This clean product was Nano dropped to check concentration

and then diluted to 25 ng/µL and checked on an agarose gel to be sure a band was present without primer dimer formations.

Sequencing

Sequencing for this project was done by Beckman, formally Agencourt, in Danvers Massachusetts. Agencourt requires 5 µL of DNA product for each sequencing run, a forward and reverse, and a chance to rerun a second time if needed, a total of 20 µL needed. Twenty-five µL quantities of each sample were sent of the 25 ng/µL concentration of clean product to be sure the company had all that was required. This had to be sent in Agencourts special barcode tubes. Each tube was labeled with the sample number. Also required was 2 µL of each primer for each reaction that may be required. The samples and primers were packed on ice and shipped to the company via Fedex.

Alignment and Tree building

Sequences were returned back within a two day period in .ab1 format. Sequences were imported into Sequencher, a software that allowed us to align the forward and reverse sections of DNA. Each sample had two pieces, forward and reverse, that had to be aligned together. The combined pieces, or contig, were trimmed of their unreadable ends and ambiguous nucleic acids were corrected manually. Once all samples were aligned and trimmed to the same size they were all aligned in Clustl W, as a FASTA file, for a preliminary view of possible tree relations. Clustal W is not used often for publishing data, but as a preliminary trial of how the tree may look.

Sequences were imported into Sequencer by clicking “File”, “Import” and “Sequences (Figure 8). This opened up a directory to allow the user the find and select the number of

samples they wish to align from the folder they saved the ab1 files in.

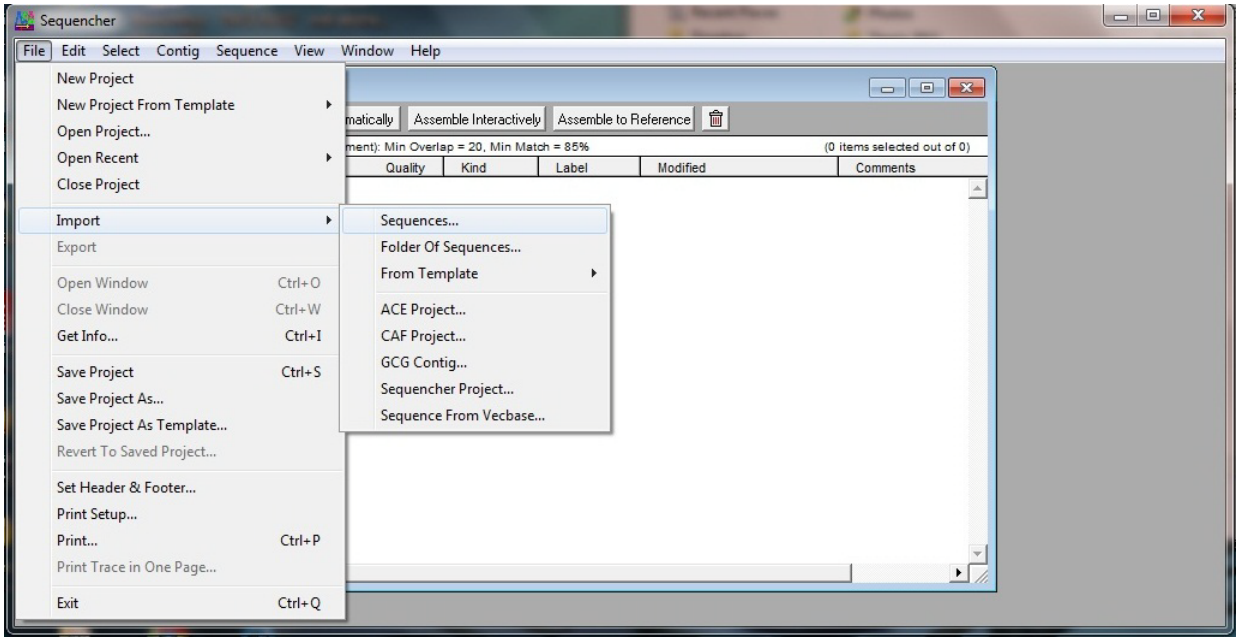


Figure 8: Importing sequences into Sequencer.

To begin aligning and correcting the sequence the user must select the two pieces of the samples sequence (Figure 9). The first sequence was selected by clicking on it, the second was selected by holding the "Ctrl" button on the keyboard and clicking the sequence.

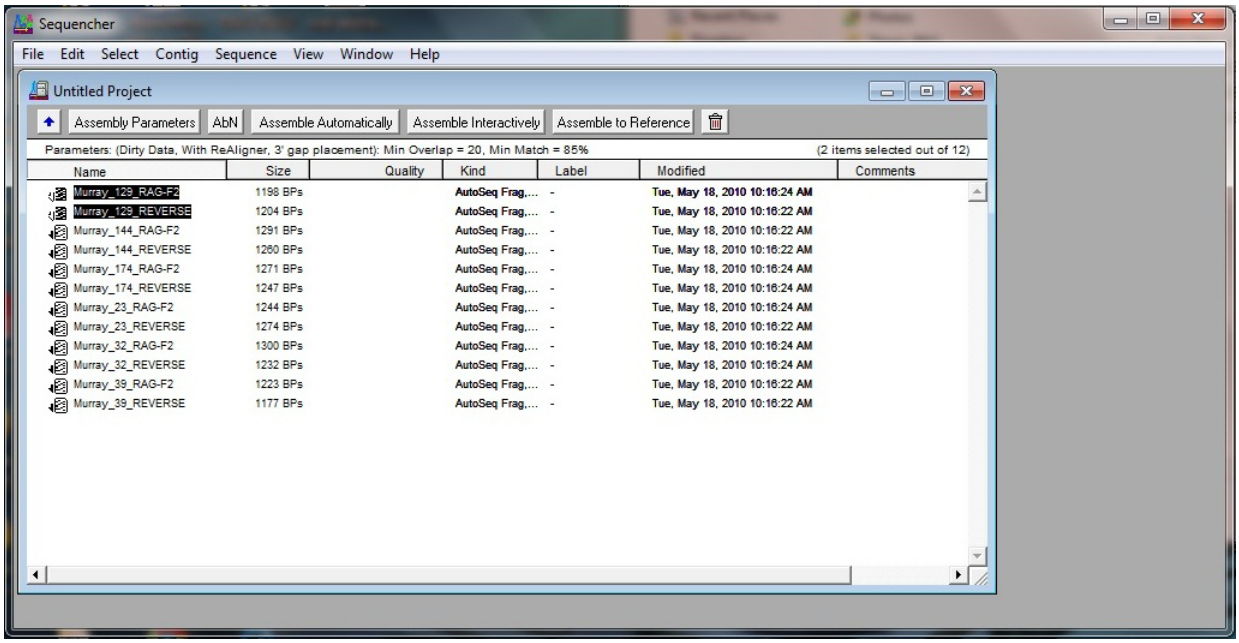


Figure 9: How to select two sequences to be aligned together.

When both pieces of the sequence were selected, “Assemble Automatically” was clicked. This caused the software to align the sequences together. A pop up window appears to let the user know whether or not the sequences were aligned. “Okay” was clicked to close the pop up. Sequencer gives the sequence a contig name. Right clicking the sequence and selecting rename allowed the user to rename the sequence in a way that makes sense to the user.

With the sequence aligned corrections were made. The contig was double clicked to open a new window (Figure 10). The button “Bases” was clicked to change the editor window to one that contained the order of the nucleic acids of the sequence. On either end there was a long strand of ‘N’ nucleic bases. These were highlighted and deleted from each end.

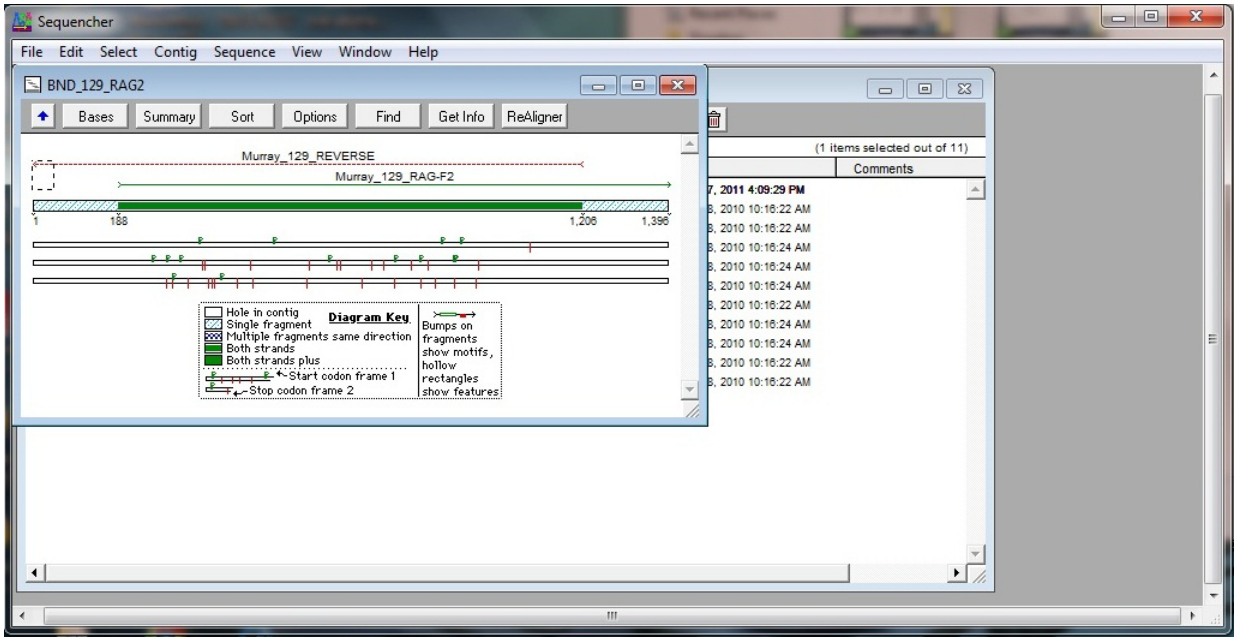


Figure 10: Editor window in Sequencher.

Figure 11: View of sequences after selecting "Show Chromatogram". The aligned sequences can be seen in the top window and the color signaling of the sequencing can be seen in the bottom window.

The button “Show Chromatograms” was clicked to open a second window. This window contained the visual signaling information from the sequence (Figure 11). At the beginning and end of the sequences there was an area of unreadable signaling. This area was where the primers were joining to the DNA during the sequencing process. These areas were removed using our best judgment of where the readable sequence information started.

Using the left and right keyboard area the sequence was checked for questionable areas. These areas were marked with a “+” below the sequence in the top window. Figure 12 demonstrates an area that caused the computer to question the sequences. The top sequence in the bottom window is not as strong as the bottom sequence. In the bottom sequence two clear peaks are seen, but in the top one only a long peak is seen. The stronger sequences were often used to make corrections to the sequence. All of these areas were corrected in this manner. When all areas were corrected the editor window was closed and the project was saved using the save command. These steps were repeated for all samples.

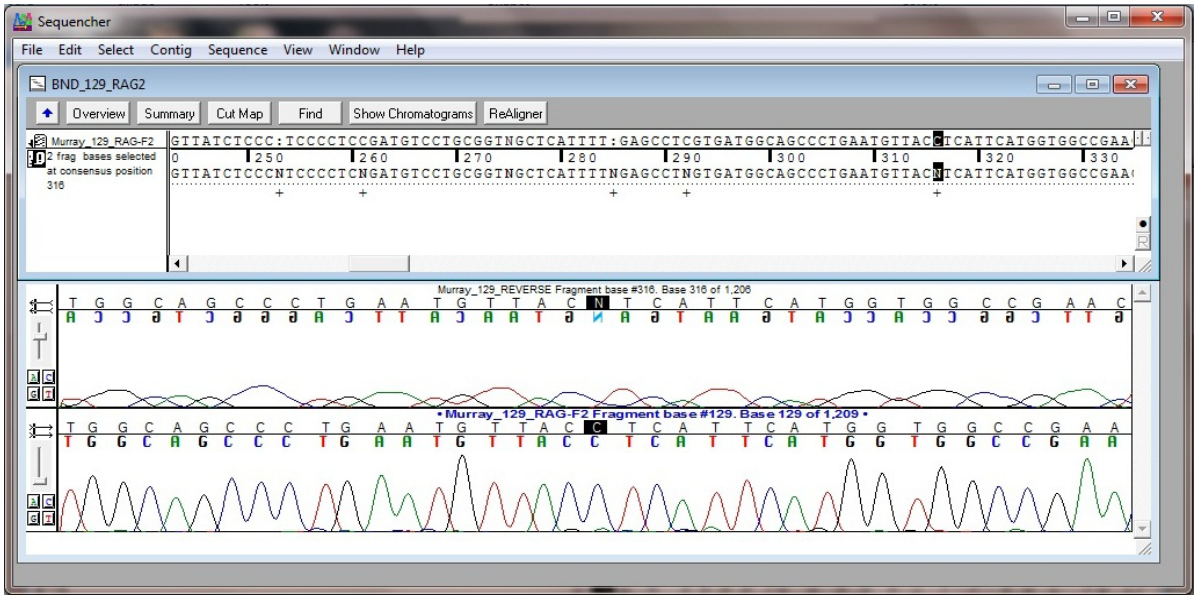


Figure 12: Finding questioning areas within a sequence.

*BEAST

The program *BEAST was used for data collection. *BEAST is a free online software program that will run sequences together multiple times and calculate for the most-likely tree for the data imported. Each set of sequences were loaded separately into BEAUti, which comes with the *BEAST software, as FASTA files. Under File select “Import Data…” (Figure 13)

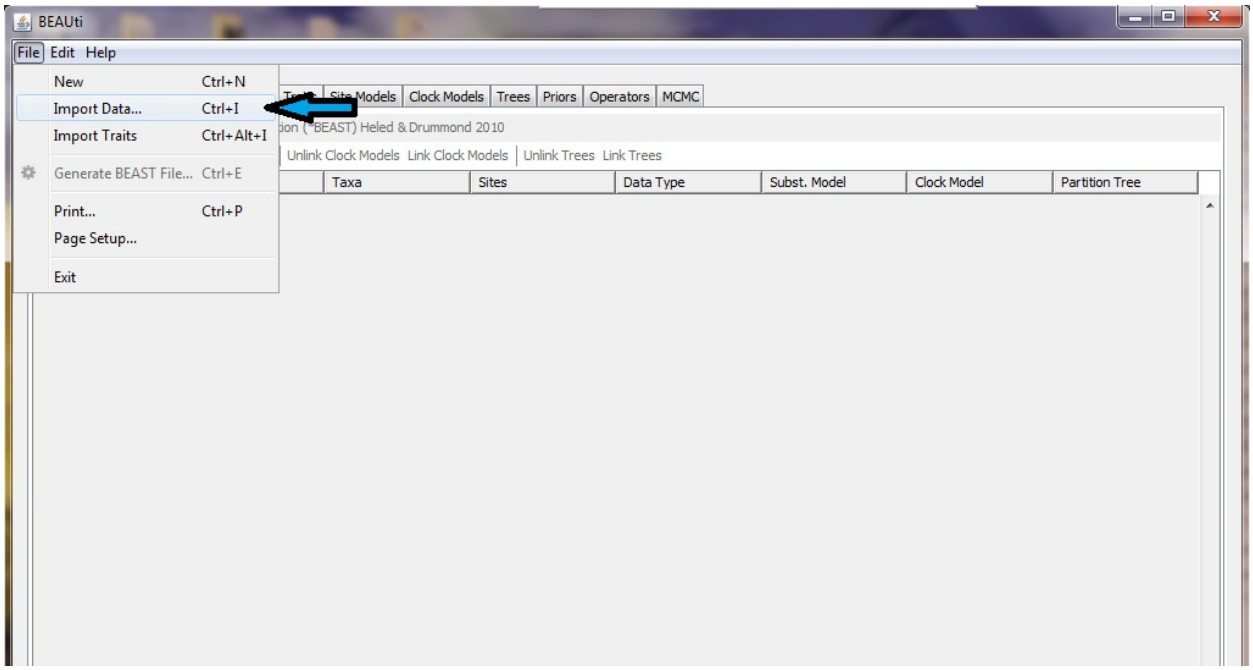


Figure 13: Importing data into BEAUti.

This caused a pop up window to open. The folder "DACE MIO" contained the mitochondrial cytochrome *b* FASTA file of all the sequences used in this study. The file "Mito FASTA…" was selected and "Open" was clicked to import the file into BEAUti (Figure 14). Once the file had up loaded the "Generate BEAST File" button was clicked (Figure 15)

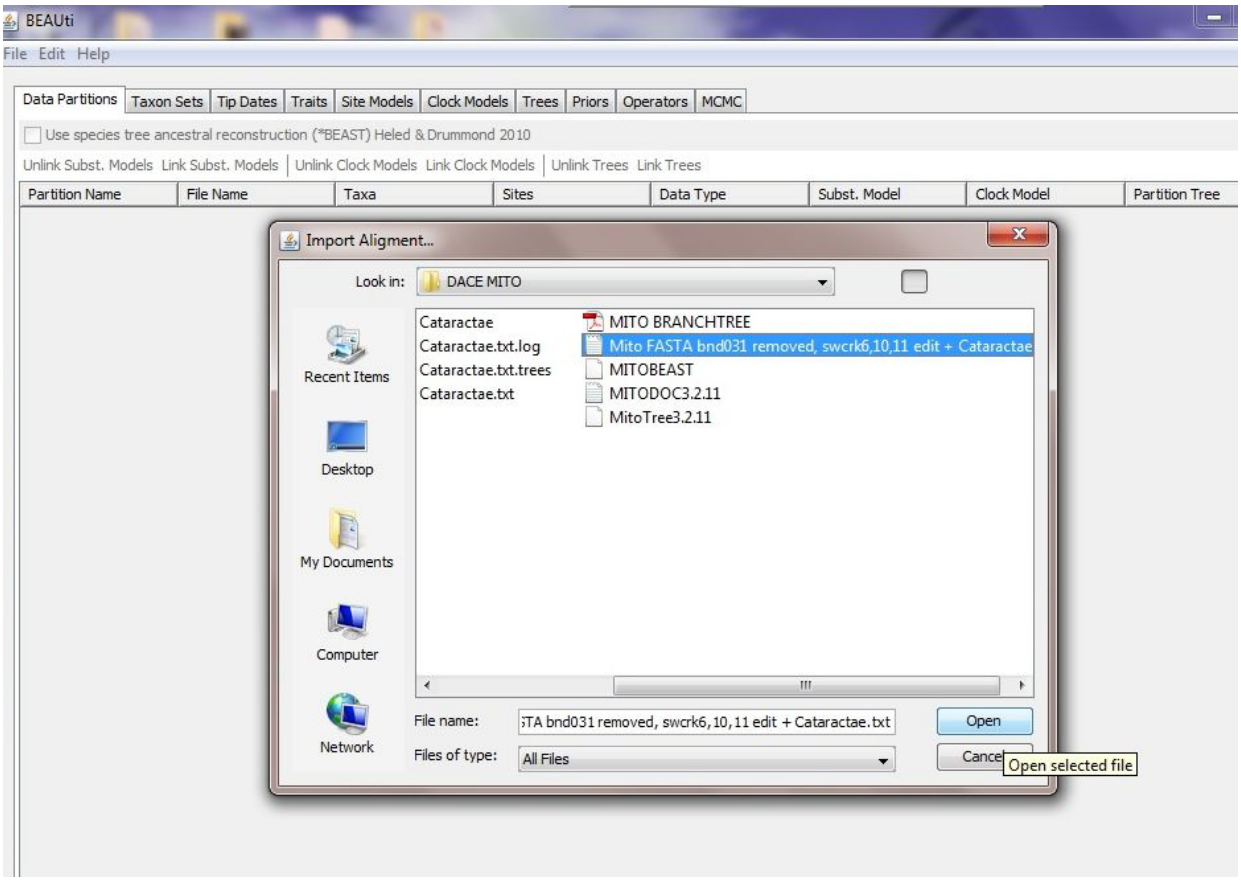


Figure 14: Opening the Mitochondrial cytochrome *b* FASTA file into BEAUti.

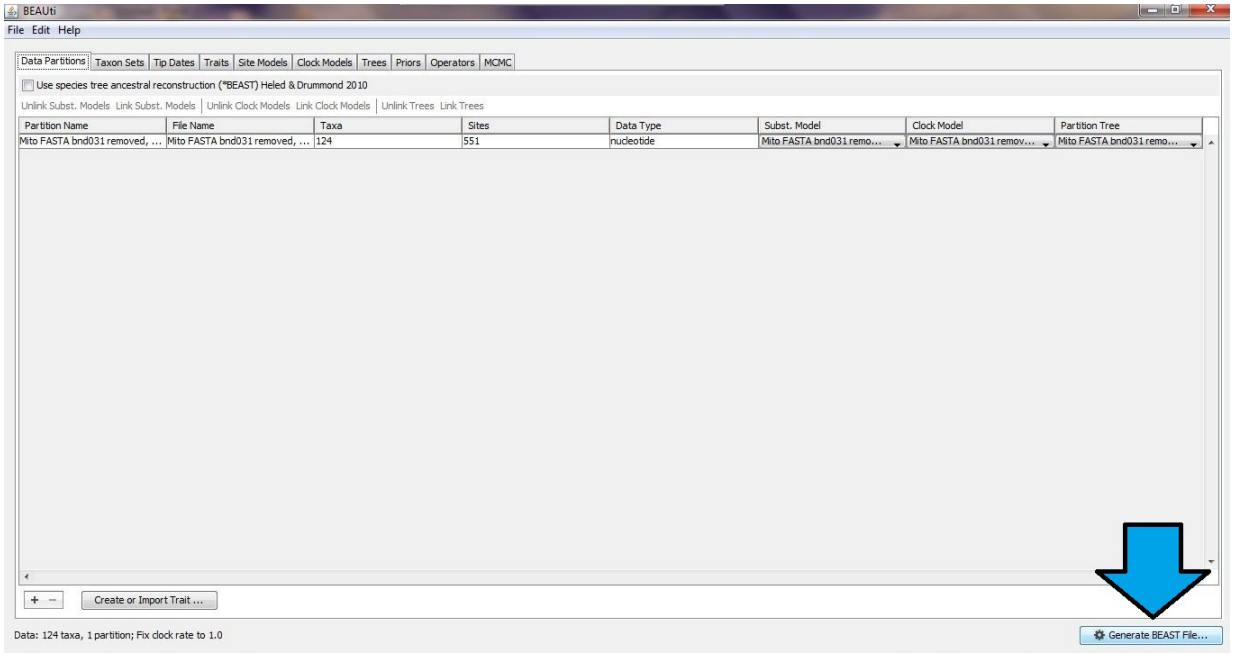


Figure 15: Selecting the "Generate BEAST file…" button after uploading the sequence data.

BEAUti can be used to add traits, time clocks, and other characteristics that are useful in making evolutionary based trees. Because all of our samples were collected in the same time frame and we only wanted to sort the data according to the sequences collected, the default settings were used (Figure 16). The "Continue" button was clicked and BEAUti created an XML file within the "DACE MITO" folder. This XML file was needed to run in *BEAST.

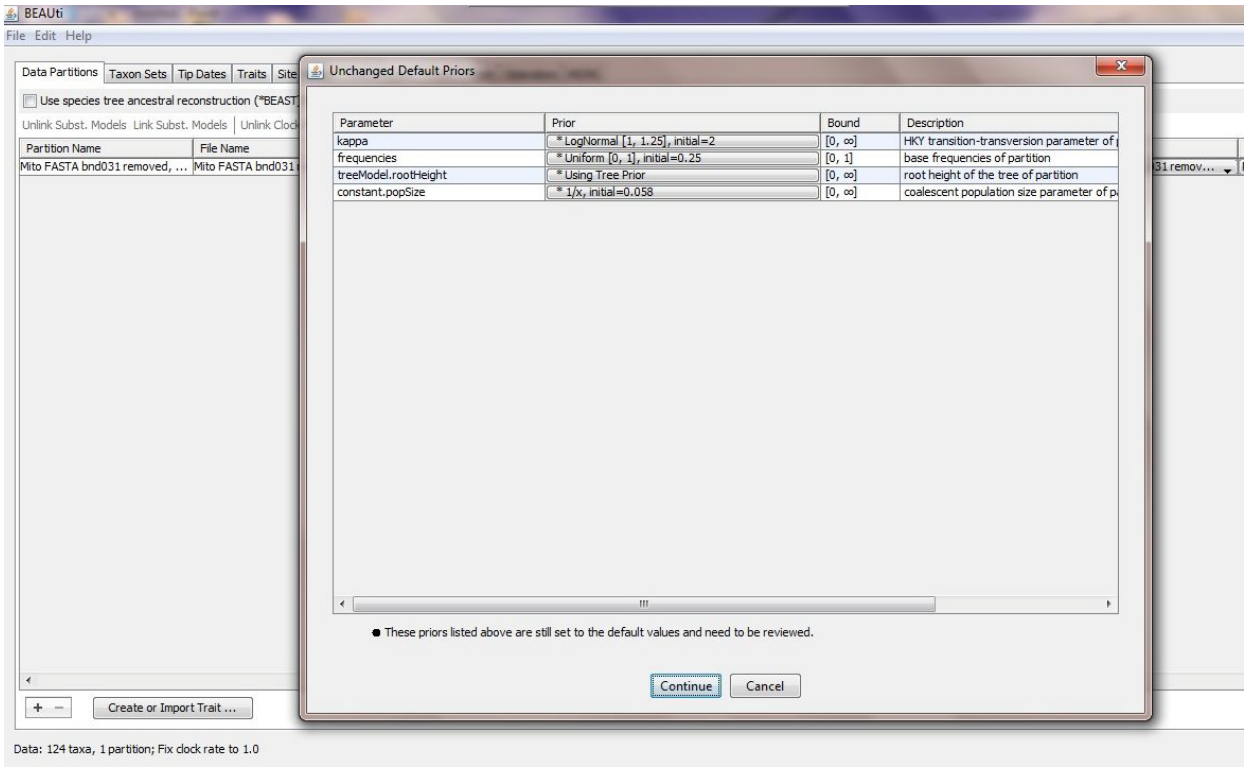


Figure 16: Default Priors for BEAST files.

*BEAST was opened and the file just created was uploaded into the Run selection. The Run button was clicked and *BEAST began to run. Each file ran for just over an hour within *BEAST. After *BEAST was finished, Tree Annotator was used to sort through all trees that *BEAST had made. Tree Annotator was opened and the .tree file that was created in *BEAST was opened into the program and a final tree was saved by this program. The program Fig Tree was then used to open the tree file created in Tree Annotator. Each tree was color coded for easy reading. Branches were color coded according to stream location in Fig Tree.

Maps

To help to visualize data collected, maps were made using Microsoft Access and ArcGIS ArcMap™ version 9.3.1. A map of West Virginia's borders and river locations were imported into ArcMap from Marshall's GIS folder. The boarders shape file was stored in the "WV_Shape_files" folder, and the rivers were in the "water_resources" folder (Figure 17).

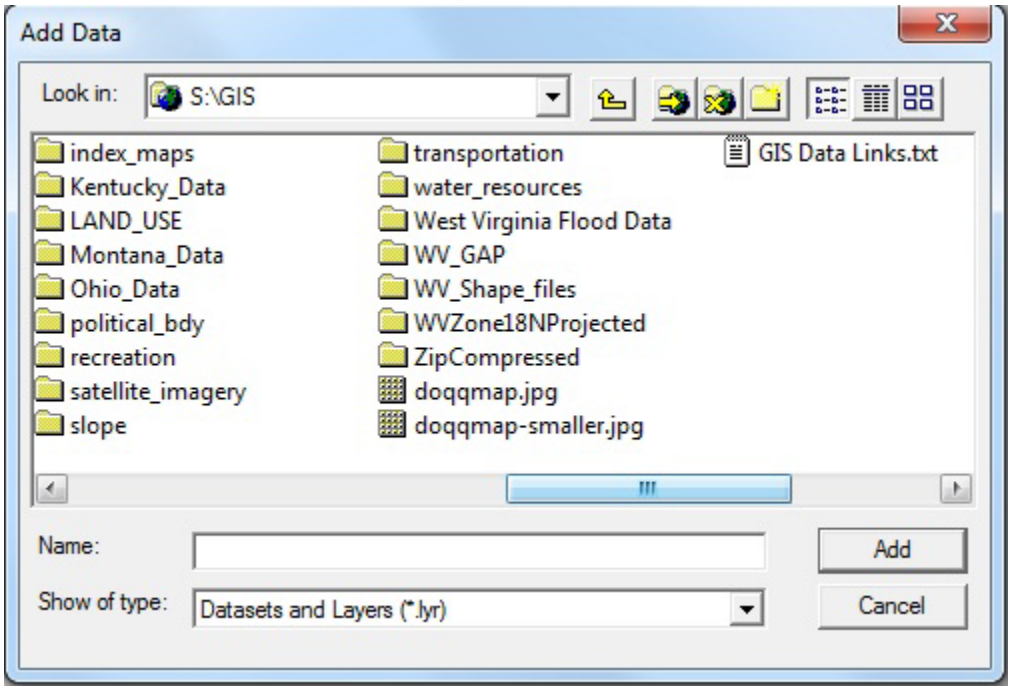


Figure 17: Folders used in the S:\GIS folder to make the stream maps of West Virginia.

For the first map, the distribution map (see results), areas were selected using the draw tool. This created shaped features that could be color coded according to the fish species to be found in an area.

For maps to display biological data, the Site Information Access table was imported using the Add Data button, under the File tab or the icon composed of a'+' with a yellow diamond behind it. The table was then located in the table of contents on the left of the program window. Right-clicking the table opened a drop down window and the "Display XY Data" option was listed. Selecting the "Display XY Data" option opened a pop up window. The "Y Field" was

changed to “North” and the “Coordinate System of Input Coordinates” were changed using the “Edit button. Select the “Select…” button in the newly opened “Spatial Reference Properties” window. The “Projected Coordinate Systems” folder was opened, then the “UTM” folder, “NAD 1983” folder, and the “NAD 1982 UTM Zone 17N” projection was selected and the “Add” button was clicked. This listed the detail of the coordinate system in the “Spatial Reference Properties” window. After this the “OK” button was clicked on both windows. This table plotted the site locations where samples were collected. The SiteInformationEvent table then had a point icon to show what represents the points, and points have been plotted on the map (Figure 18).

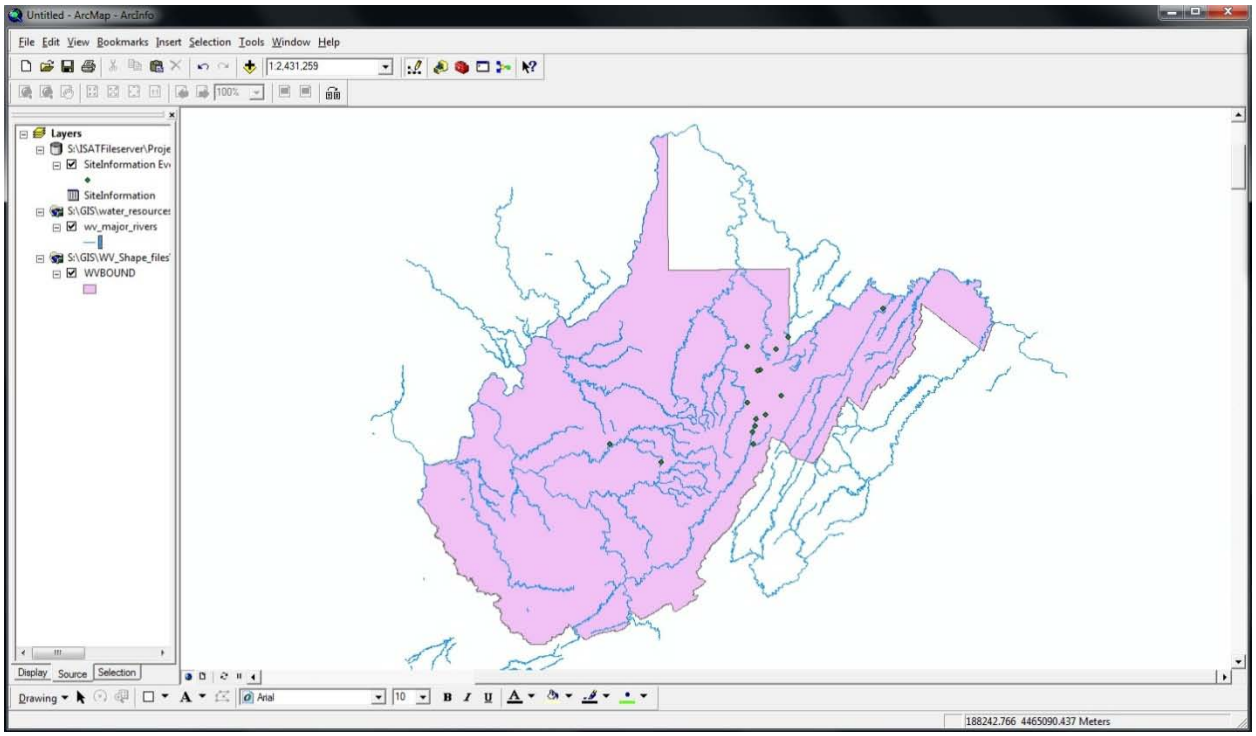


Figure 18: Plotted points in a map of West Virginia using the Site Information table.

A separate map was made for the physically recorded characteristics, mitochondrial DNA data, and RAG2 DNA data, and amino acid changes in RAG2 DNA. For each map the

corresponding Access table was imported using the “Add Data” button and joined to the Site Information table. This was done by right-clicking the Site Information table and selecting “Joins and Relates” option and selecting “Join”. In fields 1 and 3 the “Stream Code” label was used to join the two tables. Field 2 was to have the name of the table that was being joined to the Site Information table. The symbology of the Site Information layer was changed from features with a single symbol to pie charts. The Site Information table was right-clicked and “Properties” was selected from the drop down box. The symbology tab was selected and in the list on the left of the window “Charts” and “Pie” was selected. From the field selection list the three species were selected, in the RAG2 differences map the fields with the number of differences were selected, and the amino acid changes were selected for a second RAG2 map. These selections were moved to the right-side window and colors were selected to represent each species and amino acid by right-clicking the colored rectangles and selecting the new color (Figure 19).

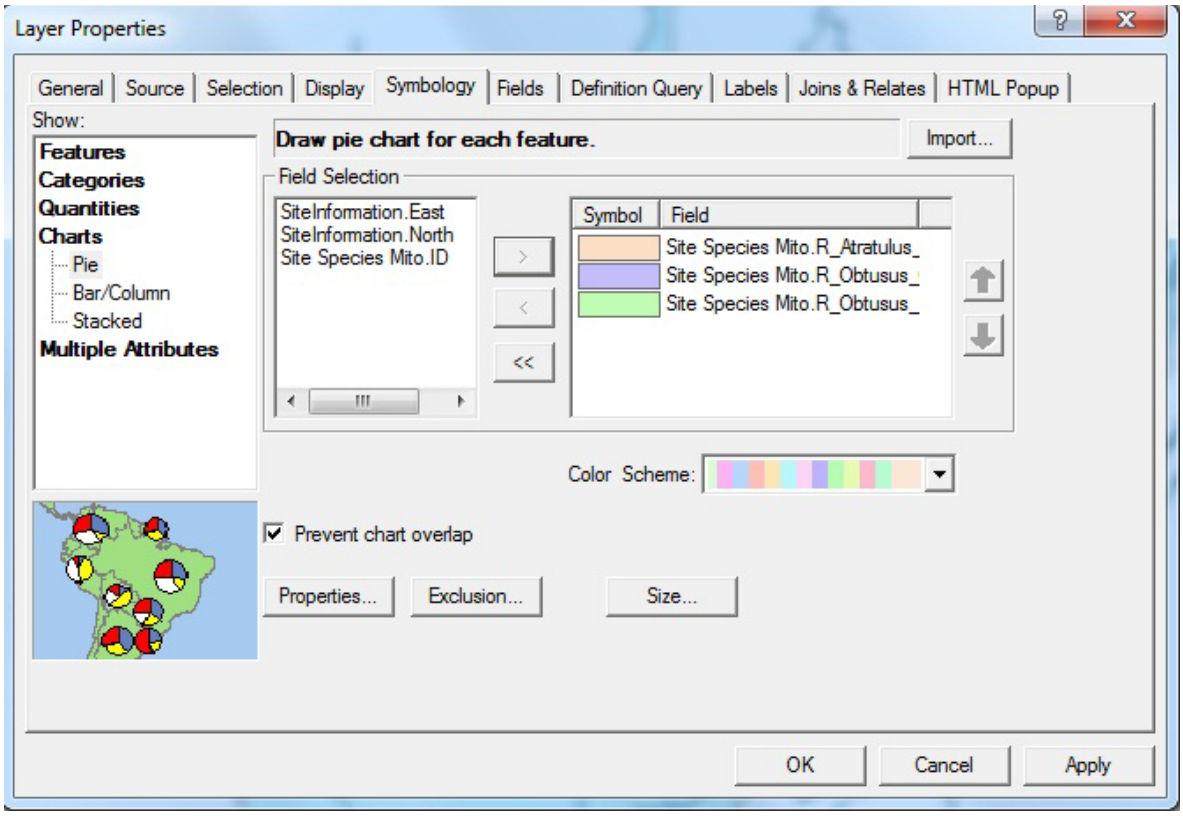


Figure 19: Window display for pie chart creation. Species have been moved, but new colors have not been selected yet.

Chapter 3: Results

Fish Collection

A total of 123 fish were collected for this study. Geoff Smith collected 91 fish for his initial study in areas with and without mixed populations of the species complex. Dr. Jones, Tyler Hern, Paul Hugg, and I collected 13 in a second collection of areas with mixed populations. Tyler Hern obtained 19 from Swan Creek in Maryland as a control group for the *Rhinichthys atratulus atratulus* species. The second and third collections were done late in the summer, when the fish do not display their nuptial colorations. Physical coloration data was not collected for those samples.

Geoff Smith did a study of the relation of several of his collected fish's physical characteristics and the resulting mitochondrial sequences. He kept a very detailed table of the traits shown in each fish, Table 3. The second and third collection fish were added to the table, but many of the fish did not express their nuptial coloration traits, so NA was entered into their row of the table.

The total number of fish collected from each site is listed in Table 4. Sites were chosen by Smith (2007) for his initial study of the blacknose dace and their molecular relationship.

> This study aimed to focus on the relationships of these members of the three forms of the blacknose dace in a zone of contact in the Appalachian Mountains of West Virginia. Important in this study were the areas of known stream capture events, anomalous distributions, and areas along the present day Eastern Continental Divide that separates the Atlantic from the Ohio River drainages, the major factor in the separation of the newly defined species status (Smith, 2007)

Fish were also collected according species location. *R. atratulus atratlusus* species were suppose to be found in the eastern drainages going to the Atlantic Ocean. *R. obtusus obtusus*

were to be found in the southern part of the state in watersheds to the lower Ohio River. *R. obtusus meleagris* were to be found in the norhtern portion of the state and drainages to the upper Ohio River.

An Access table was created (Table 5) according to Smith's collection information. A column with each site was made. Then column headers of the fish species were listed. Smith listed the possible physical classification with the fish number as well as where the fish came from. Using that data the total number of each species was counted for each site location and listed in the table.

Table 3: Fish Information according to physical characteristics

FishCode	FieldIdentity	StreamCode	LateralLineColor	LLBandWidth	ColorBelowLateral
BFORK_1	NA	Brushy	NA	NA	NA
BFORK_2	NA	Brushy	NA	NA	NA
BFORK_3	NA	Brushy	NA	NA	NA
MU_BND_001	R. obtusus meleagris	Johnny	Orange	Double	Orange
MU_BND_002	R. obtusus meleagris	Johnny	Orange	Double	Orange
MU_BND_003	R. atratulus atratulus	Cove	Cinnamon	Confined	Orange
MU_BND_005	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_006	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_008	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_009	R. atratulus atratulus	Horseshoe	Cinnamon	Confined	White
MU_BND_010	R. obtusus meleagris	Pheasant	Orange	Double	Orange
MU_BND_011	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_016	R. obtusus meleagris	UWFGR	Orange	Wide	Orange
MU_BND_017	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_019	R. atratulus atratulus	Pheasant	Cinnamon	Confined	Yellow
MU_BND_021	R. obtusus obtusus	OLR	Orange	Confined	White
MU_BND_023	R. obtusus meleagris	WFGR	Orange	Wide	Orange
MU_BND_027	R. atratulus atratulus	Horseshoe	Cinnamon	Confined	Yellow
MU_BND_028	R. obtusus obtusus	Files	Orange	Double	Orange
MU_BND_029	R. obtusus obtusus	OLR	Orange	Confined	White
MU_BND_030	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_031	R. atratulus atratulus	Pheasant	Cinnamon	Confined	Yellow

FishCode	FieldIdentity	StreamCode	LateralLineColor	LLBandWidth	ColorBelowLateral
MU_BND_032	R. obtusus obtusus	OLR	Orange	Confined	White
MU_BND_033	R. obtusus meleagris	UWFGR	Cinnamon	Wide	Orange
MU_BND_034	R. obtusus obtusus	Files	Rust	Confined	Yellow
MU_BND_035	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_036	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_037	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_038	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_039	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_042	R. obtusus obtusus	Files	Scarlet	Confined	White
MU_BND_043	R. obtusus meleagris	WFGR	Cinnamon	Wide	Orange
MU_BND_044	R. obtusus obtusus	BIRCH2	Cinnamon	Confined	White
MU_BND_045	R. obtusus meleagris	UWFGR	Cinnamon	Double	Orange
MU_BND_046	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_047	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_048	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_049	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_053	R. obtusus meleagris	UWFGR	Cinnamon	Double	Orange
MU_BND_054	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_055	R. obtusus meleagris	Birch River	Cinnamon	Double	Orange
MU_BND_056	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_057	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_058	R. obtusus obtusus	Pheasant	Orange	Confined	Yellow
MU_BND_062	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_065	R. obtusus obtusus	BIRCH2	Orange	Confined	Orange

FishCode	FieldIdentity	StreamCode	LateralLineColor	LLBandWidth	ColorBelowLateral
MU_BND_066	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_067	R. atratulus atratulus	Abernathy	Cinnamon	Confined	Yellow
MU_BND_068	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_069	R. obtusus obtusus	Files	Orange	Confined	Orange
MU_BND_070	R. obtusus obtusus	Files	Cinnamon	Confined	White
MU_BND_071	R. obtusus obtusus	BIRCH1	Orange	Confined	White
MU_BND_073	R. obtusus obtusus	OLR	Orange	Confined	White
MU_BND_074	R. obtusus obtusus	Files	Orange	Wide	Orange
MU_BND_075	R. atratulus atratulus	Pheasant	Cinnamon	Confined	Orange
MU_BND_076	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_077	R. obtusus meleagris	WFGR	Orange	Wide	Orange
MU_BND_078	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_079	R. obtusus meleagris	Pheasant	Orange	Wide	Orange
MU_BND_080	R. obtusus meleagris	WFGR	Orange	Wide	Orange
MU_BND_081	R. obtusus meleagris	WFGR	Cinnamon	Double	Orange
MU_BND_082	R. obtusus obtusus	BIRCH1	Orange	Confined	White
MU_BND_083	R. atratulus atratulus	UYR	Cinnamon	Confined	Yellow
MU_BND_084	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_085	R. obtusus obtusus	BIRCH1	Orange	Confined	White
MU_BND_086	R. obtusus meleagris	WFGR	Orange	Double	Orange
MU_BND_087	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_088	R. obtusus meleagris	WFGR	Orange	Wide	Orange
MU_BND_089	R. obtusus obtusus	Pheasant	Cinnamon	Confined	Yellow
MU_BND_090	R. obtusus obtusus	BIRCH2	Orange	Confined	White

FishCode	FieldIdentity	StreamCode	LateralLineColor	LLBandWidth	ColorBelowLateral
MU_BND_091	R. atratulus atratulus	DryFork	Cinnamon	Confined	Yellow
MU_BND_128	R. obtusus meleagris	Laurel	Cinnamon	Wide	Orange
MU_BND_129	R. obtusus meleagris	Laurel	Orange	Wide	Orange
MU_BND_130	R. obtusus meleagris	Laurel	Orange	Double	Orange
MU_BND_131	R. atratulus atratulus	Laurel	Cinnamon	Confined	Yellow
MU_BND_132	R. obtusus meleagris	Laurel	Orange	Double	Orange
MU_BND_140	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_141	R. obtusus obtusus	Files	Rust	Confined	little or no color below
MU_BND_142	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_143	R. obtusus obtusus	Files	Cinnamon	Confined	White
MU_BND_144	R. obtusus obtusus	Files	Orange	Confined	White
MU_BND_166	R. atratulus atratulus	DryFork	Cinnamon	Confined	Yellow
MU_BND_174	R. atratulus atratulus	DryFork	Cinnamon	Confined	Yellow
MU_BND_175	R. atratulus atratulus	DryFork	Cinnamon	Uncertain	Orange
MU_BND_179	R. obtusus meleagris	Laurel	Orange	Wide	Orange
MU_BND_183	R. obtusus meleagris	Laurel	Orange	Wide	Orange
MU_BND_184	R. atratulus atratulus	DryFork	Cinnamon	Confined	Yellow
MU_BND_185	R. atratulus atratulus	DryFork	Cinnamon	Confined	Yellow
MU_BND_187	NA	NA	NA	NA	NA
MU_BND_188	NA	NA	NA	NA	NA
MU_BND_202	NA	NA	NA	NA	NA
MU_BND_209	NA	NA	NA	NA	NA
MU_BND_222	NA	NA	NA	NA	NA

FishCode	FieldIdentity	StreamCode	LateralLineColor	LLBandWidth	ColorBelowLateral
MU_BND_258	NA	NA	NA	NA	NA
PRUN_1	NA	Pheasant	NA	NA	NA
PRUN_10	NA	Pheasant	NA	NA	NA
PRUN_2	NA	Pheasant	NA	NA	NA
PRUN_3	NA	Pheasant	NA	NA	NA
PRUN_4	NA	Pheasant	NA	NA	NA
PRUN_5	NA	Pheasant	NA	NA	NA
PRUN_6	NA	Pheasant	NA	NA	NA
PRUN_7	NA	Pheasant	NA	NA	NA
PRUN_8	NA	Pheasant	NA	NA	NA
PRUN_9	NA	Pheasant	NA	NA	NA
SWCRK_10	NA	Swan	NA	NA	NA
SWCRK_11	NA	Swan	NA	NA	NA
SWCRK_12	NA	Swan	NA	NA	NA
SWCRK_13	NA	Swan	NA	NA	NA
SWCRK_14	NA	Swan	NA	NA	NA
SWCRK_15	NA	Swan	NA	NA	NA
SWCRK_16	NA	Swan	NA	NA	NA
SWCRK_17	NA	Swan	NA	NA	NA
SWCRK_18	NA	Swan	NA	NA	NA
SWCRK_19	NA	Swan	NA	NA	NA
SWCRK_2	NA	Swan	NA	NA	NA
SWCRK_20	NA	Swan	NA	NA	NA
SWCRK_3	NA	Swan	NA	NA	NA
SWCRK_4	NA	Swan	NA	NA	NA
SWCRK_5	NA	Swan	NA	NA	NA
SWCRK_6	NA	Swan	NA	NA	NA
SWCRK_7	NA	Swan	NA	NA	NA
SWCRK_8	NA	Swan	NA	NA	NA
SWCRK_9	NA	Swan	NA	NA	NA

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
BFORK_1	NA	NA	NA	NA	NA
BFORK_2	NA	NA	NA	NA	NA
BFORK_3	NA	NA	NA	NA	NA
MU_BND_001	Yellow	Opaque	Present	Many	Orange
MU_BND_002	Yellow	Opaque	Present	Many	Orange
MU_BND_003	Yellow	Opaque	Present	Many	Orange
MU_BND_005	Yellow	Opaque	Present	Few	Orange
MU_BND_006	Yellow	Opaque	Present	Many	Yellow
MU_BND_008	Yellow	Opaque	Present	Few	Yellow
MU_BND_009	Yellow	Orange	Not Present	None	White
MU_BND_010	Yellow	Opaque	Present	Very Few	White
MU_BND_011	Yellow	Opaque	Present	Many	Yellow
MU_BND_016	Yellow	Opaque	Present	Few	Orange
MU_BND_017	Yellow	Orange	Present	Very Few	White
MU_BND_019	Yellow	Orange	Present	Many	Yellow
MU_BND_021	Yellow	Opaque	Present	Many	White
MU_BND_023	Yellow	Opaque	Present	Many	Orange
MU_BND_027	Yellow	Orange	Present	Few	Yellow
MU_BND_028	Yellow	Orange	Present	Few	Orange
MU_BND_029	Yellow	Opaque	Present	Few	Orange
MU_BND_030	Orange	Orange	Present	Very Few	Yellow
MU_BND_031	Yellow	Yellow	Present	Many	Yellow
MU_BND_032	Yellow	Opaque	Present	Many	Orange

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
MU_BND_033	Yellow	Opaque	Present	Many	Orange
MU_BND_034	Orange	Orange	NA	NA	NA
MU_BND_035	Yellow	Opaque	Present	Many	Orange
MU_BND_036	Orange	Orange	Present	Few	Yellow
MU_BND_037	Orange	Orange	Present	Few	Yellow
MU_BND_038	Orange	Orange	Not Present	None	Yellow
MU_BND_039	Orange	Orange	Present	Very Few	Yellow
MU_BND_042	Yellow	Opaque	Present	Many	White
MU_BND_043	Yellow	Opaque	Present	Many	Orange
MU_BND_044	Clear	Opaque	Present	Many	Orange
MU_BND_045	Yellow	Opaque	Present	Many	Orange
MU_BND_046	Orange	Orange	Present	Many	Yellow
MU_BND_047	Yellow	Orange	Present	Many	Yellow
MU_BND_048	Yellow	Orange	Present	Few	Yellow
MU_BND_049	Yellow	Opaque	Present	Very Few	Orange
MU_BND_053	Yellow	Opaque	Present	Few	Orange
MU_BND_054	Orange	Orange	Present	Many	Yellow
MU_BND_055	Yellow	Opaque	Present	Very Few	Orange
MU_BND_056	Yellow	Opaque	Present	Many	Orange
MU_BND_057	Yellow	Orange	Present	Very Few	Yellow
MU_BND_058	Yellow	Opaque	Present	Few	Orange
MU_BND_062	Yellow	Orange	Present	Few	Yellow
MU_BND_065	Yellow	Opaque	Present	Many	Orange

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
MU_BND_066	Yellow	Orange	Present	Few	Yellow
MU_BND_067	Yellow	Orange	Present	Very Few	Yellow
MU_BND_068	Yellow	Opaque	Present	Many	Orange
MU_BND_069	Yellow	Orange	Present	Few	Orange
MU_BND_070	Yellow	Opaque	Present	Few	Orange
MU_BND_071	Yellow	Opaque	Present	Many	Orange
MU_BND_073	Yellow	Opaque	Present	Few	Orange
MU_BND_074	Yellow	Opaque	Present	Many	Orange
MU_BND_075	Yellow	Orange	Present	Many	Yellow
MU_BND_076	Yellow	Opaque	Present	Many	Orange
MU_BND_077	Yellow	Opaque	Present	Many	Orange
MU_BND_078	Yellow	Opaque	Present	Many	Yellow
MU_BND_079	Yellow	Opaque	Present	Many	Orange
MU_BND_080	Yellow	Opaque	Present	Few	Orange
MU_BND_081	Yellow	Opaque	Present	Many	Orange
MU_BND_082	Yellow	Opaque	Present	Very Few	Orange
MU_BND_083	Yellow	Orange	Present	Very Few	Yellow
MU_BND_084	Yellow	Opaque	Present	Many	Orange
MU_BND_085	Yellow	Opaque	Present	Many	Orange
MU_BND_086	Yellow	Opaque	Present	Few	Orange
MU_BND_087	Yellow	Opaque	Present	Many	Yellow
MU_BND_088	Yellow	Opaque	Present	Many	Orange
MU_BND_089	Yellow	Opaque	Present	Many	Yellow

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
MU_BND_090	Yellow	Opaque	Present	Many	Orange
MU_BND_091	Yellow	Orange	Not Present	None	Yellow
MU_BND_128	Yellow	Opaque	Present	Many	Orange
MU_BND_129	Orange	Opaque	Not Present	None	Orange
MU_BND_130	Yellow	Opaque	Present	Many	Orange
MU_BND_131	Yellow	Orange	Present	Many	Yellow
MU_BND_132	Yellow	Opaque	Present	Many	Orange
MU_BND_140	Yellow	Opaque	Present	Many	Orange
MU_BND_141	Yellow	Opaque	NA	NA	NA
MU_BND_142	Yellow	Opaque	Present	Many	Yellow
MU_BND_143	Yellow	Opaque	Present	Few	Yellow
MU_BND_144	Yellow	Opaque	Present	Many	Yellow
MU_BND_166	Orange	Orange	Present	Few	Yellow
MU_BND_174	Orange	Orange	Present	Many	Yellow
MU_BND_175	Orange	Orange	Present	Many	Orange
MU_BND_179	Yellow	Orange	Present	Few	Orange
MU_BND_183	Yellow	Opaque	Present	Many	Orange
MU_BND_184	Clear	Orange	Present	Many	Yellow
MU_BND_185	Yellow	Orange	Present	Very Few	Yellow
MU_BND_187	NA	NA	NA	NA	NA
MU_BND_188	NA	NA	NA	NA	NA
MU_BND_202	NA	NA	NA	NA	NA
MU_BND_209	NA	NA	NA	NA	NA

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
MU_BND_222	NA	NA	NA	NA	NA
MU_BND_258	NA	NA	NA	NA	NA
PRUN_1	NA	NA	NA	NA	NA
PRUN_10	NA	NA	NA	NA	NA
PRUN_2	NA	NA	NA	NA	NA
PRUN_3	NA	NA	NA	NA	NA
PRUN_4	NA	NA	NA	NA	NA
PRUN_5	NA	NA	NA	NA	NA
PRUN_6	NA	NA	NA	NA	NA
PRUN_7	NA	NA	NA	NA	NA
PRUN_8	NA	NA	NA	NA	NA
PRUN_9	NA	NA	NA	NA	NA
SWCRK_10	NA	NA	NA	NA	NA
SWCRK_11	NA	NA	NA	NA	NA
SWCRK_12	NA	NA	NA	NA	NA
SWCRK_13	NA	NA	NA	NA	NA
SWCRK_14	NA	NA	NA	NA	NA
SWCRK_15	NA	NA	NA	NA	NA
SWCRK_16	NA	NA	NA	NA	NA
SWCRK_17	NA	NA	NA	NA	NA
SWCRK_18	NA	NA	NA	NA	NA
SWCRK_19	NA	NA	NA	NA	NA

FishCode	FinColor	NuptualPadColor	Melanophores	MelanophoreDensity	ChinColor
SWCRK_2	NA	NA	NA	NA	NA
SWCRK_20	NA	NA	NA	NA	NA
SWCRK_3	NA	NA	NA	NA	NA
SWCRK_4	NA	NA	NA	NA	NA
SWCRK_5	NA	NA	NA	NA	NA
SWCRK_6	NA	NA	NA	NA	NA
SWCRK_7	NA	NA	NA	NA	NA
SWCRK_8	NA	NA	NA	NA	NA
SWCRK_9	NA	NA	NA	NA	NA

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
BFORK_1	NA	NA	NA
BFORK_2	NA	NA	NA
BFORK_3	NA	NA	NA
MU_BND_001	Yellow	Yes	Partial
MU_BND_002	Orange	No	Not Obliterated
MU_BND_003	Yellow	No	Not Obliterated
MU_BND_005	Orange	Yes	Partial
MU_BND_006	Yellow	Yes	Complete
MU_BND_008	Yellow	Yes	Complete
MU_BND_009	White	No	Not Obliterated
MU_BND_010	Yellow	Yes	Complete
MU_BND_011	Yellow	Yes	Complete

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
MU_BND_016	Orange	Yes	Complete
MU_BND_017	Yellow	No	Not Obliterated
MU_BND_019	Orange	Yes	Partial
MU_BND_021	Yellow	Yes	Partial
MU_BND_023	Orange	Yes	Complete
MU_BND_027	Orange	No	Not Obliterated
MU_BND_028	Orange	Yes	Complete
MU_BND_029	Orange	Yes	Complete
MU_BND_030	Yellow	No	Not Obliterated
MU_BND_031	Yellow	No	Not Obliterated
MU_BND_032	Orange	Yes	Complete
MU_BND_033	Orange	No	Not Obliterated
MU_BND_034	NA	NA	NA
MU_BND_035	Orange	Yes	Partial
MU_BND_036	Yellow	No	Not Obliterated
MU_BND_037	Yellow	No	Not Obliterated
MU_BND_038	Yellow	No	Not Obliterated
MU_BND_039	Yellow	Yes	Partial
MU_BND_042	White	Yes	Complete
MU_BND_043	Orange	No	Not Obliterated
MU_BND_044	Yellow	No	Not Obliterated
MU_BND_045	Orange	Yes	Partial
MU_BND_046	Yellow	No	Not Obliterated

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
MU_BND_047	Yellow	No	Not Obliterated
MU_BND_048	Yellow	No	Not Obiterated
MU_BND_049	Orange	No	Not Obliterated
MU_BND_053	Orange	No	Not Obliterated
MU_BND_054	Yellow	No	Not Obliterated
MU_BND_055	Orange	No	Not Obliterated
MU_BND_056	Orange	Yes	Complete
MU_BND_057	Yellow	No	Not Obliterated
MU_BND_058	Yellow	Yes	Complete
MU_BND_062	Orange	No	Not Obliterated
MU_BND_065	Orange	Yes	Complete
MU_BND_066	Yellow	No	Not Obliterated
MU_BND_067	Yellow	No	Not Obliterated
MU_BND_068	Orange	Yes	Partial
MU_BND_069	Orange	Yes	Partial
MU_BND_070	Orange	Yes	Partial
MU_BND_071	Orange	Yes	Complete
MU_BND_073	Orange	Yes	Complete
MU_BND_074	Orange	Yes	Partial
MU_BND_075	Orange	Yes	Partial
MU_BND_076	Orange	Yes	Complete
MU_BND_077	Orange	Yes	Partial
MU_BND_078	Yellow	Yes	Partial

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
MU_BND_079	Orange	Yes	Complete
MU_BND_080	Orange	Yes	Complete
MU_BND_081	Orange	No	Not Obliterated
MU_BND_082	Yellow	Yes	Complete
MU_BND_083	Yellow	No	Not Obliterated
MU_BND_084	Orange	Yes	Partial
MU_BND_085	Yellow	Yes	Complete
MU_BND_086	Orange	Yes	Partial
MU_BND_087	Yellow	Yes	Partial
MU_BND_088	Orange	Yes	Partial
MU_BND_089	Yellow	Yes	Partial
MU_BND_090	Orange	Yes	Complete
MU_BND_091	Yellow	No	Not Obliterated
MU_BND_128	Orange	No	Not Obliterated
MU_BND_129	Orange	No	Not Obliterated
MU_BND_130	Orange	Yes	Partial
MU_BND_131	Yellow	No	Not Obliterated
MU_BND_132	Orange	Yes	Partial
MU_BND_140	Orange	Yes	Complete
MU_BND_141	Yellow	NA	NA
MU_BND_142	Yellow	Yes	Obliterated
MU_BND_143	Yellow	No	Not Obliterated
MU_BND_144	Yellow	Yes	Partial

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
MU_BND_166	Yellow	No	Not Obliterated
MU_BND_174	Yellow	No	Not Obliterated
MU_BND_175	Orange	No	Not Obliterated
MU_BND_179	Orange	Yes	Complete
MU_BND_183	Orange	Yes	Partial
MU_BND_184	Yellow	No	Not Obliterated
MU_BND_185	Yellow	No	Not Obliterated
MU_BND_187	NA	NA	NA
MU_BND_188	NA	NA	NA
MU_BND_202	NA	NA	NA
MU_BND_209	NA	NA	NA
MU_BND_222	NA	NA	NA
MU_BND_258	NA	NA	NA
PRUN_1	NA	NA	NA
PRUN_10	NA	NA	NA
PRUN_2	NA	NA	NA
PRUN_3	NA	NA	NA
PRUN_4	NA	NA	NA
PRUN_5	NA	NA	NA
PRUN_6	NA	NA	NA
PRUN_7	NA	NA	NA
PRUN_8	NA	NA	NA
PRUN_9	NA	NA	NA

FishCode	CheekColor	ObliterationLatLineStripe	AmtObliteration
SWCRK_10	NA	NA	NA
SWCRK_11	NA	NA	NA
SWCRK_12	NA	NA	NA
SWCRK_13	NA	NA	NA
SWCRK_14	NA	NA	NA
SWCRK_15	NA	NA	NA
SWCRK_16	NA	NA	NA
SWCRK_17	NA	NA	NA
SWCRK_18	NA	NA	NA
SWCRK_19	NA	NA	NA
SWCRK_2	NA	NA	NA
SWCRK_20	NA	NA	NA
SWCRK_3	NA	NA	NA
SWCRK_4	NA	NA	NA
SWCRK_5	NA	NA	NA
SWCRK_6	NA	NA	NA
SWCRK_7	NA	NA	NA
SWCRK_8	NA	NA	NA
SWCRK_9	NA	NA	NA

Table 4: Total fish collected from each stream.

Stream	Total Fish
Laurel	8
Pheasant	17
UNT to Youghiogheny River (UYR)	7
Cove	1
Files	17
Horseshoe	2
DryFork	6
West Fork Greenbrier (WFGR)	13
UNT to West Fork Greenbrier (UWFGR)	4
Johnny	2
Otter Lick Run (OLR)	4
Abernathy	8
BIRCH2	3
BIRCH1	4
Brushy	3
Swan	19
Unknown	8

Table 5: Total number of a species collected in each stream according to physical identification.

StreamCode	*R_atratulus_atratulus*	*R_obtusus_obtusus*	*R_obtusus_meleagris*
Horseshoe	2	0	0
Pheasant	5	1	0
Files	0	16	1
UYR	7	0	0
Abernathy	8	0	0
DryFork	6	0	0
BIRCH1	0	1	0
BIRCH2	0	2	0
Johnny	0	0	2
OLR	0	1	0
UWFGR	0	0	4
WFGR	0	0	10
Laurel	0	0	6
Cove	1	0	0

DNA Collection and Purification

The initial extraction of DNA from fish tissue was a simple task. Both procedures worked well. The salt method first used took several days and used hazardous materials. The kit from QIAGEN took only two days and did not have to include the hazardous materials. The gel ran after DNA purification was expected to look like those in Figure 20. The gel showed how high of a molecular weight and how intact the DNA was. Large molecular weight DNA was needed to run the PCR amplification, and if samples did not show a similar streak of larger pieces of DNA it was redone. After the gel check, DNA concentrations were checked using the Nanodrop 1000. Resulting graph should have had a strong peak near 260, seen in Figure 21.

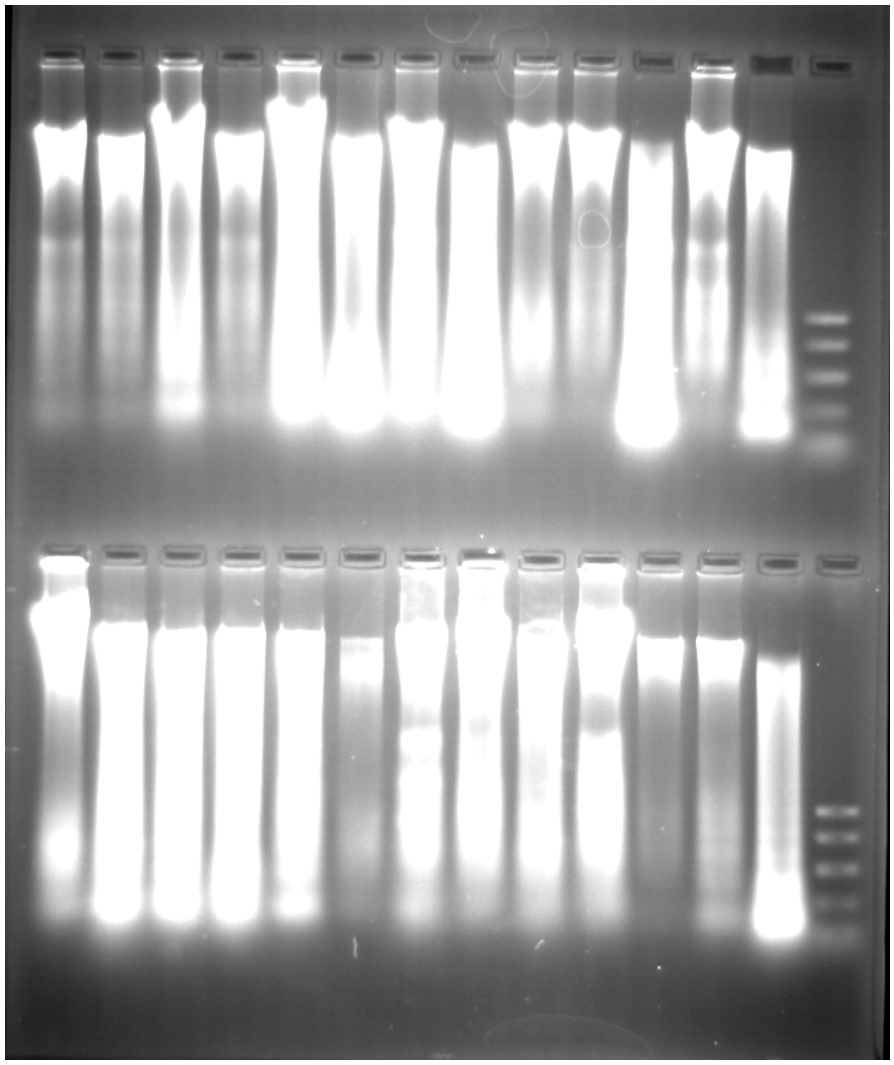


Figure 20: Gel of fish DNA.

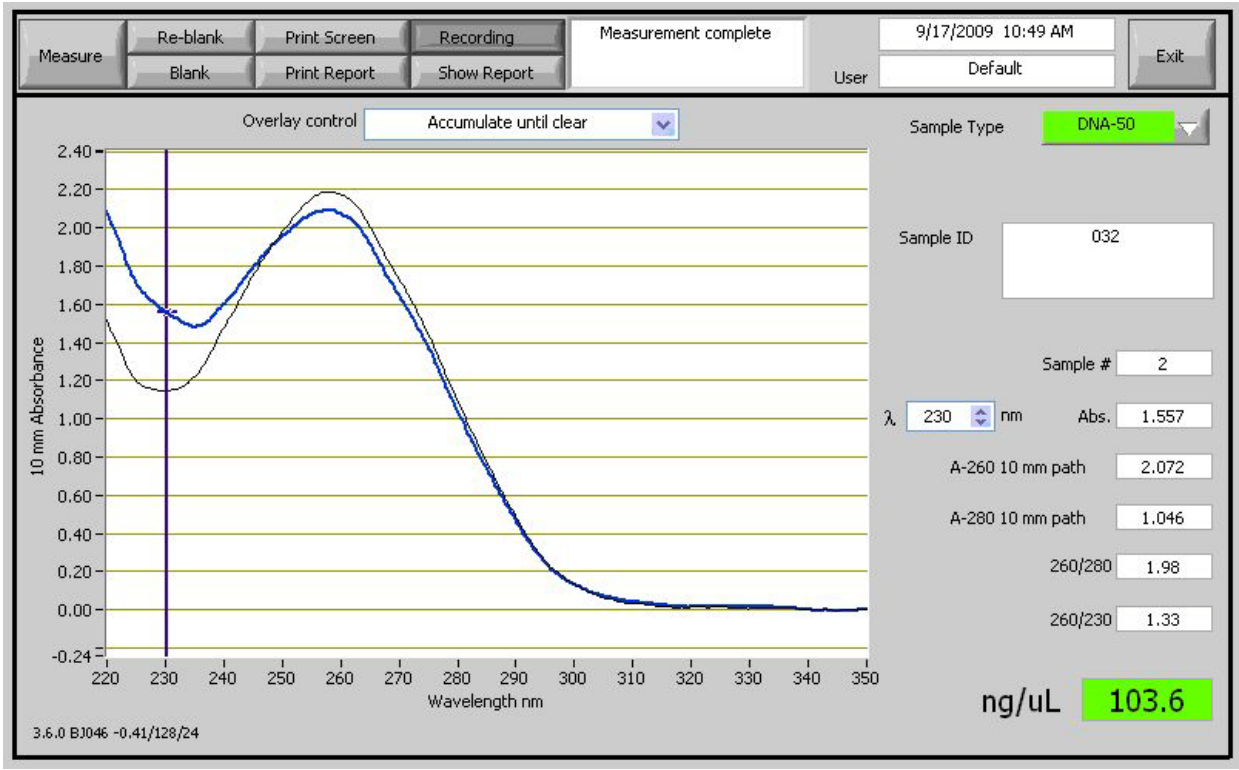


Figure 21: Example of Nanodrop data.

PCR Amplification of mitochondrial cytochrome *b*

After the DNA was nano-dropped and diluted to 100 ng/µL it went through the first cytochrome b PCR amplification that was used by Pfrender, as described in Methods. This initial series of amplification experiments was only successful for 44 of 91 fish collected. A large majority of those fish were physically classified as *R. obtusus meleagris*. It was not until primers L112509 and H112509 were created with Primer 3, that the remaining cytochrome *b* DNA samples were successfully amplified. By comparing the DNA sequences at the ends of each of the cytochrome *b* sequences that had been collected, it was noted that there were changes in specific bases that were not allowing the primers to amplify as well as they should have been. Using the online program Primer3, new primers were made that had mixed nucleotide bases in specific spots (Table 6). The temperature gradient that was used to test the new primers worked

well throughout, though 46.0˚C was chosen as the annealing temperature for this PCR reaction.

These new primers amplified every sample except sample 029, which was never amplified. The resulting amplicons were of high concentration and very little to no primer dimers were formed (Figure 22). Sequences were easily acquired with these products. All resulting sequences can be seen in FASTA format in Appendix B.

Table 6: Primers used to obtain mitochondrial cytochrome *b* gene.

Name	Sequence (5 →3)	Strand	Reference
L15162	TTCTTCCATGAGGACAAATAT	L	Pfrender (2002)
H15915A	CCTCCGTCTTCCGGATTACAAGAC	H	Pfrender (2002)
H15915B	CCTCCGTCTTCCGGATTACAAGAT	H	This study
L112509	TYTCAGCARTYCCCTATAT	L	This study
H112509	CAAGCTCATTTCARTGCTTTATT	H	This study

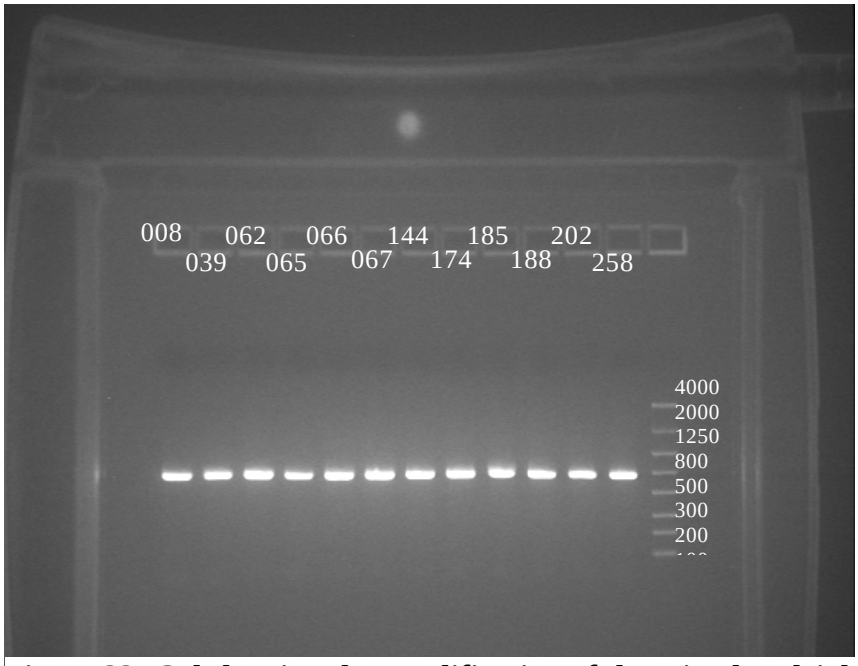


Figure 22: Gel showing the amplification of the mitochondrial cytochrome *b* gene. Amplicon is 760 base pairs.

Both Rag 2 and cytochrome b sequences were then compared to *R. atratalus* sequences that were stored on NCBI's database (DQ367018 and AF452078). In Sequencer each sequence

was aligned to the reference and the numbers of differences were recorded. For cytochrome *b* sequence, both the *R. atratalus* and *R. obtusus* sequences were identical (AF452078 and DQ990250) within the database. The cytochrome *b* sequences from the database most closely aligned with the cytochrome *b* haplotype of fish in this study classified as *R. obtusus obtusus*. *R atratalus* fish from the Potomac River watershed in this study were distinctly different in cytochrome *b* mitochondrial sequence, suggesting that the *R. atratulus atratulus* fish (AF452078) was mischaracterized. Interestingly, a single fish (SWCRK_12) was found in the Potomac watershed which had a mitochondrial haplotype more characteristic of *R. obtusus obtusus*.

The NCBI reference sequence used for the cytochrome *b* gene in *Rhinichthys* was 1140 base pairs long. The fish analyzed in this study were trimmed to 551 base pairs in Sequencher. Fish that had 0-10 changes in cytochrome *b* haplotype from the reference sequence were most often classified as *R. obtusus obtusus* physically. Fish that had 11-18 changes were classified as *R. obtusus meleagris*, and 50 or more changes were classified as *R. atratalus atratalus*. A table was made for this data within the Access file for this project. Each site was listed in a column. Then, a column for each species classification was made. The number of each species for each site was then recorded in the coordinating box (Table 7).

Table 7: Total number of a species collected in each stream according to mitochondrial DNA data.

StreamCode	*R_atratulus_atratulus*	*R_obtusus_obtusus*	*R_obtusus_meleagris*
Abernathy	5	0	0
BIRCH1	0	2	0
BIRCH2	0	2	0
Cove	1	0	0
DryFork	6	0	0
Files	0	0	12
Horseshoe	2	0	0
Johnny	0	0	2
Laurel	1	0	5
OLR	0	1	0
Pheasant	6	0	9
UWFGR	0	0	4
UYR	6	0	1
WFGR	0	0	10
Brushy	0	3	0
Swan	18	1	0

Clustal W Mitochondrial Tree

All sequences were aligned using Clustal W. Clustal W only places sequences that are closely related to none another together, it does not use any algorithms to determine a best-fit tree for the samples. This gives us only a basic result of how these fish could be related and allows all aligned sequences to be trimmed to the same length.

The resulting Clustal W tree (Figure 23) gave us three distinct groupings. Each group contained a majority of one species and similar stream groupings. Colored bars to the right of a specimen indicates that it is of the wrong species for the grouping it has been placed. Red bars indicate *R. atratulus atratulus,* green bars are *R. obtusus obtusus,* and blue bars are *R. obtusus meleagris.* Also it can be seen that three sites are in different groups as well. Samples from Pheasant Run are seen in all three groupings, and Laurel Creek and Youghiogheny River are seen in the second and third groupings.

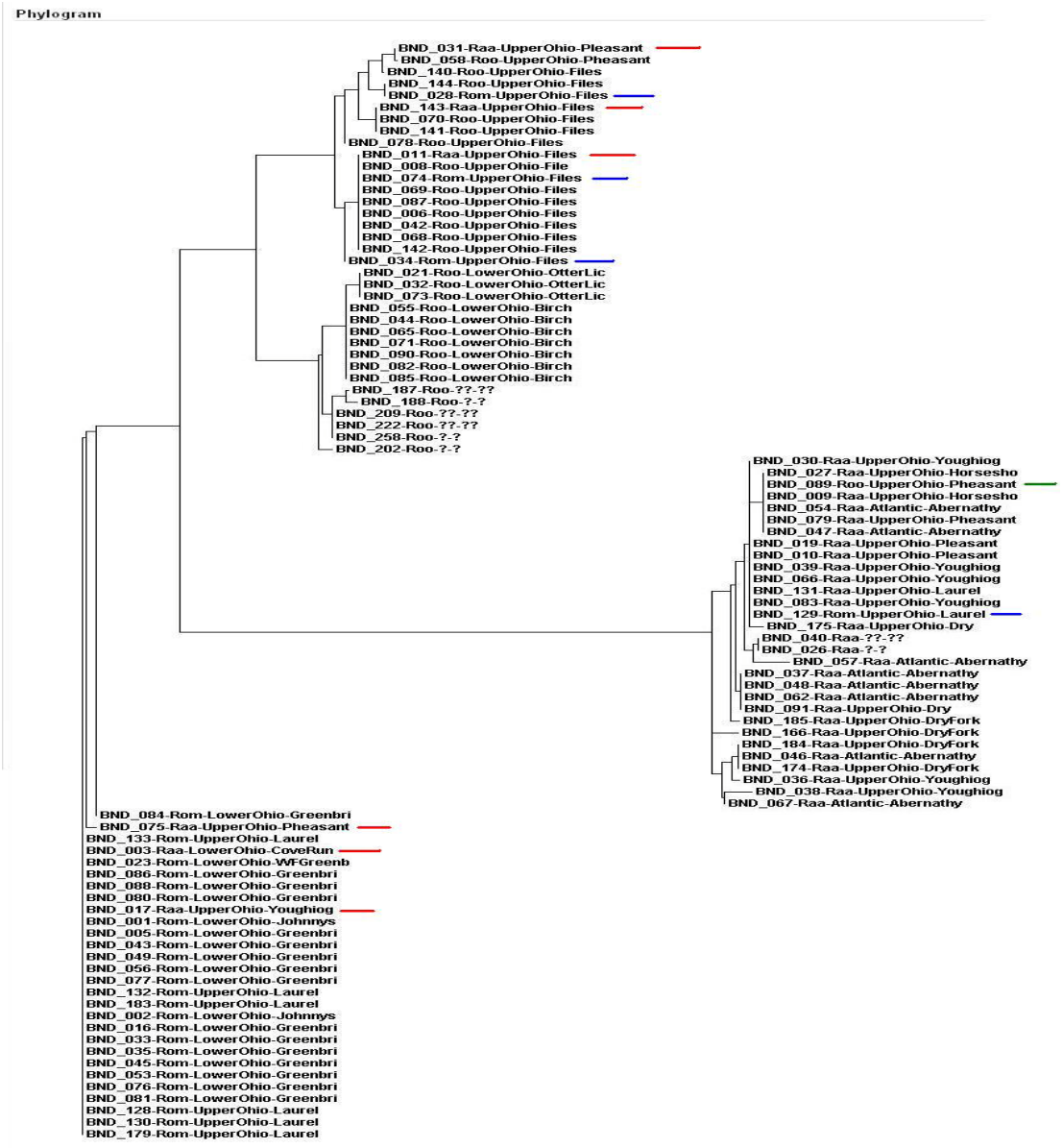


Figure 23: Mitochondrial cytochrome *b* tree formed in Clustal W. Bars indicate species grouped in different groups than how they were classified physically.

Mitochondrial Cytochrome *b* Trees using *BEAST

A second software package was used to generate trees according to algorithms that computed the tree with the highest likelihood. It needs to be understood that likelihood does not mean the tree is true. It is the probability that the tree has given rise from the data that was collected (Page and Holmes, 1998). In this study the data for the mitochondrial cytochrome *b* tree, the branch breaks into two distinct groups (Figure 24). The top group contains a large majority of the *R. atratulus atratulus* species with a few of the other two species mixed in, Figure 25. The second branch is separated into two sub branches. The first is composed of most of the *R. obtusus meleagris* species, Figure 26. Three of the fish in that branch were classified as *R. atratulus atratulus*. The second group also breaks into two sub groups, Figure 27. The first subgroup comes only from Files Creek and Pheasant Run. The second subgroup contains fish from the Birch River, Otter Lick Run, Brushy Fork, and an unclassified stream. It also contained one outlying *R. atratulus atratulus.* A table was created in Microsoft Excel listing all the sequences used to create the *BEAST tree in the order they appear in the tree (Appendix D). This table was created to record all changes within the sequences.

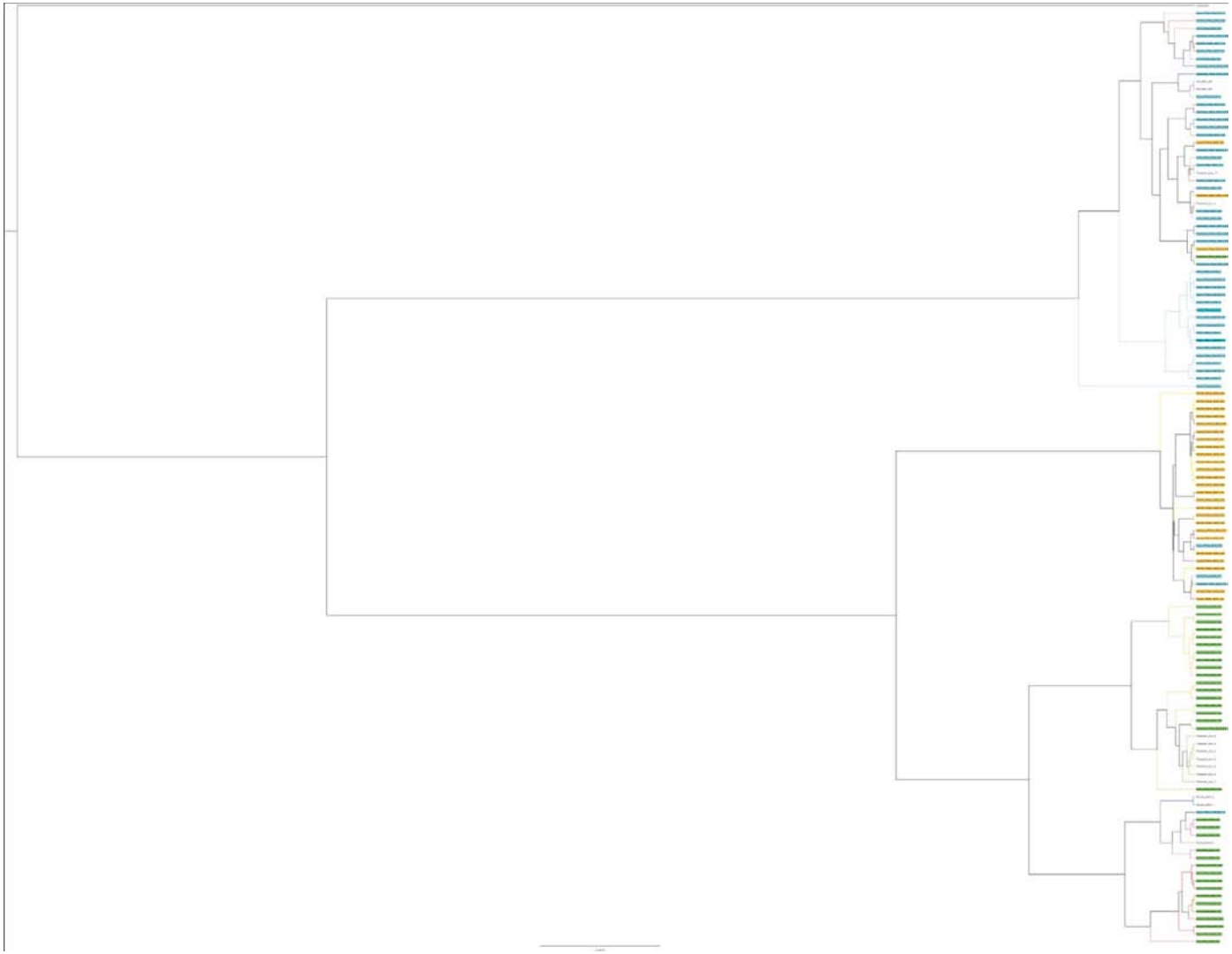


Figure 24: Mitochondrial tree made in *BEAST. Branches are color coded according the stream and fish labels are colored according to physical classification.

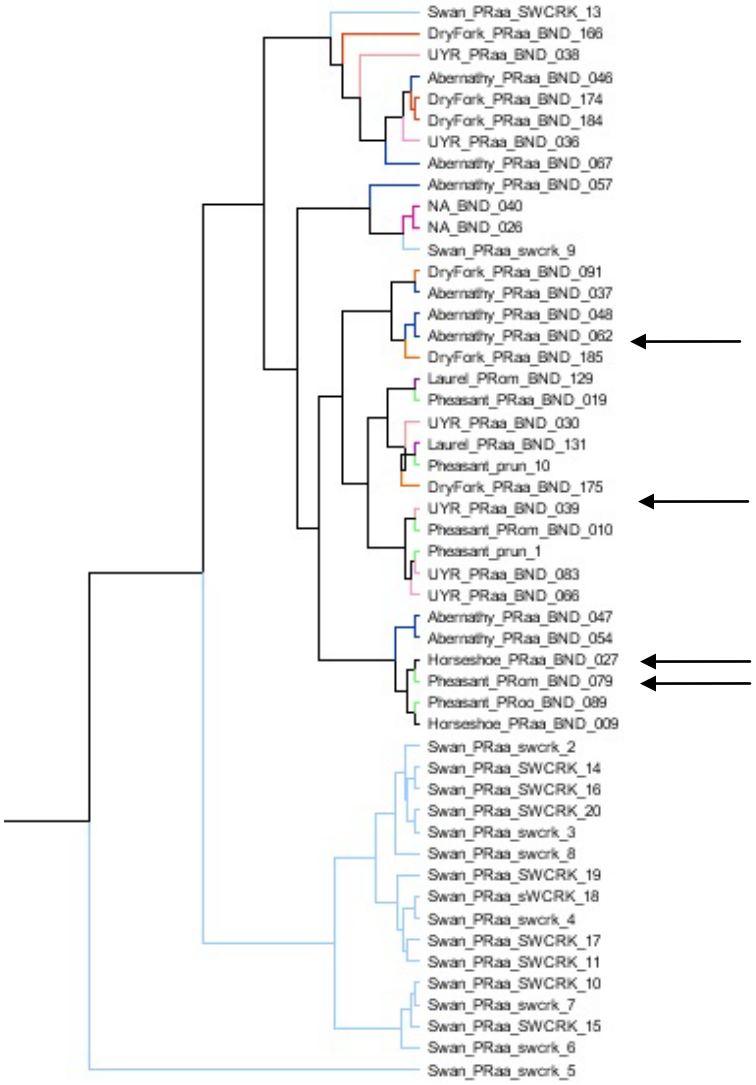


Figure 25: Top branch of mitochondrial cytochrome *b* gene *BEAST tree. Arrows indicate fish that physically do not match the *R. atratulus atratulus* mitochondrial DNA suggested with 51-58 changes.

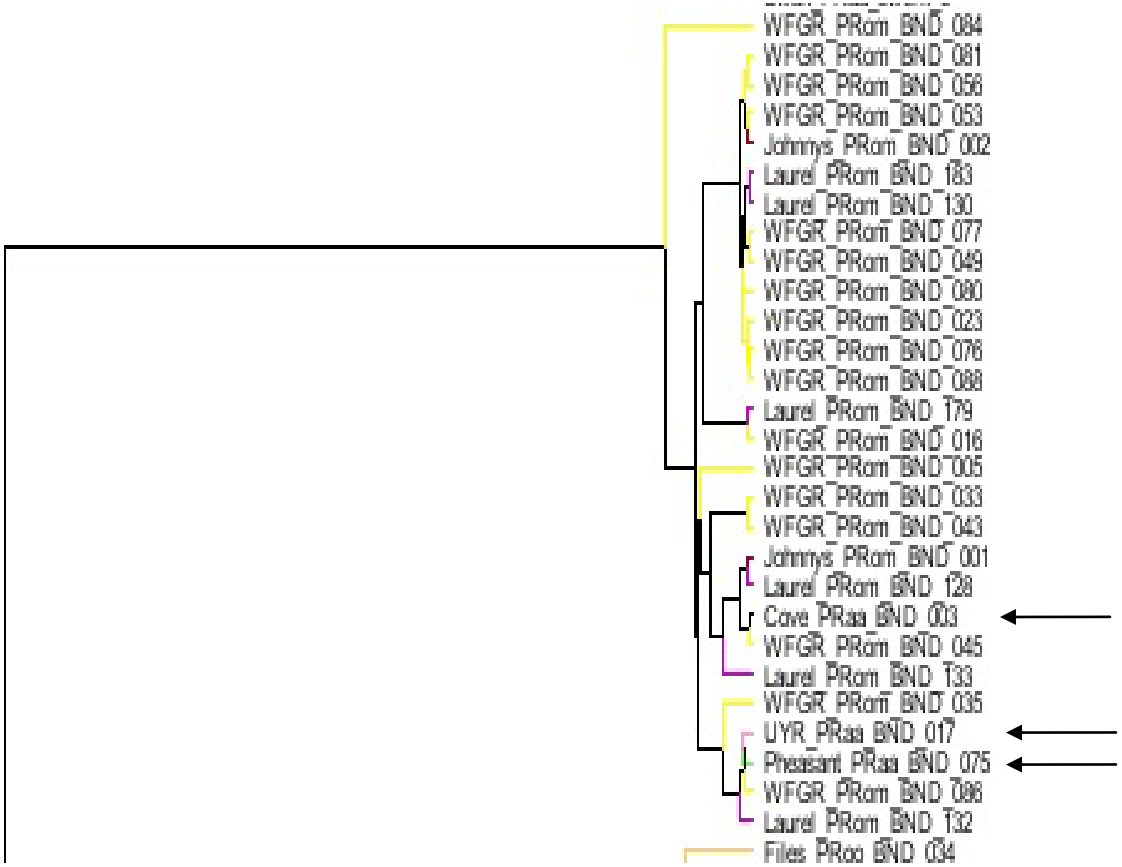


Figure 26: First branch of the *R. obtusus* grouping within the mitochondrial cytochrome *b* tree. This branch consisted mostly of physically classified *R. obtusus meleagris* and had 19-23 changes.

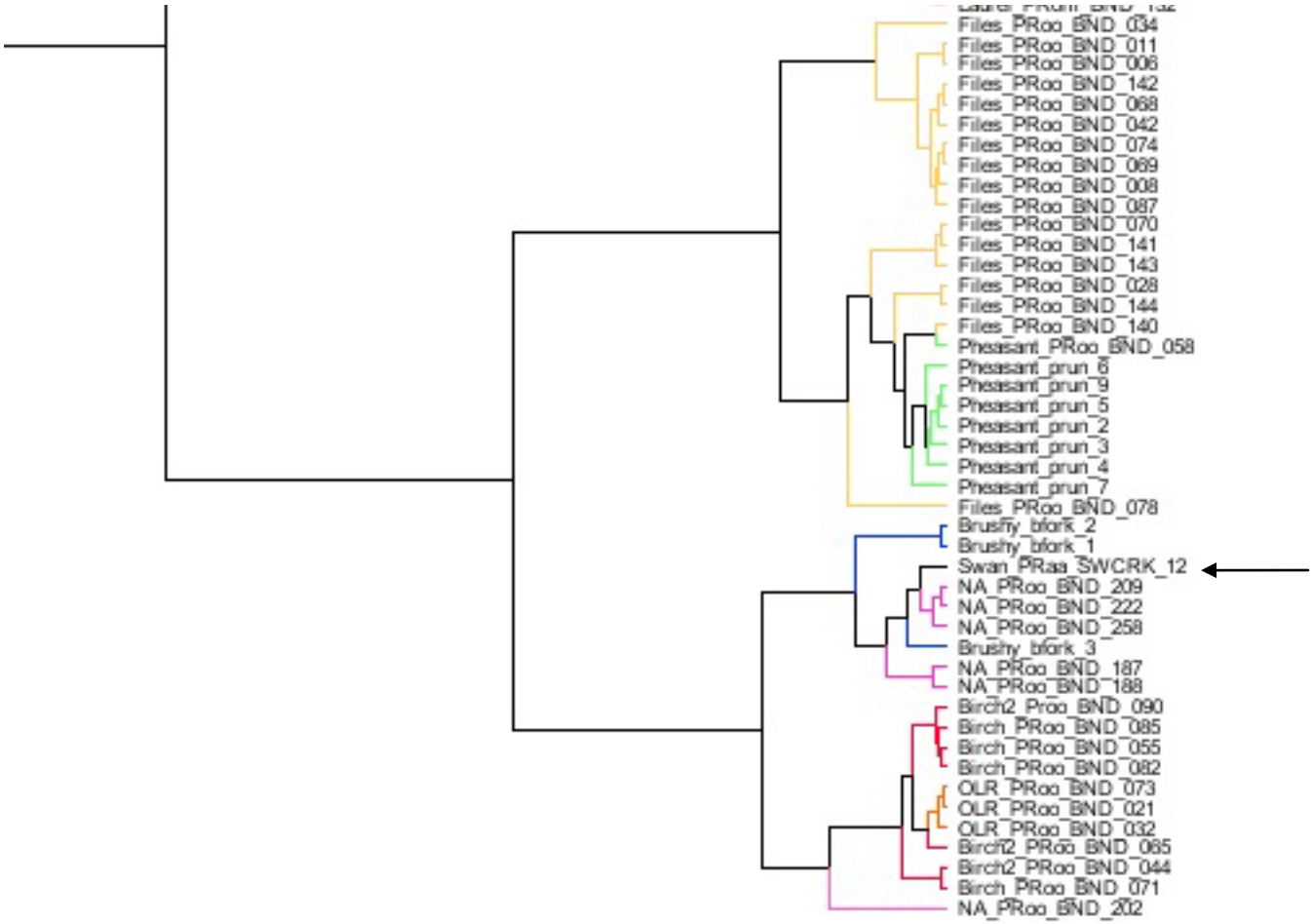


Figure 27: Second *R. obtusus* branch of the mitochondrial cytochrome *b* gene. Top section is from specific streams, with 13-19 changes. Bottom section contained fish with 0-5 changes.

RAG2 PCR Amplification

The initial primers used by Lovejoy (2001) did not work as well as hoped. One sample worked in the first trial. With this sequence new primers were made using Primer3, RhinAtratRAG2 Forward and Reverse (Table 8). A test was done to try to find the best primer combination between our primers and Lovejoy's primers. Six samples were used to test three primer combinations: RhinAtratRAG2Forward/RhinAtratRAG2Reverse, RhinAtratRAG2Forward/R4, and F2/ RhinAtratRAG2Reverse (Figure 28). It was decided that the F2/RhinAtratRAG2Reverse combination gave the best results with the least primer dimer. Samples were amplified using these primers to produce a 784 base pair amplicon. All sequences produced can be viewed in their FASTA format in Appendix C.

Table 8: Primers used to obtain genomic RAG2 gene.

Name	Sequence (5 →3)	Strand	Reference
F1	TTTGGRARAAGGGCTGGCC	L	Lovejoy(2001)
F2	ARACGCTCMTGTCCMACTGG	L	Lovejoy(2001)
R4	GTRGARTAGTAGGGCTCCCA	H	Lovejoy(2001)
R6	TGRTCCARGCAGAAGTACTTG	H	Lovejoy(2001)
RhinAtratRAG2Forward	TATCTCCCTCCACTCCGATG	L	This study
RhinAtratRAg2Reverse	CTCCATCGCTGCTAAACTCC	H	This study

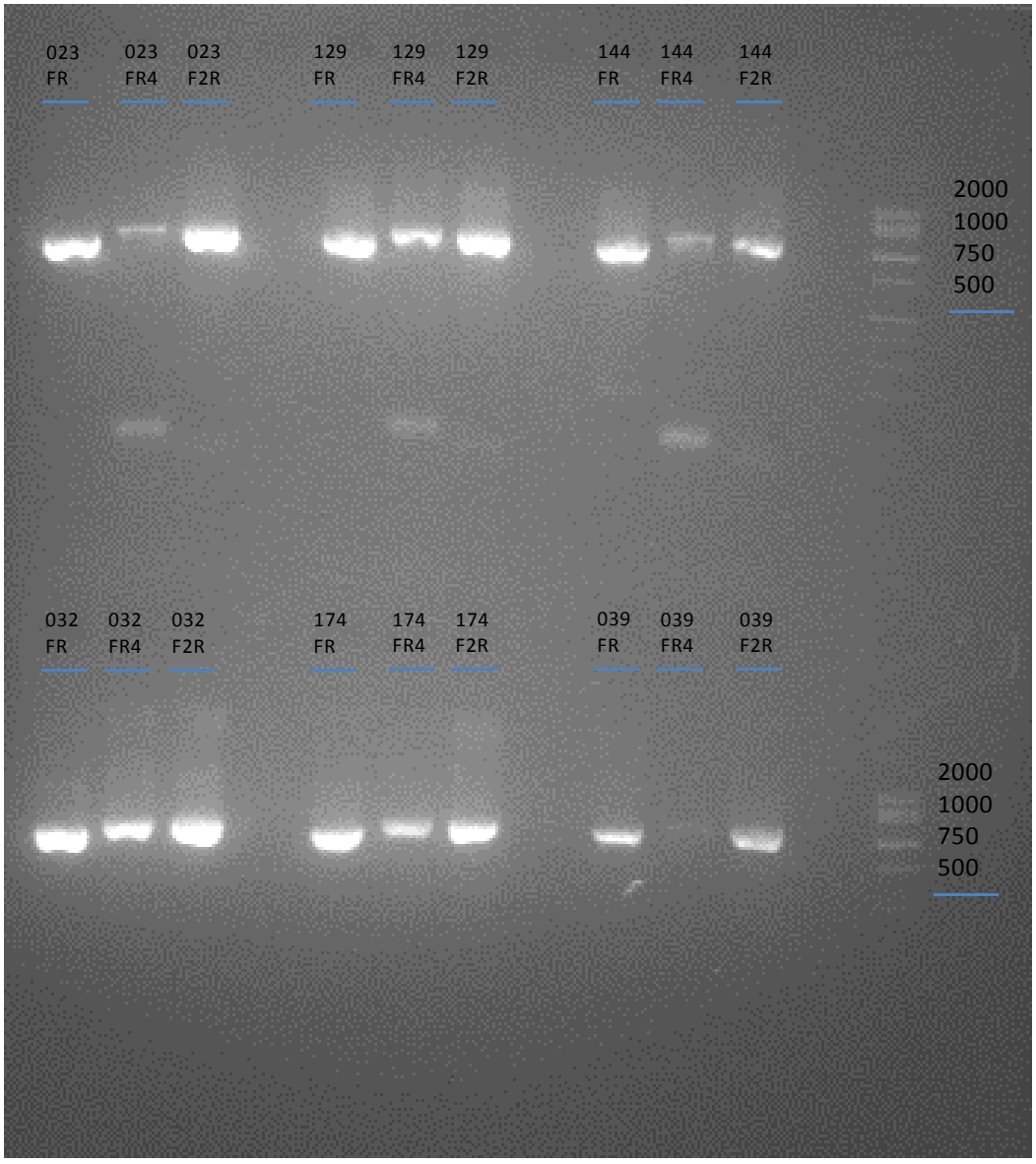


Figure 28: Primer combinations gel. FR= RhinAtratRAG2Forward/RhinAtratRAG2Reverse, FR4= RhinAtratRAG2Forward/R4, and F2R= F2/ RhinAtratRAG2Revers

RAG2 sequences were compared to a *R. atratulus atratlulus* (DQ367018) species that was also found in NCBI's database. The reference sequence was 1230 base pairs. Sequences from this study were trimmed to 784 base pairs. The number of differences within the sequences was much smaller and not specific to a species. The Access table made for this section used the site location and the number of changes that had been seen. The number of species with specific numbers of changes was recorded for each site (Table 9). A table was also made for the amino acid translation differences that were changed due to sequencing changes in RAG2 genotype (Table 10).

Table 9: Total number of samples collected with a specific number of differences in the RAG2 gene from each stream.

Stream	Samples_with_0_diff	Samples_with_1_diff	Samples_with_2_diff	Samples_with_3_diff	Samples_with_4_diff
Abernathy	0	0	0	0	4
BIRCH1	2	3	0	0	0
BIRCH2	2	0	0	0	0
Cove	1	0	0	0	0
DryFork	0	0	0	1	4
Files	7	4	1	0	0
Horseshoe	0	0	0	0	2
Johnny	0	0	0	0	0
Laurel	2	2	0	0	1
OLR	2	1	0	0	0
Pheasant	3	8	0	0	3
UWFGR	2	0	0	0	0
UYR	0	0	0	0	3
WFGR	5	1	0	0	0
Brushy	1	0	0	0	2
Swan	0	0	0	0	15

Table 10: Total fish that have amino acid changes in RAG2 from each stream

StreamCode	Site_304-I	Site_304-V	Site_304-I/V	Site_466-V	Site_466-L	Site_466-V/L
Laurel	1	6	1	8	0	0
Pheasant	2	12	3	17	0	0
UYR	5	2	0	7	0	0
Cove	0	1	0	1	0	0
Files	0	17	1	17	0	0
Horseshoe	2	0	0	2	0	0
DryFork	3	3	0	6	0	0
WFGR	0	13	0	13	0	0
UWFGR	0	4	0	4	0	0
Johnny	0	2	0	2	0	0
OLR	0	4	0	3	0	1
Abernathy	7	1	0	8	0	0
BIRCH2	0	3	0	2	1	0
BIRCH1	0	4	0	3	2	0
Brushy	0	1	2	3	0	0
Swan	16	3	0	19	0	0

Clustal W RAG2 Tree

A tree was made in Clustal W using RAG2 sequences collected, Figure 29. The resulting tree was broken into two branches. The top branch is composed of mainly *R. atratulus atratulus*, *R. obtusus obtusus* and *R. obtusus meleagris* are indicated by the green and blue bars. The second branch is a fairly stable branch. There were not many sequences that did not vary from one another on this branch, there are two sections that break off slightly. All but two fish are physically classified as either *R. obtusus obtusus* or *R. obtusus meleagris*.

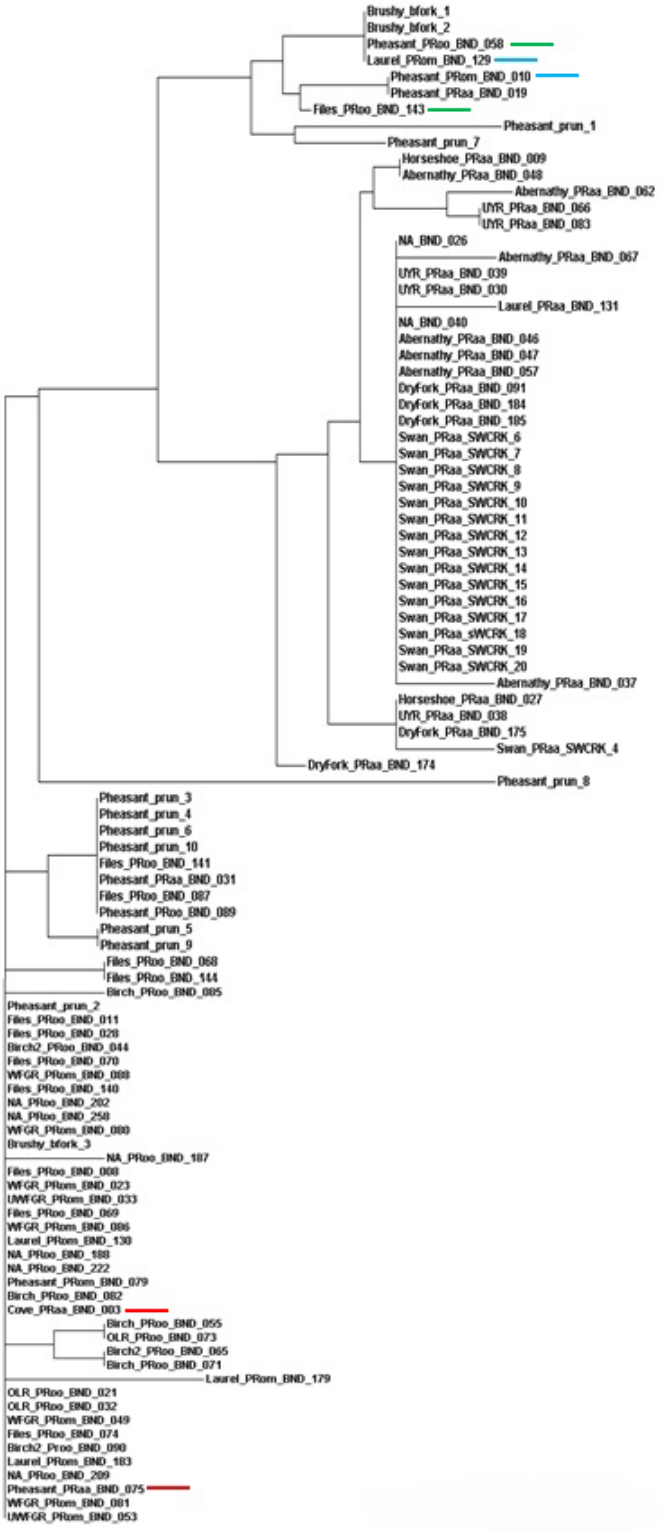


Figure 29: Clustal W tree for RAG2. Bars indicate fish that are physically different than where they were placed in the tree.

RAG2 *BEAST Tree

The RAG 2 *BEAST tree was separated into two branches also (Figure 30). The top branch (Figure 31) contained a mixture of all three species, though only four of *R. atratulus atratulus*. No groupings of the *R. obtusus* species was seen like there was in the mitochondrial tree. The bottom branch contained all *R. atratulus atratulus*. Neither branch expressed groupings according to stream locations. A table was recorded with all changes within the RAG2 sequences in the order that the sequences appear in the *BEAST tree (Appendix E)

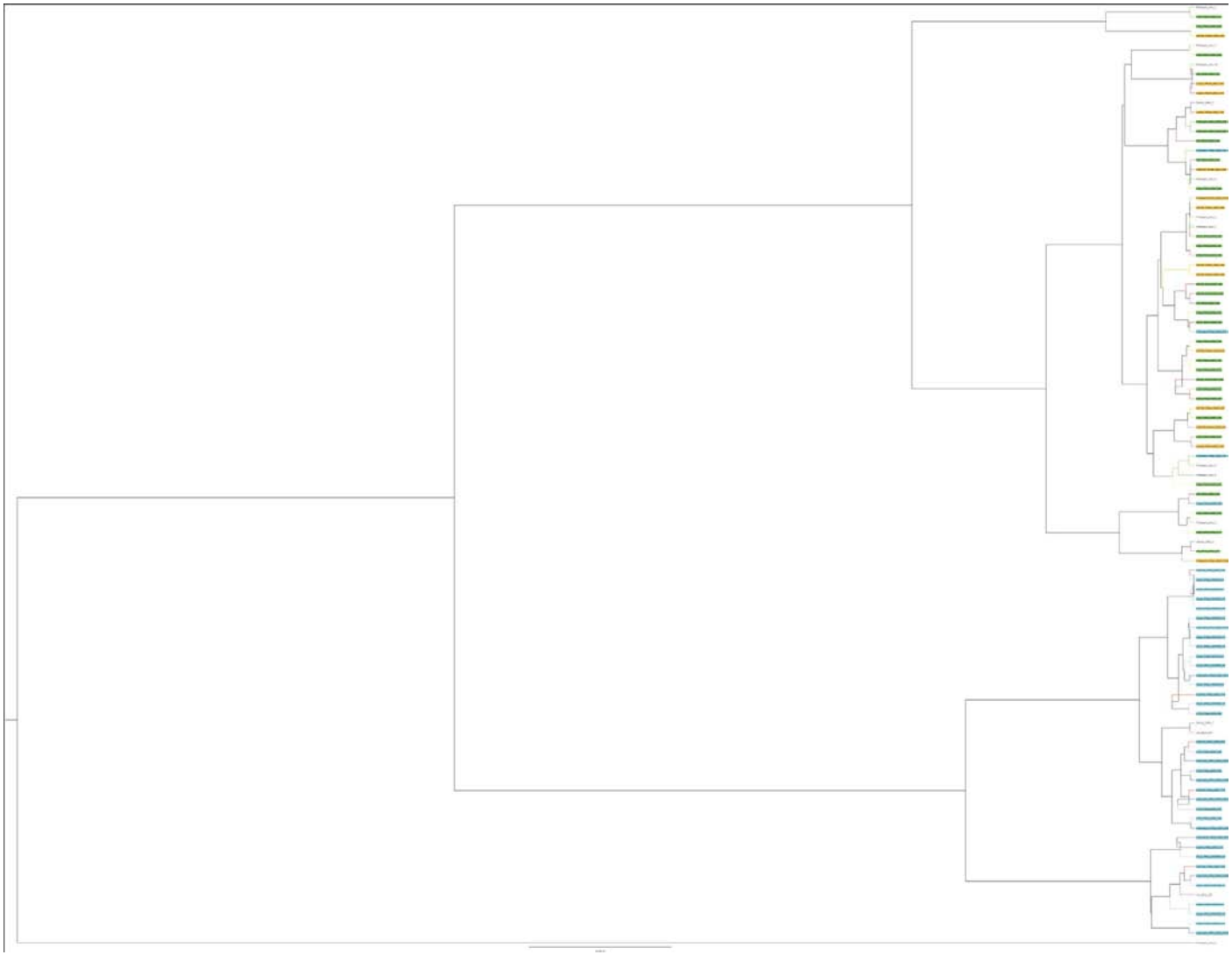


Figure 30: RAG2 tree made with *BEAST. Branches are colored according to stream and labels are colored by physical classification.

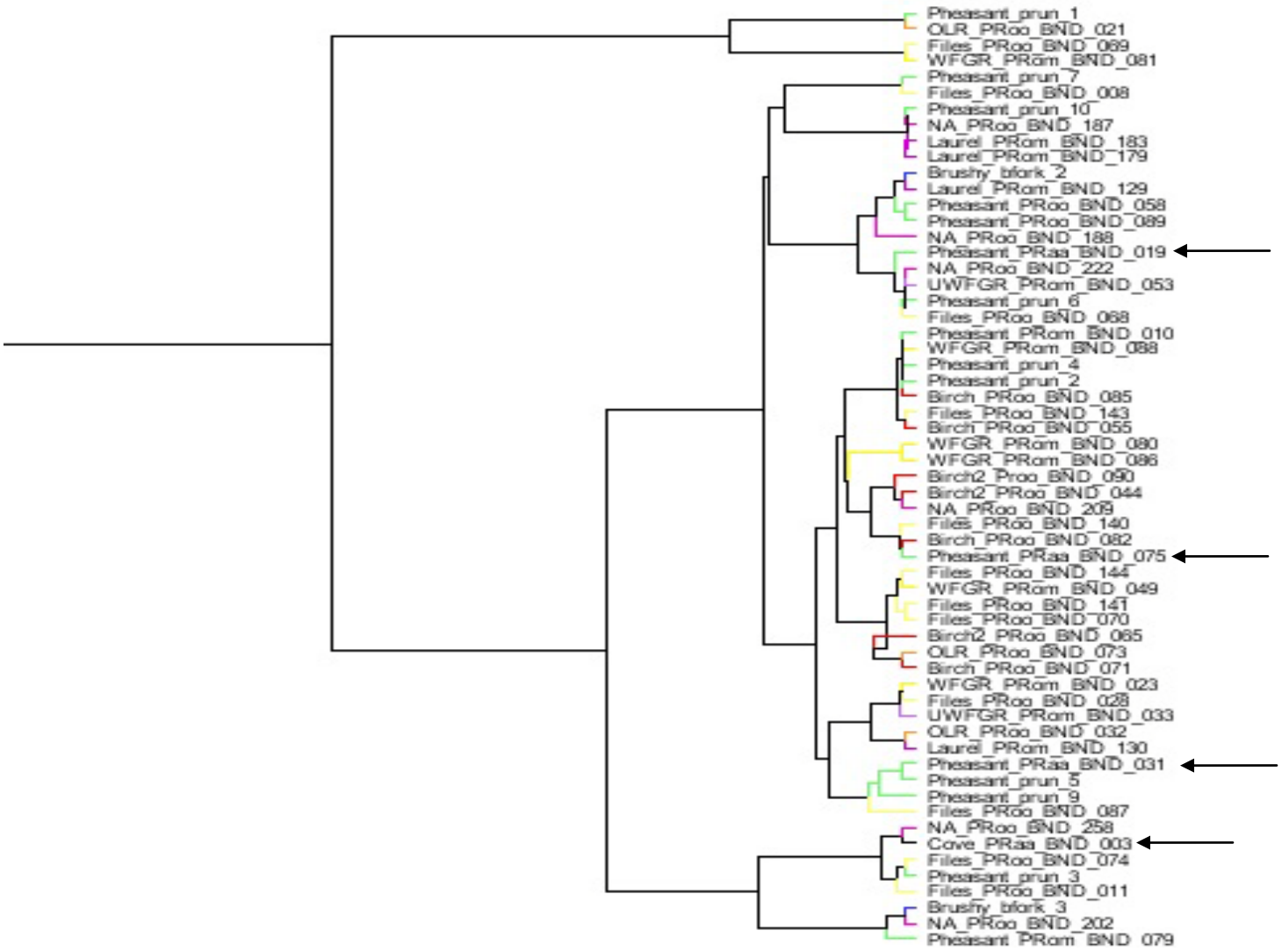


Figure 31: First RAG2 branch containing mostly physically characterized fish of *R. obtusus obtusus* and *R. obtusus meleagris.*

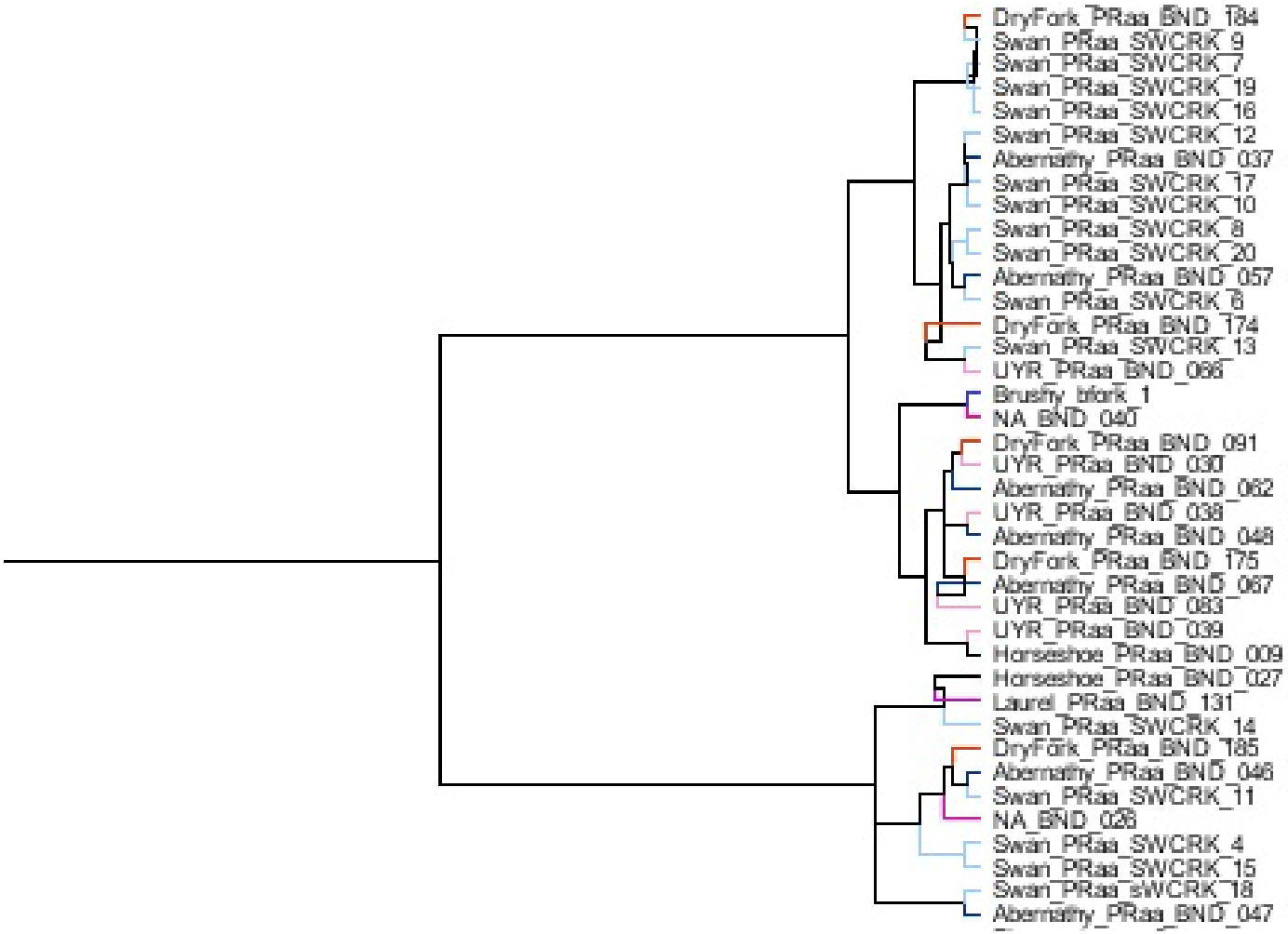


Figure 32: Second branch of RAG2.

Maps in ArcMap

The physical and mitochondrial maps (Figure 34 and Figure 35) display the distribution of the fish species according to either their physical characteristics, as defined by Geoff Smith, or their mitochondrial cytochrome *b* DNA differences. Both maps are labeled with *R. atratulus atratulus* as blue, *R. obtusus obtusus* as green, and *R. obtusus meleagris* as orange. The resulting maps help to give a strong visual comparison of the two ways of classifying species. Two sites did not have mating coloration and are not displayed on the physical map, Brushy Fork and Swan Creek. It is assumed that Brushy Fork would contain fish with the physical characteristics of *R. obtusus meleagris* and Swan Creek would have *R. atratulus atratulus* according to the regional distribution of the fish.

The first RAG2 map (Figure 36) is organized by the number of differences seen within the sequences of the samples. It was hoped that each species may display a certain number of differences as was seen with the mitochondrial DNA. It was noted that there were two nucleic acid positions that displayed heterogeneity within the study fish population, leading to variation in amino acids in the RAG2 protein positions. Images of all heterogeneity locations can be seen in Appendix F. Maps were made for these two amino acid positions, Figure 37 and Figure 38. Amino acid at nucleotide position 304 had a large number of changes from the references sequence amino acid of Valine to Isoleucine. Seven fish had the heterogeneity for both amino acids (Figure 33).

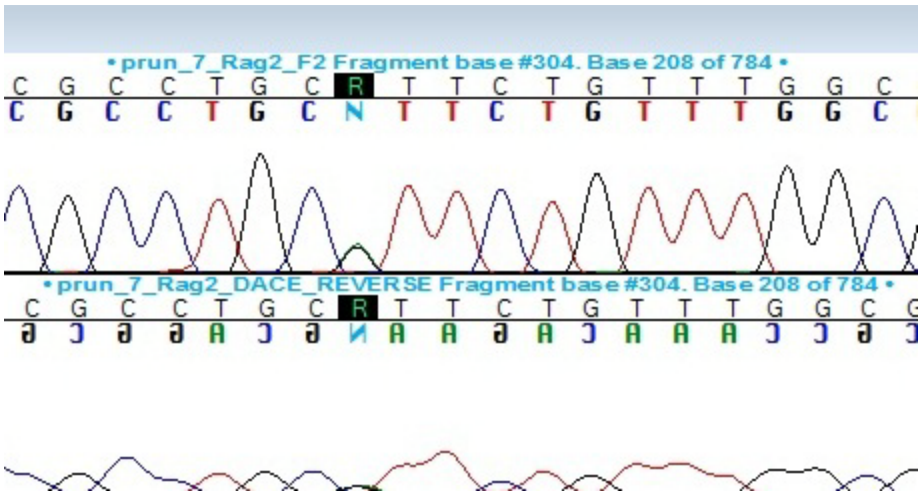


Figure 33: Heterogeneity of amino acid at nucleotide position 304 of reference, possibly expressing both Valine and Isoleucine amino acids within the fish.

The second amino acid position, 466 did not have as many fish with changes. Four fish had changes from the references sequence which substituted Valine at that position. Three of the changes made Leucine and one had coding for both amino acids. Figure 37 and Figure 38 show the distribution of fish with these amino acid substitutions throughout the streams.

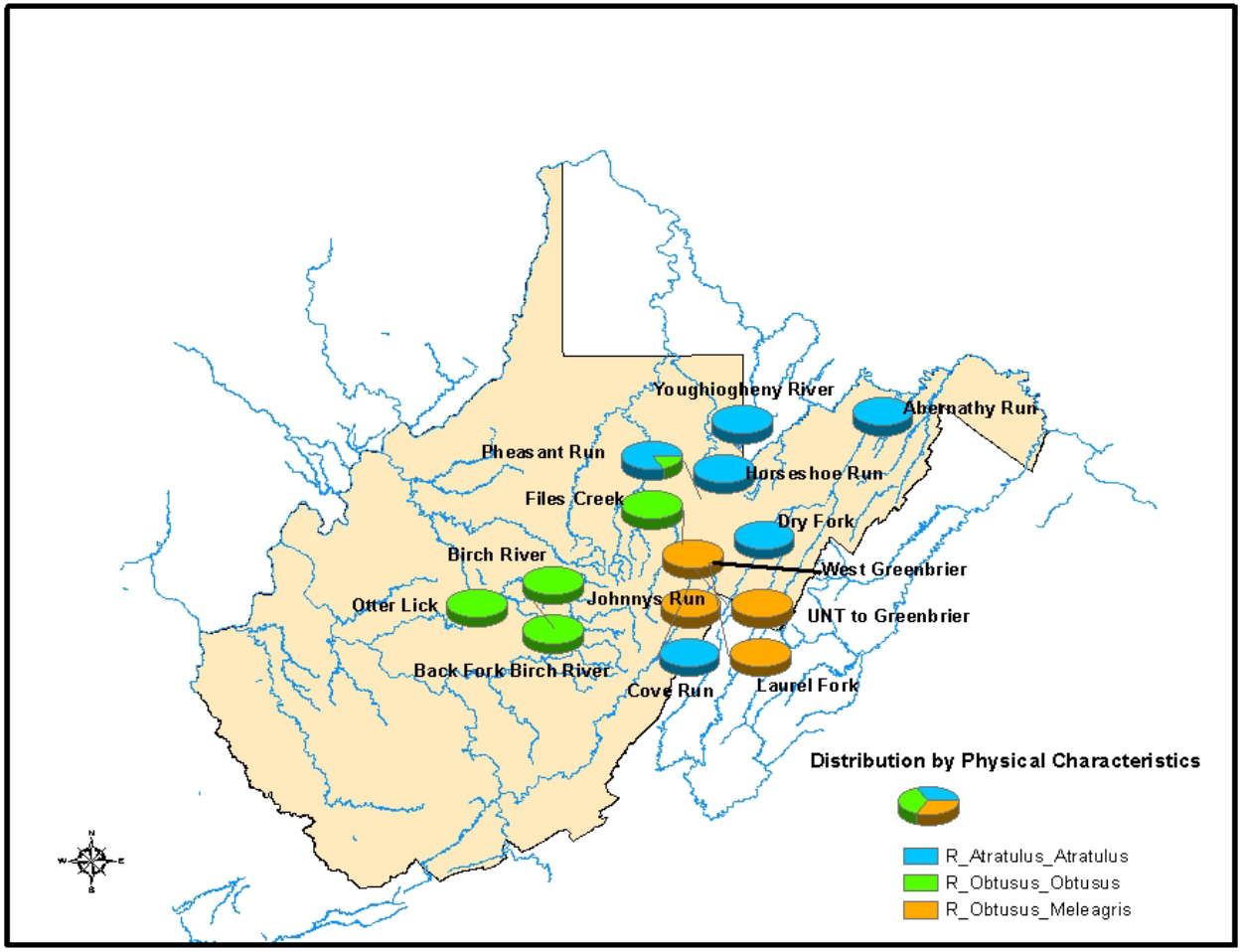


Figure 34: Distribution of species according to physical characteristics of fish collected.

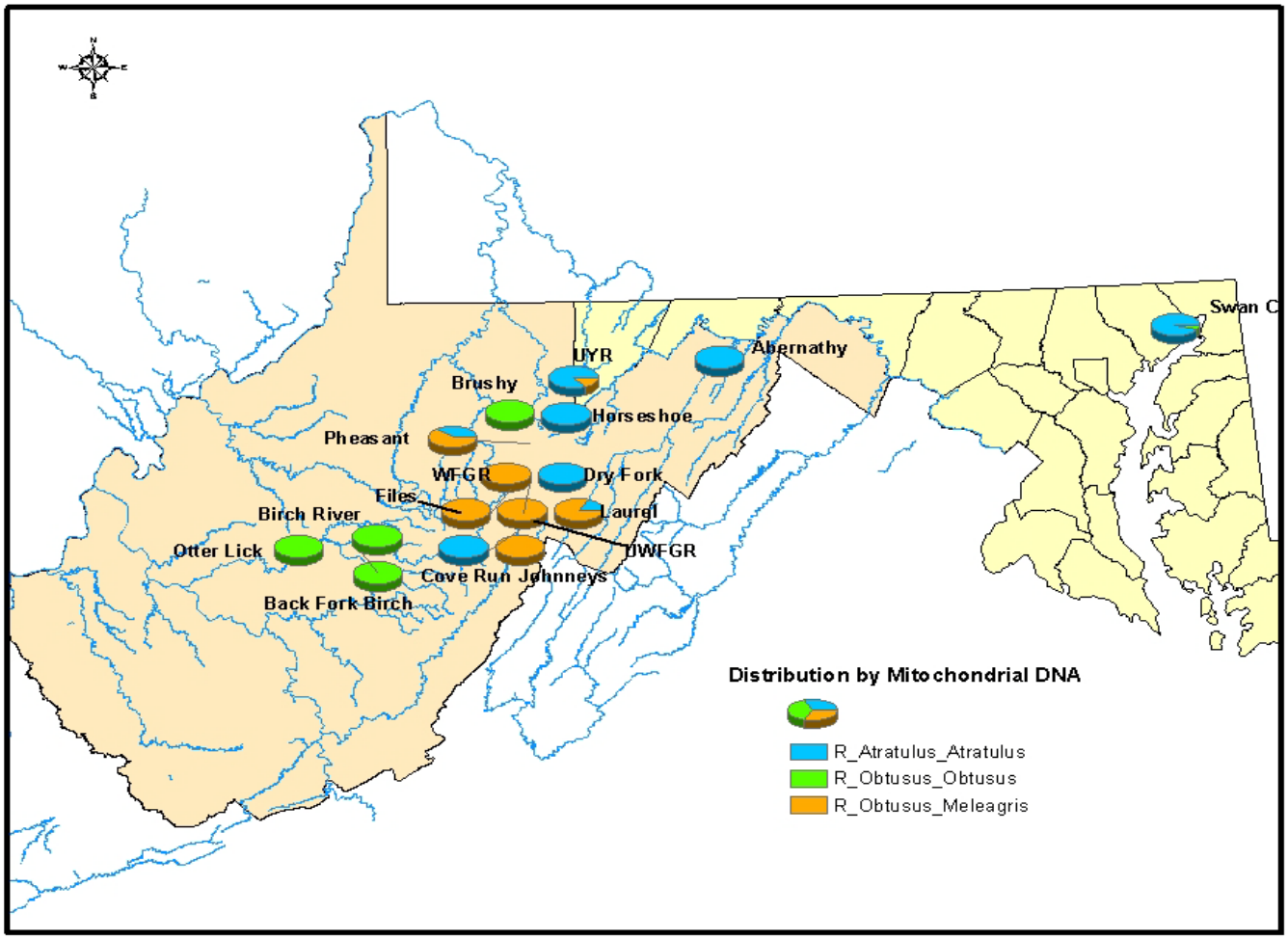


Figure 35: Distribution of species according to the mitochondrial DNA data collected from each fish.

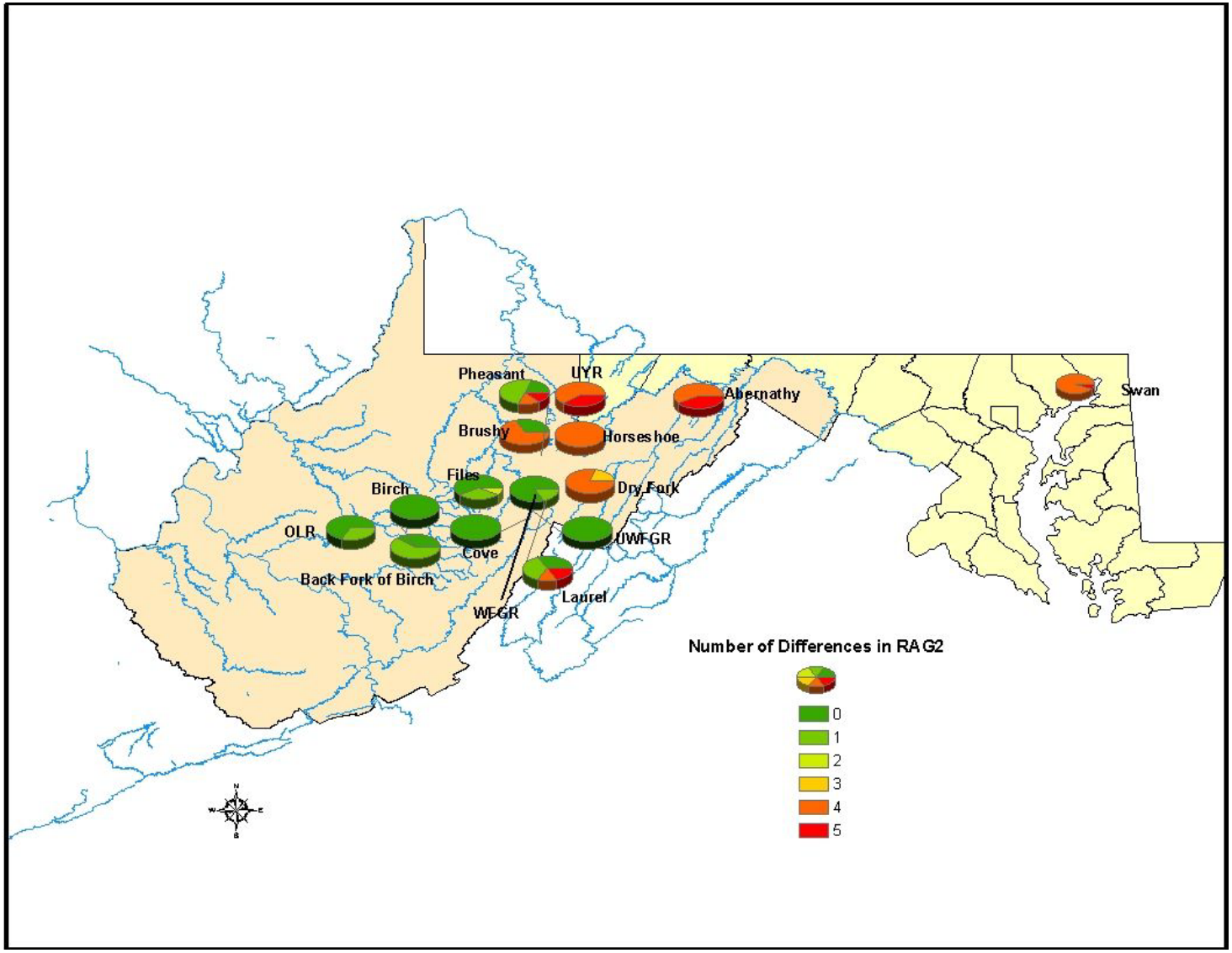


Figure 36: Distribution of RAG2 differences in fish samples.

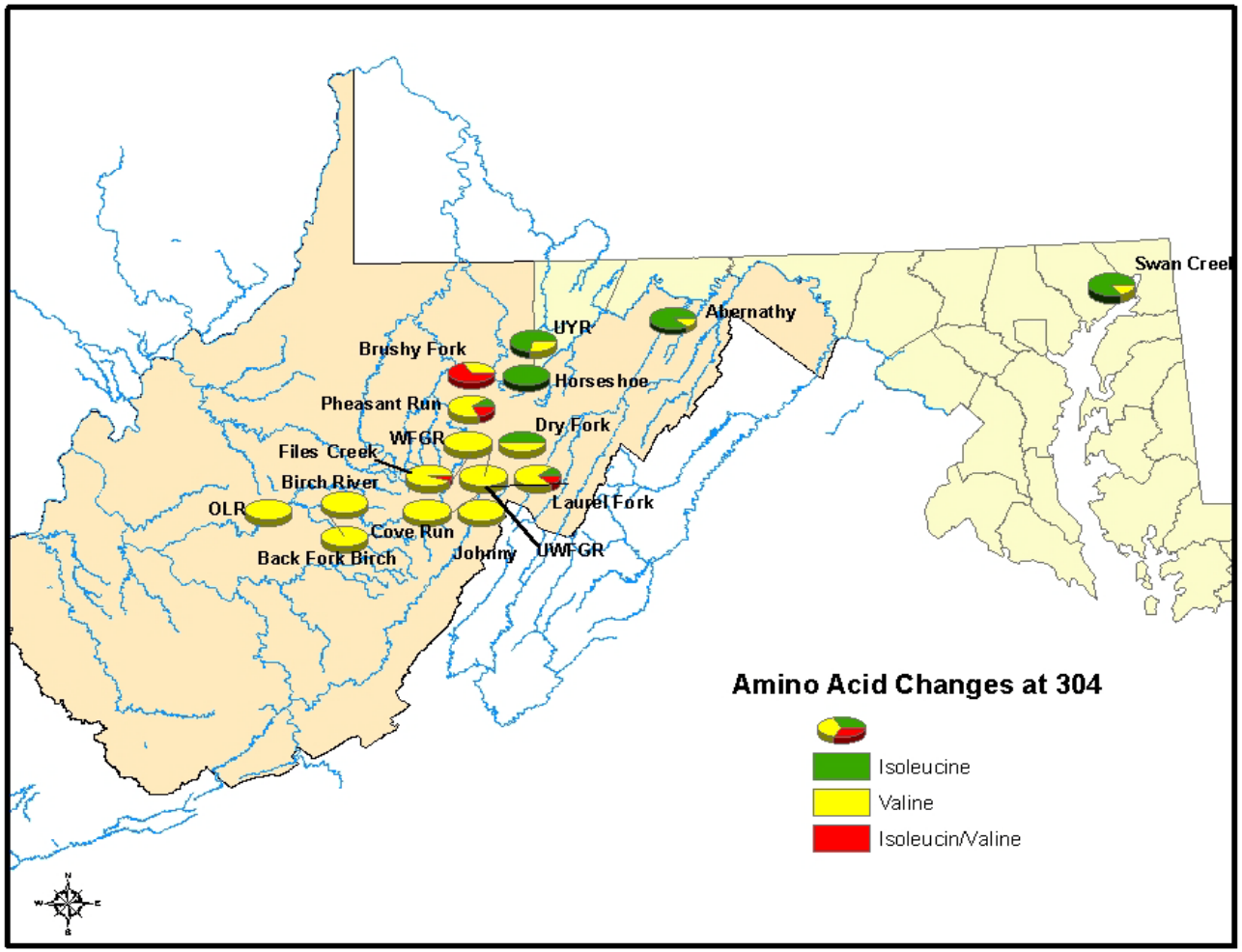


Figure 37: Changes in amino acid formation due to changes in RAG2 sequences at site 304.

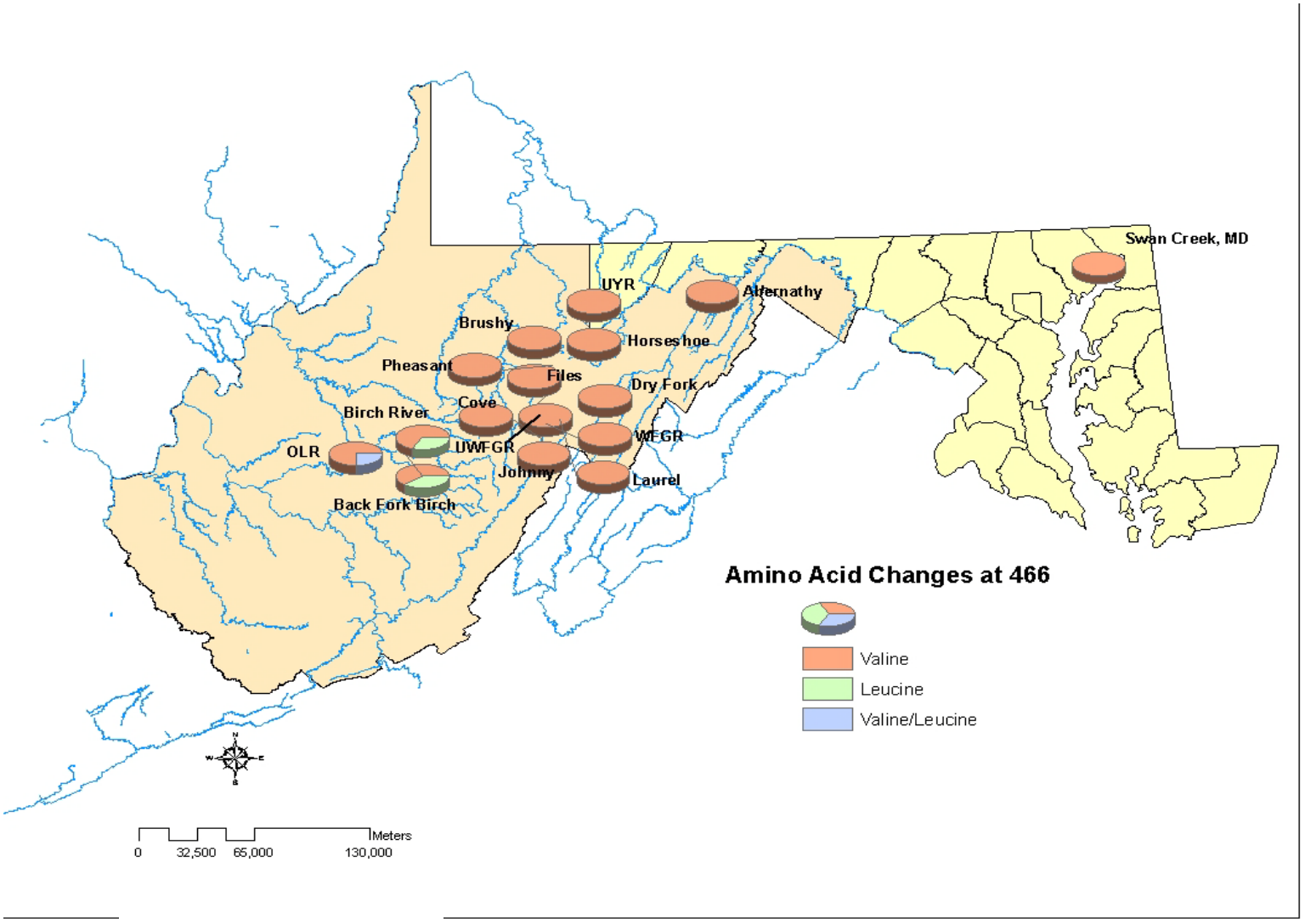


Figure 38: Amino Acid change at site 466 due to changes in RAG2 sequencing.

Chapter 4: Conclusions/Discussion

Mitochondrial Cytochrome *b* *BEAST tree

Of 123 specimens captured, only one did not amplify and was not included, were used in the creation of the mitochondrial cytochrome *b* tree made in *BEAST (Figure 24 ch3). As discussed in the results the tree has two branches, one of which is separated into two sub-branches. The top branch contains 50 samples. This branch is seen as the *R. atratulus atratulus* branch, because of those 50, 44 were recorded as *R. atratulus atratulus*. The remaining six fish were either not physically classified or were classified as one of the other species. All samples had 51-58 differences from the reference sequence used. The finial cytochrome *b* sequences were 551 base pairs long. The change within these fish is 9-11% of the reference sequences.

Of the six fish that had different classifications, only four had pictures. These pictures (Figure 39, Figure 40, Figure 41, and Figure 42) are used to show that their physical characteristics are indeed different from their mitochondrial DNA classification. Fish MU_BND_010 (Figure 39) has completely lost the dark lateral line pigmentation that defines a *R. atratulus atratulus*. It also has light red pigmentation on its lower belly, giving it a physical classification of *R. obtusus meleagris*. The next fish, MU_BND_089 (Figure 40) was physically classified as *R. obtusus obtusus* because of the disappearance of the dark lateral line. No other colorations appeared to classify it as either of the other species. Fish MU_BND_079 (Figure 41) was given the classification of *R. obtusus meleagris*. It has lost the dark coloration of the lateral line and has mating colors into its cheek and underbelly. MU_BND_129's picture is questionable (Figure 42). Clearly the fish is *R. obtusus meleagris* because of the coloration on the cheek and throughout the belly. What is interesting is the dark lateral line is still visible.

This could be because the fish was understress while taking the photo after its capture. Some fish will quickly lose the mating coloration when too much stress is put upon them.

Figure 39: MU_BND_010: Physical classification *R. obtusus meleagris*, mitochondrial classification *R. atratulus atratulus*.

Figure 40: MU_BND_089: Physical classification *R. obtusus obtusus*, mitochondrial classification *R. atratulus atratulus*.

Figure 41: MU_BND_079: Physical classification *R. obtusus meleagris*, mitochondrial classification *R. atratulus atratulus*.

Figure 42: MU_BND_129: Physical classification *R. obtusus meleagris*, mitochondrial classification *R. atratulus atratulus*.

The first subbranch of the second branch of the mitochondiral cytochrome *b* *BEAST tree contains 25 *R. obtusus meleagris* and 3 *R. atratlus atratulus*. This is the branch of *R. obtusus meleagris*. The base pair differences from the reference sequence in this grouping ranged from 19-23 differences, 3-4% of the reference sequence

First of the three *R. atratlus atratulus* fish in this grouping is MU_BND_003 (Figure 43). This fish was physically classified as *R. atratulus atratulus*. The image of this sample is not the best for coloration. The fish may have been stressed and lost its nuptial coloration. The lateral line is intact, but there is a redish pigment in the area of the nuptial fin pad, a color characteristic of *R. atratulus atratulus*. The second fish, MU_BND_017 is clearly in the nuptial coloration for *R. atratulus atratulus* (Figure 44). The lateral line is still intact with red coloration along the top and part of the lateral line and the nuptial fin pad red coloration. The final fish, also *R. atratulus atratulus*, has again lost most of its mating coloration. Near the front of its body there is a slight coloration. It appears that the dark lateral line is being replaced by the red mating color, rather than overlaying it. Yet, there is also a red coloration in the nuptial fin pad area (Figure 45).

The second subbranch is itself divided into another set of subbranches. The first section contains only fish from Files Creek and Pheasant Run. The differences from the reference sequence range from 13-19, 2-3% of the reference sequence All fish in this group are classified as *R. obtusus obtusus*. The second grouping has a range of 0-5, 0%, differences from the reference sequenes that was used. Streams that appear to be closely realated by looking at the mitochondrial tree in this section are Otter Lick Run and the Birch River collection sites. These sites only contain 4-5 changes, while the other sites are only 0-3 changes. The only outlying stream in this portion of the tree is SWCRK_12. There is no picture for this fish because it was caught out of mating season, however, it was assumed that all samples from Swan Creek would

have been *R. atratulus ataratulus* because of the location of the drainage in Maryland.

SWCRK_12 only had 2 base pair changes, making it *R. obtusus obtusus* mitochondrially.

Figure 43: MU_BND_003: Physical classification *R. atratulus atratulus*, mitochondrial classification *R. obtusus meleagris*.

Figure 44: MU_BND_017: Physical classification *R. atratulus atratulus*, mitochondrial classification *R. obtusus meleagris*.

Figure 45: MU_BND_075: Physical classification *R. atratulus atratulus*, mitochondrial classification *R. obtusus meleagris*.

Genomic RAG2 gene *BEAST tree

The tree formed for the RAG2 gene sequences has been more difficult to interpret since there is less distinctive and deep branching of species than in the cytochrome *b* discussed above. The tree has two branches (Figure 30, ch3). The first branch contains a mixture of all three species. The majority of it is *R. obtusus obtusus* and *R. obtusus meleagris*, but there are at least four fish that were physically identified as *R. atratulus atratulus* within that group. Throughout the entire sequence there are at maximum only 5 base pair changes, less than 1% change to the reference sequence. The *R. atratulus atratulus* within this grouping range from 0-4 changes. The second branch is composed of *R. atratulus atratulus* as the majority, though there is one within the group from Brusy Fork which had mitocondrial DNA for *R. obtusus obtusus.* This tree clearly differentiates between *R. atratulus atratulus* and the two *R. obtusus* species. It does not distinguish *R. obtusus obtusus* and *R. obtusus meleagris* as separate groupings.

Mitochondrial Distribution in ESRI ArcMap

The ESRI maps made in ArcMap are helpful in visualizing what species are in each stream. Figure 35, ch3, shows the distribution of the fish according to the mitochondrial cytochrome *b* gene. Key interest streams that stand out are Pheasant Run, Laurel Creek, and UNT to Youghiogheny River. For the Pheasant and Laurel Creek sites, there is a strong possiblity that these streams have had stream capture events with nearby streams based on the diversity mitochondrial haplogroups found in fish captured from these streams. Pheasant Run and the Greenbrier are separated by a very small amount of land. A large rain can cause the streams to flood and join together. This allows fish to travel from one watershed to the other. Once the streams return to their normal flow, the fish are trapped in a new steam. Pheasant Run drains into Shavers Fork and is located in an area that should be *R. atratulus atratulus.* It is very

possible for a stream capture even to happen between Pheasant Run the Greenbrier to transfer *R. obtusus meleagris*. The same conclusion can be seen with Laurel Creek from and a possible capture with Dry Fork.

It is also important to compare the mitochondrial map with the map made of the physical characteristics (Figure 34, ch3). In the case of Pheasant Run, physically the fish found there can be classified as *R. atratulus atraulus* and *R. obtusus obtusus* based on nuptual coloration. Based on mitochondrial DNA, a large portion of the fish have *R. obtusus meleagris* lineage mixed in with some of *R. atratulus atratulus*. Being able to see these comparisons further suggests that there is an exchange of genes occurring that allows the physical characteristics of one species to be seen and the mitochondrial DNA of another, possibly due to interspecific hybridization. Other streams such as Laurel Creek, Files Creek, and the Youghiogheny, show simular data, of mitochondrial DNA of one species hiding silent behind the physical characteristics of another.

RAG2 Distribution in ESRI ArcMap

Several conclusions could be made comparing the mitochondrial and RAG2 maps. First it is very possible the the larger number of differences could belong to *R. atratulus* species and those with 0-2 differences are *R.obtusus.* Having such a close range though, makes it difficult to decifer what is a natural genetic mutation that possibly could be species related, or those that are only fish specific variation. It isn't clear that there are enough distinctive RAG2 variants to separate distinctive Rhinichythese species, compared with mitochondrial differences.

It could be misleading that some of the mitochondrial pie charts look very similar to the RAG 2 pie charts on the ESRI maps. It could be assumed that the similar distributions of variation would mean there may be a relationship, but additional data on other genomic sequences with the same pattern of variation in these streams would be helpful to test the

possibility that there is interspecific mixing. The mitochondrial chart for Swan Creek shows one *R. obtusus obtusus*. The RAG2 chart also shows one fish differing from the rest of the group. However there is not a single mixed fish but two different ones from each of the variants. The *R. obtusus obtusus* contains only four differences within its RAG2 gene. It is a *R. atratulus atratulus* that displays one extra difference than the rest in RAG2.

In Pheasant Run something similar is observed. The two pie charts (Rag 2 and cytochrome *b*) reflect genetic variation within this stream. However, considering the individual fish with the number of changes in each the mitochondrial and RAG2 changes, the species types do not match up. The two fish that have 5 differences only one had only on difference between their mitochondrial haplotypes and neither had physical data for coloration. Therefore mitochondrial assignment of the fish did match with the *R. atratulus* origin for RAG2 variation. There were 3 fish with 4 changes that had a mixture of information. Two of the three had physical traits recorded. One was classified as *R.atratlus atratlus* and the other *R. obtusus meleagris*, both had mitochondrial changes for *R. atratlus.* The conflicting *R. obtusus meleagris* fish (Figure 39) had the mitochondrial change for *Ratratulus* but physically it displays characteristics of *R. obtusus meleagris.* The third fish fell into the *R. obutus obtusus* catogory for mitochondrial DNA. The majority, eight, of this area had 1 base pair change. The mitochondrial sequence assignment for these fish were eight *R. obutus obtusus* and two *R. atratulus atratalus.* Only one of these fish had physical characteristics available, Figure 40. It had physical characteristics of *R. obtusus obtusus* and the mitochondrial changes more likely to occur in *R. atratulus atratulus*. The three fish that have no changes are very interesting. Fish MU_BND_079 was physically classified as *R. obtusus meleagris* with the mitochondrial DNA for *R. atratulus atratalus*, Figure 41. The second fish, MU_BND_075, Figure 45, had the physical

characteristics of *R. atratulus atratalus* and the mitochondrial DNA look like that of *R. obtusus meleagris*. These two fish, identified physically and mitochondrially, were very different, but the RAG2 genomic DNA was identaical to one another. The third fish was caught out of mating season, but its mitochondrial DNA suggests that it was *R.obtusus obtusus*. This suggests that all three species, either from physical characteristics or from mitochondrial data, could have the exact same RAG2 DNA.

Another possibility is that the RAG2 gene has mutated in separate regions. It can be seen as a gradation of changes from the western part of West Virginia to the Atlantic Ocean. As fish are analyzed west to east, the number of changes within the genomic RAG2 gene increase. Streams in the central part of the state can be seen as boundry lines of where these changes are taking place and possibly mixing among each other.

Amino Acid changes in RAG2

It is also possible that there could be some selective advantage to the RAG2 amino acid changes, although much more data is required to support this possibility. Only changes which alter the coding region could affect the function of the protein. Because using just the nucleic acid differences provided less clear information on the maps, the effects of the base pair changes on amino acid translation was investigated. Of the 5 possible changes only 2 had a possiblity of changing the amino acid produced. These were at nucleotide positions 304 (Figure 37, ch3) and 466 (Figure 38, ch3) in the RAG2 reference sequence. The change at 304 was very common, changing Valine to Isoleucine. Of the 97 fish that had results for RAG2, 39 had this change. It appears to be more prevalent in the *R. atratlus* species, although two exceptions were both *R. obtusus*. Seven fish in the study displayed the heterogeneity to produce both amino acids. These fish had classifications of all three species in both physical and mitochondrial areas. The

mutation at site 466 only occured in four fish, all of which were *R.obtusus obtusus* in both physical and mitochondrial characteristics. The streams in which they occurred were sites that were possibly fully *R. obutus obtusus* in both physical and mitochondrial analysis.

Overall Conclusion

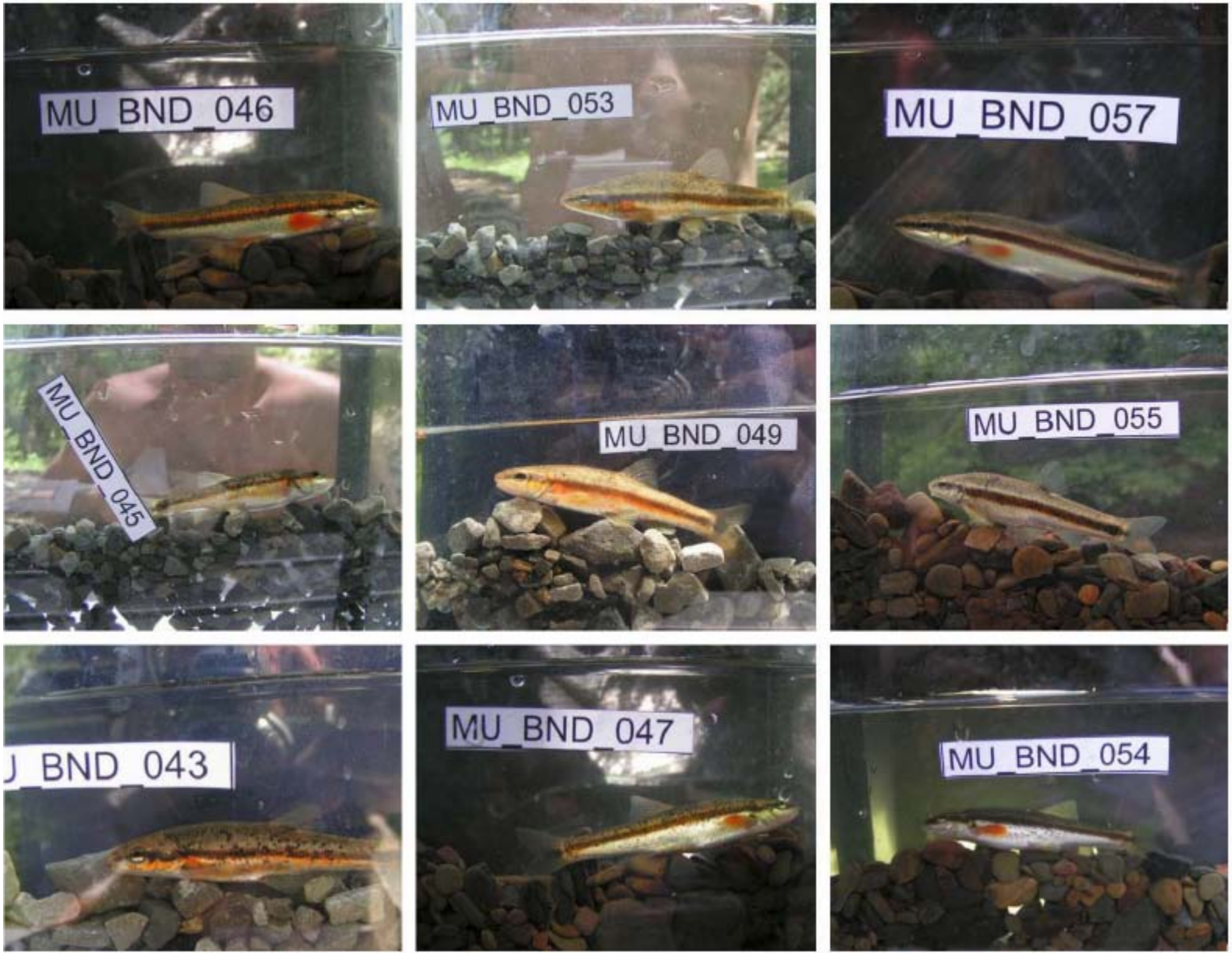
Analyzing all data collected: fish characteristics, mitochondria cytochrobe *b* gene tree, genomic RAG2 gene tree, and maps of all characteristics, the data supports the taxonomic classification of the blacknose dace species complex (*Rhinichthys*) proposed by Smith, (1985; from three subspecies of *Rhinichthys atratulus* into two separate species and a subspecies, *Rhinichthys atratulus* and *Rhinichthys obtusus* with subspecies *Rhinichthys obtusus meleagris,* (Smith, 1985). Both the Clustal W and BEAST trees for cytochrome *b* showed distinct branches between most *Rhinichthys atratulus* haplotypes, with over 50 nucleotide differences from the *Rhinichthys obtusus* reference sequence. The mitochondrial haplotypes for *Rhinichthys obtusus obtusus* with subspecies *Rhinichthys obtusus meleagris* formed a large grouping on the BEAST tree with several branches which often associated with individual streams. In general, Geoffrey Smith's categorization of the fish to a species based on nuptial coloration characteristics correlated well with their mitochondrial DNA categorization. However, it is possible to conclude that there is likely to be interbreeding occuring between these species.

This study shows that there are some fish that have the physical appearance of one species of *Rhinichthys* and the mitochondrial DNA of another. This 'ghosting' in hybrids prevents simple use of mitochondrial information to replace physical classification of nuptual color in data collections. Though this study did not have any distinct samples of hybridization of physical traits, such as has been seen in the field in past studies, it does suggest distinct mixture of genetic and physical traits could be used to distinguish between distinctive taxonomic units.

A larger scale study would benefit this project greatly. A larger collection of fish, and more genomic and mitochondrial markers could help in finding exact areas that cause the mating coloration differences. Those gene markers would help in figureing out possible methods for clearly identifying these species in the field and in the lab.

Appendix A: Photos of fish in coloration


MU_BND_031
MU_BND_036
MU_BND_042
MU_BND_028
ND_035
MU_BND_039
MU_BND_027
MU_BND_032
MU_BND_037


MU_BND_046
MU_BND_053
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MU_BND_045
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BND_043
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MU_BND_054


MU_BND_066
MU_BND_070
MU_BND_074
MU_BND_062
MU_BND_069
MU_BND_073
MU_BND_058
MU_BND_068
MU_BND_071


MU_BND_077
MU_BND_084
MU_BND_087
MU_BND_076
MU_BND_079
MU_BND_086
MU_BND_075
MU_BND_078
MU_BND_085


MU_BND_090
MU_BND_130
MU_BND_133
MU_BND_089
MU_BND_129
MU_BND_132
MU_BND_088
MU_BND_128
MU_BND_131


MU_BND_143


MU_BND_183


MU_BND_142


MU_BND_175


MU_BND_140


MU_BND_174


MU_BND_185


MU_BND_019
MU_BND_029
MU_BND_038
MU_BND_011
U_BND_023
MU_BND_033
MU_BND_010
MU_BND_021
MU_BND_030


MU_BND_056
MU_BND_080
MU_BND_083
MU_BND_048
MU_BND_067
MU_BND_082
MU_BND_044
BND_065
MU_BND_081


MU_BND_144


MU_BND_184


MU_BND_091


MU_BND_179


MU_BND_089


MU_BND_166

Appendix B: Mitochondria cytochrome *b* sequences in FASTA format

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Appendix C: Genomic RAG2 sequences in FASTA format

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Appendix D: Table of Changes in mitochondrial cytochrome *b* sequence in BEAST Tree Order

FishCode	FieldIdentity	StreamCode	558	576	579	585	592	606	612	615
Reference			G	A	A	G	T	G	G	A
SWCRK_13		Swan	A	G	C	C	C		A	G
MU_BND_166	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_038	R. atratulus atratulus	UYR	A	G	C	C	C		A	G
MU_BND_046	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_174	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_184	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_036	R. atratulus atratulus	UYR	A	G	C	C	C		A	G
MU_BND_067	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_057	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_026			A	G	C	C	C		A	G
SWCRK_9		Swan	A	G	C	C	C		A	G
MU_BND_091	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_037	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_048	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_062	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_185	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_129	R. obtusus meleagris	Laurel								
MU_BND_019	R. atratulus atratulus	Pheasant	A	G	C	C	C		A	G
MU_BND_131	R. atratulus atratulus	Laurel	A	G	C	C	C		A	G
PRUN_10		Pheasant2	A	G	C	C	C		A	G
MU_BND_175	R. atratulus atratulus	DryFork	A	G	C	C	C		A	G
MU_BND_039	R. atratulus atratulus	UYR	A	G	C	C	C		A	G
MU_BND_010	R. obtusus meleagris	Pheasant	A	G	C	C	C		A	G
PRUN_1		Pheasant2	A	G	C	C	C		A	G
MU_BND_083	R. atratulus atratulus	UYR	A	G	C	C	C		A	G

FishCode	FieldIdentity	StreamCode	558	576	579	585	592	606	612	615
MU_BND_066	R. atratulus atratulus	UYR	A	G	C	C	C		A	G
MU_BND_047	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_054	R. atratulus atratulus	Abernathy	A	G	C	C	C		A	G
MU_BND_027	R. atratulus atratulus	Horseshoe								
MU_BND_079	R. obtusus meleagris	Pheasant	A	G	C	C	C		A	G
MU_BND_089	R. obtusus obtusus	Pheasant	A	G	C	C	C		A	G
MU_BND_009	R. atratulus atratulus	Horseshoe	A	G	C	C	C		A	G
SWCRK_2		Swan	A	G	C	C	C		A	G
SWCRK_14		Swan	A	G	C	C	C		A	G
SWCRK_16		Swan	A	G	C	C	C		A	G
SWCRK_20		Swan	A	G	C	C	C		A	G
SWCRK_3		Swan	A	G	C	C	C		A	G
SWCRK_8		Swan	A	G	C	C	C		A	G
SWCRK_19		Swan	A	G	C	C	C		A	G
SWCRK_18		Swan	A	G	C	C	C		A	G
SWCRK_4		Swan	A	G	C	C	C		A	G
SWCRK_17		Swan	A	G	C	C	C		A	G
SWCRK_11		Swan	A	G	C	C	C		A	G
SWCRK_10		Swan	A	G	C	C	C		A	G
SWCRK_7		Swan	A	G	C	C	C		A	G
SWCRK_15		Swan	A	G	C	C	C		A	G
SWCRK_6		Swan	A	G	C	C	C		A	G
SWCRK_5		Swan	A	G	C	C	C		A	G
MU_BND_084	R. obtusus meleagris	WFGR	A				C			
MU_BND_081	R. obtusus meleagris	WFGR	A				C			
MU_BND_056	R. obtusus meleagris	WFGR	A				C			
MU_BND_053	R. obtusus meleagris	UWFGR	A				C			

FishCode	FieldIdentity	StreamCode	558	576	579	585	592	606	612	615
MU_BND_002	R. obtusus meleagris	Johnny	A				C			
MU_BND_183	R. obtusus meleagris	Laurel	A				C			
MU_BND_130	R. obtusus meleagris	Laurel	A				C			
MU_BND_077	R. obtusus meleagris	WFGR	A				C			
MU_BND_049	R. obtusus meleagris	WFGR	A				C			
MU_BND_080	R. obtusus meleagris	WFGR	A				C			
MU_BND_023	R. obtusus meleagris	WFGR	A				C			
MU_BND_076	R. obtusus meleagris	WFGR	A				C			
MU_BND_088	R. obtusus meleagris	WFGR	A				C			
MU_BND_179	R. obtusus meleagris	Laurel	A				C			
MU_BND_016	R. obtusus meleagris	UWFGR	A				C			
MU_BND_005	R. obtusus meleagris	WFGR	A				C			
MU_BND_033	R. obtusus meleagris	UWFGR	A				C			
MU_BND_043	R. obtusus meleagris	WFGR	A				C			
MU_BND_001	R. obtusus meleagris	Johnny	A				C			
MU_BND_128	R. obtusus meleagris	Laurel	A				C			
MU_BND_003	R. atratulus atratulus	Cove	A				C			
MU_BND_045	R. obtusus meleagris	UWFGR	A				C			
MU_BND_133	R. obtusus meleagris	Laurel	A				C			
MU_BND_035	R. obtusus meleagris	WFGR	A				C			
MU_BND_017	R. atratulus atratulus	UYR	A				C			
MU_BND_075	R. atratulus atratulus	Pheasant	A							
MU_BND_086	R. obtusus meleagris	WFGR	A				C			
MU_BND_132	R. obtusus meleagris	Laurel	A				C			
MU_BND_034	R. obtusus obtusus	Files								
MU_BND_011	R. obtusus obtusus	Files								
MU_BND_006	R. obtusus obtusus	Files								

FishCode	FieldIdentity	StreamCode	558	576	579	585	592	606	612	615
MU_BND_142	R. obtusus obtusus	Files								
MU_BND_068	R. obtusus obtusus	Files								
MU_BND_042	R. obtusus obtusus	Files								
MU_BND_074	R. obtusus obtusus	Files								
MU_BND_069	R. obtusus obtusus	Files								
MU_BND_008	R. obtusus obtusus	Files								
MU_BND_087	R. obtusus obtusus	Files								
MU_BND_070	R. obtusus obtusus	Files								
MU_BND_141	R. obtusus obtusus	Files								
MU_BND_143	R. obtusus obtusus	Files								
MU_BND_028	R. obtusus obtusus	Files								
MU_BND_144	R. obtusus obtusus	Files								
MU_BND_140	R. obtusus obtusus	Files								
MU_BND_058	R. obtusus obtusus	Pheasant								
PRUN_6		Pheasant2								
PRUN_9		Pheasant2								
PRUN_5		Pheasant2								
PRUN_2		Pheasant2								
PRUN_3		Pheasant2								
PRUN_4		Pheasant2								
PRUN_7		Pheasant2								
MU_BND_078	R. obtusus obtusus	Files								
BFORK_2		Brushy								
BFORK_1		Brushy								
SWCRK_12		Swan								
BND_209										
BND_222										

FishCode	FieldIdentity	StreamCode	558	576	579	585	592	606	612	615
BND_258										
BFORK_3		Brushy						A		
BND_187										
BND_188										
MU_BND_090	R. obtusus obtusus	BIRCH2								
MU_BND_085	R. obtusus obtusus	BIRCH1								
MU_BND_055	R. obtusus obtusus	Birch River								
MU_BND_082	R. obtusus obtusus	BIRCH1								
MU_BND_073	R. obtusus obtusus	OLR								
MU_BND_021	R. obtusus obtusus	OLR								
MU_BND_032	R. obtusus obtusus	OLR								
MU_BND_065	R. obtusus obtusus	BIRCH2								
MU_BND_044	R. obtusus obtusus	BIRCH2								
MU_BND_071	R. obtusus obtusus	BIRCH1								
BND_202										

FishCode	618	621	630	631	645	666	681	690	702	709	711
Reference	C	C	G	C	G	G	G	T	T	C	A
SWCRK_13	T			T	A	A	A	C	A	T	G
MU_BND_166	T			T	A	A	A	C	A	T	G
MU_BND_038	T			T	A	A	A	C	A	T	G
MU_BND_046	T			T	A	A	A	C	A	T	G
MU_BND_174	T			T	A	A	A	C	A	T	G
MU_BND_184	T			T	A	A	A	C	A	T	G
MU_BND_036	T			T	A	A	A	C	A	T	G
MU_BND_067	T			T	A	A	A	C	A	T	G
MU_BND_057	T			T	A	A	A	C	A	T	G

FishCode	618	621	630	631	645	666	681	690	702	709	711
MU_BND_026	T			T	A	A	A	C	A	T	G
SWCRK_9	T			T	A	A	A	C	A	T	G
MU_BND_091	T			T	A	A	A	C	A	T	G
MU_BND_037	T			T	A	A	A	C	A	T	G
MU_BND_048	T			T	A	A	A	C	A	T	G
MU_BND_062	T			T	A	A	A	C	A	T	G
MU_BND_185	T			T	A	A	A	C	A	T	G
MU_BND_129											
MU_BND_019	T			T	A	A	A	C	A	T	G
MU_BND_131	T			T	A	A	A	C	A	T	G
PRUN_10	T			T	A	A	A	C	A	T	G
MU_BND_175	T			T	A	A	A	C	A	T	G
MU_BND_039	T			T	A	A	A	C	A	T	G
MU_BND_010	T			T	A	A	A	C	A	T	G
PRUN_1	T			T	A	A	A	C	A	T	G
MU_BND_083	T			T	A	A	A	C	A	T	G
MU_BND_066	T			T	A	A	A	C	A	T	G
MU_BND_047	T			T	A	A	A	C	A	T	G
MU_BND_054	T			T	A	A	A	C	A	T	G
MU_BND_027											
MU_BND_079	T			T	A	A	A	C	A	T	G
MU_BND_089	T			T	A	A	A	C	A	T	G
MU_BND_009	T			T	A	A	A	C	A	T	G
SWCRK_2	T			T	A	A	A	C	A	T	G
SWCRK_14	T			T	A	A	A	C	A	T	G
SWCRK_16	T			T	A	A	A	C	A	T	G
SWCRK_20	T			T	A	A	A	C	A	T	G

FishCode	618	621	630	631	645	666	681	690	702	709	711
SWCRK_3	T			T	A	A	A	C	A	T	G
SWCRK_8	T			T	A	A	A	C	A	T	G
SWCRK_19	T			T	A	A	A	C	A	T	G
SWCRK_18	T			T	A	A	A	C	A	T	G
SWCRK_4	T			T	A	A	A	C	A	T	G
SWCRK_17	T			T	A	A	A	C	A	T	G
SWCRK_11	T			T	A	A	A	C	A	T	G
SWCRK_10	T			T	A	A	A	C	A	T	G
SWCRK_7	T			T	A	A	A	C	A	T	G
SWCRK_15	T			T	A	A	A	C	A	T	G
SWCRK_6	T			T	A	A	A	C	A	T	G
SWCRK_5	T			T	A	A	A	C	A	T	G
MU_BND_084						A			A		
MU_BND_081						A			A		
MU_BND_056						A			A		
MU_BND_053						A			A		
MU_BND_002						A			A		
MU_BND_183						A			A		
MU_BND_130						A			A		
MU_BND_077						A			A		
MU_BND_049						A			A		
MU_BND_080						A			A		
MU_BND_023						A			A		
MU_BND_076						A			A		
MU_BND_088						A			A		
MU_BND_179						A			A		
MU_BND_016						A			A		

FishCode	618	621	630	631	645	666	681	690	702	709	711
MU_BND_005						A			A		
MU_BND_033						A			A		
MU_BND_043						A			A		
MU_BND_001						A			A		
MU_BND_128						A			A		
MU_BND_003						A			A		
MU_BND_045						A			A		
MU_BND_133						A			A		
MU_BND_035						A			A		
MU_BND_017						A			A		
MU_BND_075						A			A		
MU_BND_086						A			A		
MU_BND_132						A			A		
MU_BND_034					A	A			C		
MU_BND_011					A	A			C		
MU_BND_006					A	A			C		
MU_BND_142					A	A			C		
MU_BND_068					A	A			C		
MU_BND_042					A	A			C		
MU_BND_074					A	A			C		
MU_BND_069					A	A			C		
MU_BND_008					A	A			C		
MU_BND_087					A	A			C		
MU_BND_070					A	A			C		
MU_BND_141					A	A			C		
MU_BND_143					A	A			C		
MU_BND_028					A	A			A		

FishCode	618	621	630	631	645	666	681	690	702	709	711
MU_BND_144					A	A			C		
MU_BND_140					A	A			C		
MU_BND_058					A	A			C		
PRUN_6					A	A			C		
PRUN_9					A	A			C		
PRUN_5					A	A			C		
PRUN_2					A	A			C		
PRUN_3					A	A			C		
PRUN_4					A	A			C		
PRUN_7					A	A			C		
MU_BND_078					A	A			A		
BFORK_2											
BFORK_1											
SWCRK_12		T									
BND_209											
BND_222											
BND_258											
BFORK_3											
BND_187											
BND_188			A								
MU_BND_090			A			A					
MU_BND_085			A			A					
MU_BND_055			A			A					
MU_BND_082			A			A					
MU_BND_073			A			A					
MU_BND_021			A			A					
MU_BND_032			A			A					

FishCode	618	621	630	631	645	666	681	690	702	709	711
MU_BND_065			A			A					
MU_BND_044			A			A					
MU_BND_071			A			A					
BND_202						A					

FishCode	720	721	727	729	750	753	762	768	774	777	780
Reference	A	T	A	A	A	C	A	C	A	A	T
SWCRK_13					G	T	G			G	C
MU_BND_166					G	T	G			G	C
MU_BND_038					G	T	G		G	G	C
MU_BND_046					G	T	G			G	C
MU_BND_174					G	T	G			G	C
MU_BND_184					G	T	G			G	C
MU_BND_036					G	T	G			G	C
MU_BND_067					G	T	G			G	C
MU_BND_057					G	T	G			G	C
MU_BND_026					G	T	G			G	C
SWCRK_9					G	T	G			G	C
MU_BND_091					G	T	G			G	C
MU_BND_037					G	T	G			G	C
MU_BND_048					G	T	G			G	C
MU_BND_062					G	T	G			G	C
MU_BND_185					G	T	G			G	C
MU_BND_129											
MU_BND_019					G	T	G			G	C
MU_BND_131					G	T	G			G	C
PRUN_10					G	T				G	C

FishCode	720	721	727	729	750	753	762	768	774	777	780
MU_BND_175					G	T	G			G	C
MU_BND_039					G	T	G			G	C
MU_BND_010					G	T	G			G	C
PRUN_1					G	T	G			G	C
MU_BND_083					G	T	G			G	C
MU_BND_066					G	T	G			G	C
MU_BND_047					G	T	G			G	C
MU_BND_054					G	T	G			G	C
MU_BND_027											
MU_BND_079					G	T	G			G	C
MU_BND_089					G	T	G			G	C
MU_BND_009					G	T	G			G	C
SWCRK_2					G	T	G			G	C
SWCRK_14					G	T	G			G	C
SWCRK_16					G	T	G			G	C
SWCRK_20					G	T	G			G	C
SWCRK_3					G	T	G			G	C
SWCRK_8					G	T	G			G	C
SWCRK_19					G	T	G			G	C
SWCRK_18					G	T	G			G	C
SWCRK_4					G	T	G			G	C
SWCRK_17	G				G	T	G			G	C
SWCRK_11					G	T	G			G	C
SWCRK_10					G	T	G			G	C
SWCRK_7					G	T	G			G	C
SWCRK_15					G	T	G			G	C
SWCRK_6					G	T	G			G	C

FishCode	720	721	727	729	750	753	762	768	774	777	780
SWCRK_5					G	T	G			G	C
MU_BND_084				G		T	G	T			C
MU_BND_081				G		T	G	T			C
MU_BND_056				G		T	G	T			C
MU_BND_053				G		T	G	T			C
MU_BND_002				G		T	G	T			C
MU_BND_183				G		T	G	T			C
MU_BND_130				G		T	G	T			C
MU_BND_077				G		T	G	T			C
MU_BND_049				G		T	G	T			C
MU_BND_080				G		T	G	T			C
MU_BND_023				G		T	G	T			C
MU_BND_076				G		T	G	T			C
MU_BND_088				G		T	G	T			C
MU_BND_179				G		T	G	T			C
MU_BND_016				G		T	G	T			C
MU_BND_005				G		T	G	T			C
MU_BND_033				G		T	G	T			C
MU_BND_043				G		T	G	T			C
MU_BND_001				G		T	G	T			C
MU_BND_128				G		T	G	T			C
MU_BND_003				G		T	G	T			C
MU_BND_045				G		T	G	T			C
MU_BND_133				G		T	G	T			C
MU_BND_035				G		T	G	T			C
MU_BND_017				G		T	G	T			C
MU_BND_075				G		T	G	T			C

FishCode	720	721	727	729	750	753	762	768	774	777	780
MU_BND_086				G		T	G	T			C
MU_BND_132				G		T	G	T			C
MU_BND_034		G		G	G		G				
MU_BND_011		G		G	G		G				
MU_BND_006		G		G	G		G				
MU_BND_142		G		G	G		G				
MU_BND_068		G		G	G		G				
MU_BND_042		G		G	G		G				
MU_BND_074		G		G	G		G				
MU_BND_069		G		G	G		G				
MU_BND_008		G		G	G		G				
MU_BND_087		G		G	G		G				
MU_BND_070	G	G		G	G		G				
MU_BND_141	G	G		G	G		G				
MU_BND_143	G	G		G	G		G				
MU_BND_028	G	G		G	G		G				
MU_BND_144	G	G		G	G		G				
MU_BND_140	G	G		G	G		G				
MU_BND_058	G	G		G	G		G				
PRUN_6	G	G		G	G		G				
PRUN_9	G	G		G	G		G				
PRUN_5	G	G		G	G		G				
PRUN_2	G	G		G	G		G				
PRUN_3	G	G		G	G		G				
PRUN_4	G	G		G	G		G				
PRUN_7	G	G		G	G		G				
MU_BND_078		G		G	G		G				

FishCode	720	721	727	729	750	753	762	768	774	777	780
BFORK_2				G			G				
BFORK_1				G			G				
SWCRK_12				G							
BND_209				G							
BND_222				G							
BND_258				G							
BFORK_3				G							
BND_187				G							
BND_188				G							
MU_BND_090				G							
MU_BND_085				G							
MU_BND_055				G							
MU_BND_082				G							
MU_BND_073			G	G							
MU_BND_021			G	G							
MU_BND_032			G	G							
MU_BND_065				G							
MU_BND_044				G							
MU_BND_071				G							
BND_202				G							

FishCode	795	798	807	810	828	837	846	849	852	855	876
Reference	A	A	A	C	T	C	A	C	T	A	G
SWCRK_13		G	G		C	T				G	
MU_BND_166		G	G			T			C	G	
MU_BND_038		G	G			T				G	
MU_BND_046	G		G			T				G	

FishCode	795	798	807	810	828	837	846	849	852	855	876
MU_BND_174	G		G			T				G	
MU_BND_184	G		G			T				G	
MU_BND_036	G		G			T				G	
MU_BND_067			G			T				G	
MU_BND_057		G	G			T				G	
MU_BND_026		G	G			T				G	
SWCRK_9		G	G			T				G	
MU_BND_091		G	G			T				G	
MU_BND_037		G	G			T				G	
MU_BND_048		G	G			T				G	
MU_BND_062		G	G			T				G	
MU_BND_185		G	G			T				G	
MU_BND_129											
MU_BND_019		G	G			T				G	
MU_BND_131		G	G			T				G	
PRUN_10		G	G			T				G	
MU_BND_175		G	G			T			C	G	
MU_BND_039		G	G			T				G	
MU_BND_010		G	G			T				G	
PRUN_1		G	G			T				G	
MU_BND_083		G	G			T				G	
MU_BND_066		G	G			T				G	
MU_BND_047		G	G			T				G	
MU_BND_054		G	G			T				G	
MU_BND_027											
MU_BND_079		G	G			T				G	
MU_BND_089		G	G			T				G	

FishCode	795	798	807	810	828	837	846	849	852	855	876
MU_BND_009		G	G			T				G	
SWCRK_2		G	G			T				G	
SWCRK_14		G	G			T				G	
SWCRK_16		G	G			T				G	
SWCRK_20		G	G			T				G	
SWCRK_3		G	G			T				G	
SWCRK_8		G	G			T				G	
SWCRK_19		G	G			T				G	
SWCRK_18		G	G			T				G	
SWCRK_4		G	G			T				G	
SWCRK_17		G	G			T				G	
SWCRK_11		G	G			T				G	
SWCRK_10		G	G			T				G	
SWCRK_7		G	G			T				G	
SWCRK_15		G	G			T				G	
SWCRK_6		G	G			T				G	
SWCRK_5		G	G			T				G	
MU_BND_084				A							
MU_BND_081				A							
MU_BND_056				A							
MU_BND_053				A							
MU_BND_002				A							
MU_BND_183				A							
MU_BND_130				A							
MU_BND_077				A							
MU_BND_049				A							
MU_BND_080				A							

FishCode	795	798	807	810	828	837	846	849	852	855	876
MU_BND_023				A							
MU_BND_076				A							
MU_BND_088				A							
MU_BND_179				A							
MU_BND_016				A							
MU_BND_005				A							
MU_BND_033				A							
MU_BND_043				A							
MU_BND_001				A							
MU_BND_128				A							
MU_BND_003				A							
MU_BND_045				A							
MU_BND_133				A							
MU_BND_035				A							
MU_BND_017				A							
MU_BND_075				A							
MU_BND_086				A							
MU_BND_132				A							
MU_BND_034							G				
MU_BND_011							G				
MU_BND_006							G				
MU_BND_142							G				
MU_BND_068							G				
MU_BND_042							G				
MU_BND_074							G				
MU_BND_069							G				
MU_BND_008							G				

FishCode	795	798	807	810	828	837	846	849	852	855	876
MU_BND_087							G				
MU_BND_070											
MU_BND_141											
MU_BND_143											
MU_BND_028							G				A
MU_BND_144							G				A
MU_BND_140							G				
MU_BND_058							G				
PRUN_6							G				
PRUN_9							G				
PRUN_5							G				
PRUN_2							G				
PRUN_3							G				
PRUN_4							G				
PRUN_7							G				
MU_BND_078											
BFORK_2				C				T			
BFORK_1				C				T			
SWCRK_12											
BND_209											
BND_222											
BND_258											
BFORK_3											
BND_187									C		
BND_188									C		
MU_BND_090											
MU_BND_085											

FishCode	795	798	807	810	828	837	846	849	852	855	876
MU_BND_055											
MU_BND_082											
MU_BND_073											
MU_BND_021											
MU_BND_032											
MU_BND_065											

FishCode	882	885	894	897	900	901	904	906	909	912	918
Reference	A	A	T	G	G	T	C	G	A	C	C
SWCRK_13			C	A	A	C	T	A	G		T
MU_BND_166			C	A	A	C	T		G		T
MU_BND_038			C	A	A	C	T	A	G		T
MU_BND_046			C	A	A	C	T	A	G		T
MU_BND_174			C	A	A	C	T	A	G		T
MU_BND_184			C	A	A	C	T	A	G		T
MU_BND_036			C	A	A	C	T	A	G		T
MU_BND_067			C	A	A	C	T	A	G		T
MU_BND_057		G	C	A	A	C	T	A	G		T
MU_BND_026			C	A	A	C	T	A	G		T
SWCRK_9			C	A	A	C	T	A	G		T
MU_BND_091			C	A	A	C	T	A	G		T
MU_BND_037			C	A	A	C	T	A	G		T
MU_BND_048			C	A	A	C	T	A	G		T
MU_BND_062			C	A	A	C	T	A	G		T
MU_BND_185			C	A	A	C	T	A	G		T
MU_BND_129											
MU_BND_019			C	A	A	C	T	A	G		T

FishCode	882	885	894	897	900	901	904	906	909	912	918
MU_BND_131			C	A	A	C	T	A	G		T
PRUN_10			C	A	A	C	T	A	G		T
MU_BND_175			C	A	A	C	T	A	G		T
MU_BND_039			C	A	A	C	T	A	G		T
MU_BND_010			C	A	A	C	T	A	G		T
PRUN_1			C	A	A	C	T	A	G		T
MU_BND_083			C	A	A	C	T	A	G		T
MU_BND_066			C	A	A	C	T	A	G		T
MU_BND_047			C	A	A	C	T	A	G		T
MU_BND_054			C	A	A	C	T	A	G		T
MU_BND_027											
MU_BND_079			C	A	A	C	T	A	G		T
MU_BND_089			C	A	A	C	T	A	G		T
MU_BND_009			C	A	A	C	T	A	G		T
SWCRK_2			C	A	A	C	T	A	G		T
SWCRK_14			C	A	A	C	T	A	G		T
SWCRK_16			C	A	A	C	T	A	G		T
SWCRK_20			C	A	A	C	T	A	G		T
SWCRK_3			C	A	A	C	T	A	G		T
SWCRK_8			C	A	A	C	T	A	G		T
SWCRK_19			C	A	A	C	T	A	G		T
SWCRK_18			C	A	A	C	T	A	G		T
SWCRK_4			C	A	A	C	T	A	G		T
SWCRK_17			C	A	A	C	T	A	G		T
SWCRK_11			C	A	A	C	T	A	G		T
SWCRK_10			C	A	A	C	T	A	G		T
SWCRK_7			C	A	A	C	T	A	G		T

FishCode	882	885	894	897	900	901	904	906	909	912	918
SWCRK_15			C	A	A	C	T	A	G		T
SWCRK_6			C	A	A	C	T	A	G		T
SWCRK_5			C	A	A	C	T	A	G		T
MU_BND_084	G										
MU_BND_081	G										
MU_BND_056	G										
MU_BND_053	G										
MU_BND_002	G										
MU_BND_183	G										
MU_BND_130	G										
MU_BND_077	G										
MU_BND_049	G										
MU_BND_080	G										
MU_BND_023	G										
MU_BND_076	G										
MU_BND_088	G										
MU_BND_179	G										
MU_BND_016	G										
MU_BND_005	G										
MU_BND_033	G										
MU_BND_043	G										
MU_BND_001	G										
MU_BND_128	G										
MU_BND_003	G										
MU_BND_045	G										
MU_BND_133	G										
MU_BND_035	G										

FishCode	882	885	894	897	900	901	904	906	909	912	918
MU_BND_017	G										
MU_BND_075	G										
MU_BND_086	G										
MU_BND_132	G										
MU_BND_034	G										
MU_BND_011	G										
MU_BND_006	G										
MU_BND_142	G										
MU_BND_068	G										
MU_BND_042	G										
MU_BND_074	G										
MU_BND_069	G										
MU_BND_008	G										
MU_BND_087	G										
MU_BND_070	G									T	
MU_BND_141	G									T	
MU_BND_143	G									T	
MU_BND_028	G										
MU_BND_144	G										
MU_BND_140	G										
MU_BND_058	G										
PRUN_6	G										
PRUN_9	G										
PRUN_5	G										
PRUN_2	G										
PRUN_3	G										
PRUN_4	G										

FishCode	882	885	894	897	900	901	904	906	909	912	918
PRUN_7	G										
MU_BND_078	G										
BFORK_2											
BFORK_1											
SWCRK_12											
BND_209											
BND_222											
BND_258											
BFORK_3											
BND_187											
BND_188											
MU_BND_090											
MU_BND_085											
MU_BND_055											
MU_BND_082											
MU_BND_073											
MU_BND_021											
MU_BND_032											
MU_BND_065											
MU_BND_044											
MU_BND_071											
BND_202											
MU_BND_044											
MU_BND_071											
BND_202											

FishCode	921	924	927	933	936	939	943	948	958	963	967
Reference	A	C	C	A	A	T	T	C	A	T	T
SWCRK_13	G			G		G	C	T			
MU_BND_166	G			G		G	C	T			
MU_BND_038	G			G		G	C	T			
MU_BND_046	G			G		G	C	T			
MU_BND_174	G			G		G	C	T			
MU_BND_184	G			G		G	C	T			
MU_BND_036	G			G		G	C	T			
MU_BND_067	G			G		G	C	T			
MU_BND_057	G			G		G	C	T			C
MU_BND_026	G			G		G	C	T			
SWCRK_9	G			G		G	C	T			
MU_BND_091	G			G		G	C	T			
MU_BND_037	G			G		G	C	T			
MU_BND_048	G			G		G	C	T			
MU_BND_062	G			G		G	C	T			
MU_BND_185	G			G		G	C	T			
MU_BND_129											
MU_BND_019	G			G		G	C	T			
MU_BND_131	G			G		G	C	T			
PRUN_10	G			G		G	C	T			
MU_BND_175	G			G		G	C	T			
MU_BND_039	G			G		G	C	T			
MU_BND_010	G			G		G	C	T			
PRUN_1	G			G		G	C	T			
MU_BND_083	G			G		G	C	T			
MU_BND_066	G			G		G	C	T			

FishCode	921	924	927	933	936	939	943	948	958	963	967
MU_BND_047	G			G		G	C	T			
MU_BND_054	G			G		G	C	T			
MU_BND_027											
MU_BND_079	G			G		G	C	T			
MU_BND_089	G			G		G	C	T			
MU_BND_009	G			G		G	C	T			
SWCRK_2	G			G		G	C	T			
SWCRK_14	G			G		G	C	T			
SWCRK_16	G			G		G	C	T			
SWCRK_20	G			G		G	C	T			
SWCRK_3	G			G		G	C	T			
SWCRK_8	G			G		G	C	T			
SWCRK_19	G			G		G	C	T			
SWCRK_18	G			G		G	C	T			
SWCRK_4	G			G		G	C	T			
SWCRK_17	G			G		G	C	T			
SWCRK_11	G			G		G	C	T			
SWCRK_10	G			G		G	C	T			
SWCRK_7	G			G		G	C	T			
SWCRK_15	G			G		G	C	T			
SWCRK_6	G			G		G	C	T			
SWCRK_5	G			G		G	C	T			
MU_BND_084					G						
MU_BND_081					G						
MU_BND_056					G						
MU_BND_053					G						
MU_BND_002					G						

FishCode	921	924	927	933	936	939	943	948	958	963	967
MU_BND_183					G						
MU_BND_130					G						
MU_BND_077					G						
MU_BND_049					G						
MU_BND_080					G						
MU_BND_023					G						
MU_BND_076					G						
MU_BND_088					G						
MU_BND_179					G						
MU_BND_016					G						
MU_BND_005					G						
MU_BND_033					G						
MU_BND_043					G						
MU_BND_001					G						
MU_BND_128					G						
MU_BND_003					G						
MU_BND_045					G						
MU_BND_133					G						
MU_BND_035					G						
MU_BND_017					G						
MU_BND_075					G						
MU_BND_086					G						
MU_BND_132					G						
MU_BND_034			A								
MU_BND_011			A			C					
MU_BND_006			A			C					
MU_BND_142			A			C					

FishCode	921	924	927	933	936	939	943	948	958	963	967
MU_BND_068			A			C					
MU_BND_042			A			C					
MU_BND_074			A			C					
MU_BND_069			A			C					
MU_BND_008			A			C					
MU_BND_087			A			C					
MU_BND_070		T	A								
MU_BND_141		T	A								
MU_BND_143		T	A								
MU_BND_028		T	A								
MU_BND_144		T	A								
MU_BND_140		T	A								
MU_BND_058		T	A							C	
PRUN_6		T	A								
PRUN_9		T	A								
PRUN_5		T	A								
PRUN_2		T	A								
PRUN_3		T	A								
PRUN_4		T	A								
PRUN_7		T	A								
MU_BND_078		T	A								
BFORK_2											
BFORK_1											
SWCRK_12											
BND_209											
BND_222											
BND_258											

FishCode	921	924	927	933	936	939	943	948	958	963	967
BFORK_3											
BND_187											
BND_188											
MU_BND_090											
MU_BND_085											
MU_BND_055											
MU_BND_082											
MU_BND_073											
MU_BND_021											
MU_BND_032											
MU_BND_065											
MU_BND_044											
MU_BND_071											
BND_202									G		

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
Reference	C	A	G	G	A	C	A	A	T	G	G
SWCRK_13			C		G	T	G	G	C	A	A
MU_BND_166			C	A	G	T	G	G	C	A	A
MU_BND_038			C	A	G	T	G	G	C	A	A
MU_BND_046			C	A	G	T	G	G	C	A	A
MU_BND_174			C	A	G	T	G	G	C	A	A
MU_BND_184			C	A	G	T	G	G	C	A	A
MU_BND_036			T	A	G	T	G	G	C	A	A
MU_BND_067			C	A	G	T	G	G	C	A	A
MU_BND_057			C		G	T	G	G	C	A	A
MU_BND_026			C		G	T	G	G	C	A	A

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
SWCRK_9			C		G	T	G	G	C	A	A
MU_BND_091			C	A	G	T	G	G	C	A	A
MU_BND_037			C	A	G	T	G	G	C	A	A
MU_BND_048			C	A	G	T	G	G	C	A	A
MU_BND_062			C	A	G	T	G	G	C	A	A
MU_BND_185			C	A	G	T	G	G	C	A	A
MU_BND_129											
MU_BND_019			C	A	G	T	G	G	C	A	A
MU_BND_131			C	A	G	T	G	G	C	A	
PRUN_10			C	A	G	T	G	G	C	A	A
MU_BND_175			C	A	G	T	G	G	C	A	A
MU_BND_039			C	A	G	T	G	G	C	A	A
MU_BND_010			C	A	G	T	G	G	C	A	A
PRUN_1			C	A	G	T	G	G	C	A	A
MU_BND_083			C	A	G	T	G	G	C	A	A
MU_BND_066			C	A	G	T	G	G	C	A	A
MU_BND_047	T		C	A	G	T	G	G	C	A	A
MU_BND_054	T		C	A	G	T	G	G	C	A	A
MU_BND_027											
MU_BND_079	T		C	A	G	T	G	G	C	A	A
MU_BND_089	T		C	A	G	T	G	G	C	A	A
MU_BND_009	T		C	A	G	T	G	G	C	A	A
SWCRK_2			C	A	G	T	G	G		A	A
SWCRK_14			C	A	G	T	G	G		A	A
SWCRK_16			C	A	G	T	G	G		A	A
SWCRK_20			C	A	G	T	G	G		A	A
SWCRK_3			C	A	G	T	G	G		A	A

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
SWCRK_8			C	A	G	T	G	G		A	A
SWCRK_19			C	A	G	T	G	G		A	A
SWCRK_18			C	A	G	T	G	G		A	A
SWCRK_4			C	A	G	T	G	G		A	A
SWCRK_17			C	A	G	T	G	G		A	A
SWCRK_11			C	A	G	T	G	G		A	A
SWCRK_10			C	A	G	T	G	G		A	A
SWCRK_7			C	A	G	T	G	G		A	A
SWCRK_15			C	A	G	T	G	G		A	A
SWCRK_6			C	A	G	T	G	G		A	A
SWCRK_5			C	A	G	T	G	G		A	A
MU_BND_084				A			G				
MU_BND_081		G		A			G				
MU_BND_056		G		A			G				
MU_BND_053		G		A			G				
MU_BND_002		G		A			G				
MU_BND_183		G		A			G				
MU_BND_130		G		A			G				
MU_BND_077		G		A			G				
MU_BND_049		G		A			G				
MU_BND_080		G		A			G				
MU_BND_023		G		A			G				
MU_BND_076		G		A			G				
MU_BND_088		G		A			G				
MU_BND_179		G		A			G				
MU_BND_016		G		A			G				
MU_BND_005		G		A			G				

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
MU_BND_033		G		A			G				
MU_BND_043		G		A			G				
MU_BND_001		G		A			G				
MU_BND_128		G		A			G				
MU_BND_003		G		A			G				
MU_BND_045		G		A			G				
MU_BND_133		G		A			G				
MU_BND_035		G		A			G				
MU_BND_017		G		A			G				
MU_BND_075		G		A			G				
MU_BND_086		G		A			G				
MU_BND_132		G		A			G				
MU_BND_034				A							
MU_BND_011				A							
MU_BND_006				A							
MU_BND_142				A							
MU_BND_068				A							
MU_BND_042				A							
MU_BND_074				A							
MU_BND_069				A							
MU_BND_008				A							
MU_BND_087				A							
MU_BND_070				A							
MU_BND_141				A							
MU_BND_143				A							
MU_BND_028				A							
MU_BND_144				A							

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
MU_BND_140				A							
MU_BND_058				A							
PRUN_6				A							
PRUN_9				A							
PRUN_5				A							
PRUN_2				A							
PRUN_3				A							
PRUN_4				A							
PRUN_7				A							
MU_BND_078				A							
BFORK_2											
BFORK_1											
SWCRK_12											
BND_209											
BND_222											
BND_258											
BFORK_3											
BND_187											
BND_188											
MU_BND_090											A
MU_BND_085											A
MU_BND_055											A
MU_BND_082											A
MU_BND_073											A
MU_BND_021											A
MU_BND_032											A
MU_BND_065											A

FishCode	969	972	984	990	996	999	1005	1008	1014	1017	1023
MU_BND_044											A
MU_BND_071											A
BND_202											

FishCode	1032	1044	1045	1050	1065	1071
Reference	A	T	A	C	A	A
SWCRK_13		C	G	T	G	
MU_BND_166		C	G	T	G	
MU_BND_038		C	G	T	G	
MU_BND_046		C	G	T	G	
MU_BND_174		C	G	T	G	
MU_BND_184		C	G	T	G	
MU_BND_036		C	G	T	G	
MU_BND_067		C	G	T	G	
MU_BND_057	G	C	G	T	G	G
MU_BND_026	G	C	G	T	G	
SWCRK_9	G	C	G	T	G	
MU_BND_091	G	C		T	G	
MU_BND_037	G	C		T	G	
MU_BND_048	G	C		T	G	
MU_BND_062	G	C		T	G	
MU_BND_185	G	C		T		
MU_BND_129						
MU_BND_019	G	C	G	T	G	
MU_BND_131	G	C	G	T	G	
PRUN_10	G	C	G	T	G	
MU_BND_175	G	C	G	T	G	

FishCode	1032	1044	1045	1050	1065	1071
MU_BND_039	G	C	G	T	G	
MU_BND_010	G	C	G	T	G	
PRUN_1	G	C	G	T	G	
MU_BND_083	G	C	G	T	G	
MU_BND_066	G	C	G	T	G	
MU_BND_047	G	C	G	T	G	
MU_BND_054	G	C	G	T	G	
MU_BND_027						
MU_BND_079	G	C	G	T	G	
MU_BND_089	G	C	G	T	G	
MU_BND_009	G	C	G	T	G	
SWCRK_2	G	C	G	T	G	
SWCRK_14	G	C	G	T	G	
SWCRK_16	G	C	G	T	G	
SWCRK_20	G	C	G	T	G	
SWCRK_3	G	C	G	T	G	
SWCRK_8	G	C	G	T	G	
SWCRK_19	G	C	G	T	G	
SWCRK_18	G	C	G	T	G	
SWCRK_4	G	C	G	T	G	
SWCRK_17	G	C	G	T	G	
SWCRK_11	G	C	G	T	G	
SWCRK_10	G	C	G	T	G	
SWCRK_7	G	C	G	T	G	
SWCRK_15	G	C	G	T	G	
SWCRK_6	G	C	G	T	G	
SWCRK_5	G	C	G	T	G	

FishCode	1032	1044	1045	1050	1065	1071
MU_BND_084		C			G	G
MU_BND_081		C			G	G
MU_BND_056		C			G	G
MU_BND_053		C			G	G
MU_BND_002		C			G	G
MU_BND_183		C			G	G
MU_BND_130		C			G	G
MU_BND_077		C			G	G
MU_BND_049		C			G	G
MU_BND_080		C			G	G
MU_BND_023		C			G	G
MU_BND_076		C			G	G
MU_BND_088		C			G	G
MU_BND_179		C			G	G
MU_BND_016		C			G	G
MU_BND_005		C			G	G
MU_BND_033		C			G	G
MU_BND_043		C			G	G
MU_BND_001		C			G	G
MU_BND_128		C			G	G
MU_BND_003		C			G	G
MU_BND_045		C			G	G
MU_BND_133		C			G	G
MU_BND_035		C			G	G
MU_BND_017		C			G	G
MU_BND_075		C			G	G
MU_BND_086		C			G	G

FishCode	1032	1044	1045	1050	1065	1071
MU_BND_132		C			G	G
MU_BND_034						
MU_BND_011						
MU_BND_006						
MU_BND_142						
MU_BND_068						
MU_BND_042						
MU_BND_074						
MU_BND_069						
MU_BND_008						
MU_BND_087						
MU_BND_070						
MU_BND_141						
MU_BND_143						
MU_BND_028						
MU_BND_144						
MU_BND_140			G			
MU_BND_058			G			
PRUN_6						
PRUN_9						
PRUN_5						
PRUN_2						
PRUN_3						
PRUN_4						
PRUN_7						
MU_BND_078						
BFORK_2						

FishCode	1032	1044	1045	1050	1065	1071
BFORK_1						
SWCRK_12						
BND_209						
BND_222						
BND_258						
BFORK_3						
BND_187						
BND_188						
MU_BND_090						
MU_BND_085						
MU_BND_055						
MU_BND_082						
MU_BND_073						
MU_BND_021						
MU_BND_032						
MU_BND_065						
MU_BND_044						
MU_BND_071						
BND_202						

Appendix E: Table of Changes in RAG2 sequence in BEAST Tree Order

FishCode	FieldIdentity	StreamCode	210	258	296	304	321	381	432
Reference			G	C	C	G	G	A	C
PRUN_1		Pheasant2				GA			
MU_BND_021	R. obtusus obtusus	OLR							
MU_BND_069	R. obtusus obtusus	Files							
MU_BND_081	R. obtusus meleagris	WFGR							
PRUN_7		Pheasant2				GA			
MU_BND_008	R. obtusus obtusus	Files							
PRUN_10		Pheasant2							
BND_187									
MU_BND_183	R. obtusus meleagris	Laurel							
MU_BND_179	R. obtusus meleagris	Laurel		CT					
BFORK_2		Brushy				AG			
MU_BND_129	R. obtusus meleagris	Laurel				AG			
MU_BND_058	R. obtusus obtusus	Pheasant				AG			
MU_BND_089	R. obtusus obtusus	Pheasant							
BND_188									
MU_BND_019	R. atratulus atratulus	Pheasant				AG			
BND_222									
MU_BND_053	R. obtusus meleagris	UWFGR							
PRUN_6		Pheasant2							
MU_BND_068	R. obtusus obtusus	Files							
MU_BND_010	R. obtusus meleagris	Pheasant				AG			
MU_BND_088	R. obtusus meleagris	WFGR							
PRUN_4		Pheasant2							
PRUN_2		Pheasant2							
MU_BND_085	R. obtusus obtusus	BIRCH1							CG

FishCode	FieldIdentity	StreamCode	210	258	296	304	321	381	432
MU_BND_143	R. obtusus obtusus	Files				AG			
MU_BND_055	R. obtusus obtusus	Birch River							
MU_BND_080	R. obtusus meleagris	WFGR							
MU_BND_086	R. obtusus meleagris	WFGR							
MU_BND_090	R. obtusus obtusus	BIRCH2							
MU_BND_044	R. obtusus obtusus	BIRCH2							
BND_209									
MU_BND_140	R. obtusus obtusus	Files							
MU_BND_082	R. obtusus obtusus	BIRCH1							
MU_BND_075	R. atratulus atratulus	Pheasant							
MU_BND_144	R. obtusus obtusus	Files							
MU_BND_049	R. obtusus meleagris	WFGR							
MU_BND_141	R. obtusus obtusus	Files							
MU_BND_070	R. obtusus obtusus	Files							
MU_BND_065	R. obtusus obtusus	BIRCH2							
MU_BND_073	R. obtusus obtusus	OLR							
MU_BND_071	R. obtusus obtusus	BIRCH1							
MU_BND_023	R. obtusus meleagris	WFGR							
MU_BND_028	R. obtusus obtusus	Files							
MU_BND_033	R. obtusus meleagris	UWFGR							
MU_BND_032	R. obtusus obtusus	OLR							
MU_BND_130	R. obtusus meleagris	Laurel							
MU_BND_031	R. atratulus atratulus	Pheasant							
PRUN_5		Pheasant2							
PRUN_9		Pheasant2							
MU_BND_087	R. obtusus obtusus	Files							
BND_258									

FishCode	FieldIdentity	StreamCode	210	258	296	304	321	381	432
MU_BND_003	R. atratulus atratulus	Cove							
MU_BND_074	R. obtusus obtusus	Files							
PRUN_3		Pheasant2							
MU_BND_011	R. obtusus obtusus	Files							
BFORK_3		Brushy							
BND_202									
MU_BND_079	R. obtusus meleagris	Pheasant							
MU_BND_184	R. atratulus atratulus	DryFork							
SWCRK_9		Swan				A			
SWCRK_7		Swan				A			
SWCRK_19		Swan				A			
SWCRK_16		Swan				A			
SWCRK_12		Swan				A			
MU_BND_037	R. atratulus atratulus	Abernathy				A			CT
SWCRK_17		Swan				A			
SWCRK_10		Swan				A			
SWCRK_8		Swan				A			
SWCRK_20		Swan				A			
MU_BND_057	R. atratulus atratulus	Abernathy				A			
SWCRK_6		Swan				A			
MU_BND_174	R. atratulus atratulus	DryFork				A			
SWCRK_13		Swan				A			
MU_BND_066	R. atratulus atratulus	UYR				A			
BFORK_1		Brushy				AG			
BND_040						A			
MU_BND_091	R. atratulus atratulus	DryFork				A			
MU_BND_030	R. atratulus atratulus	UYR				A			

FishCode	FieldIdentity	StreamCode	210	258	296	304	321	381	432
MU_BND_062	R. atratulus atratulus	Abernathy				A			
MU_BND_038	R. atratulus atratulus	UYR				A			
MU_BND_048	R. atratulus atratulus	Abernathy				A			
MU_BND_175	R. atratulus atratulus	DryFork				A			
MU_BND_067	R. atratulus atratulus	Abernathy				A	A		
MU_BND_083	R. atratulus atratulus	UYR				A			
MU_BND_039	R. atratulus atratulus	UYR				A			
MU_BND_009	R. atratulus atratulus	Horseshoe				A			
MU_BND_027	R. atratulus atratulus	Horseshoe				A			
MU_BND_131	R. atratulus atratulus	Laurel			A	A			
SWCRK_14		Swan				A			
MU_BND_185	R. atratulus atratulus	DryFork							
MU_BND_046	R. atratulus atratulus	Abernathy				A			
SWCRK_11		Swan				A			
MU_BND_026						A			
SWCRK_4		Swan				A			
SWCRK_15		Swan				A			
SWCRK_18		Swan				A			
MU_BND_047	R. atratulus atratulus	Abernathy				A			
PRUN_8		Pheasant2	A					G	

FishCode	441	466	489	492	534	540	556	591	740	759	859
Reference	G	G	T	C	G	C	G	T	T	A	C
PRUN_1			TC			CG				AG	
MU_BND_021											
MU_BND_069											
MU_BND_081											

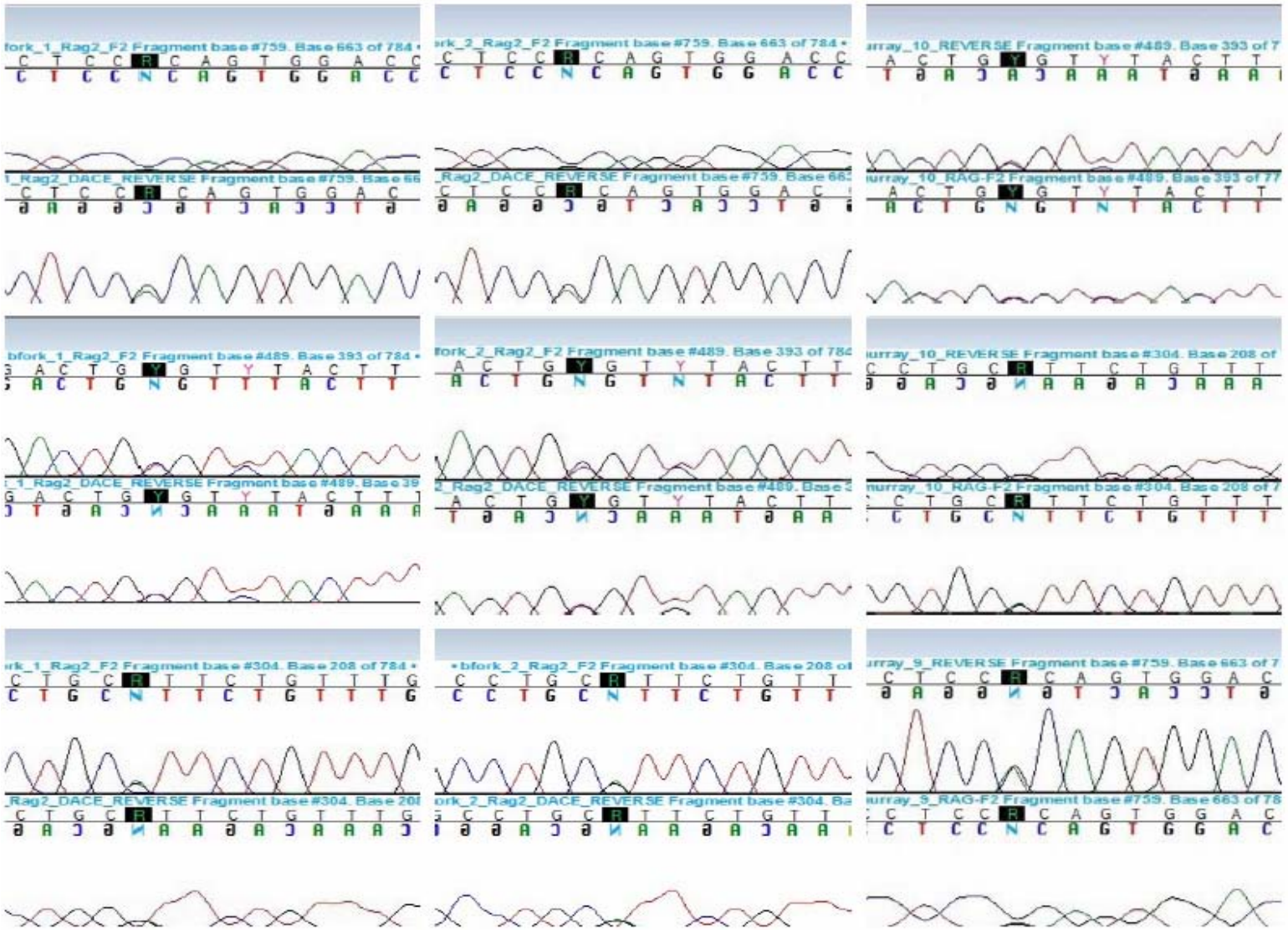
FishCode	441	466	489	492	534	540	556	591	740	759	859
PRUN_7			CT					TC		G	
MU_BND_008											
PRUN_10								TC			
BND_187											
MU_BND_183			C	T						G	
MU_BND_179											CT
BFORK_2			CT	CT						GA	
MU_BND_129			CT	CT						AG	
MU_BND_058			CT	CT						AG	
MU_BND_089								CT			
BND_188											
MU_BND_019			CT	CT				CT			
BND_222											
MU_BND_053											
PRUN_6								TC			
MU_BND_068	GA										
MU_BND_010			CT	CT				CT			
MU_BND_088											
PRUN_4								CT			
PRUN_2											
MU_BND_085											
MU_BND_143			CT								
MU_BND_055		C									
MU_BND_080											
MU_BND_086											
MU_BND_090											
MU_BND_044											

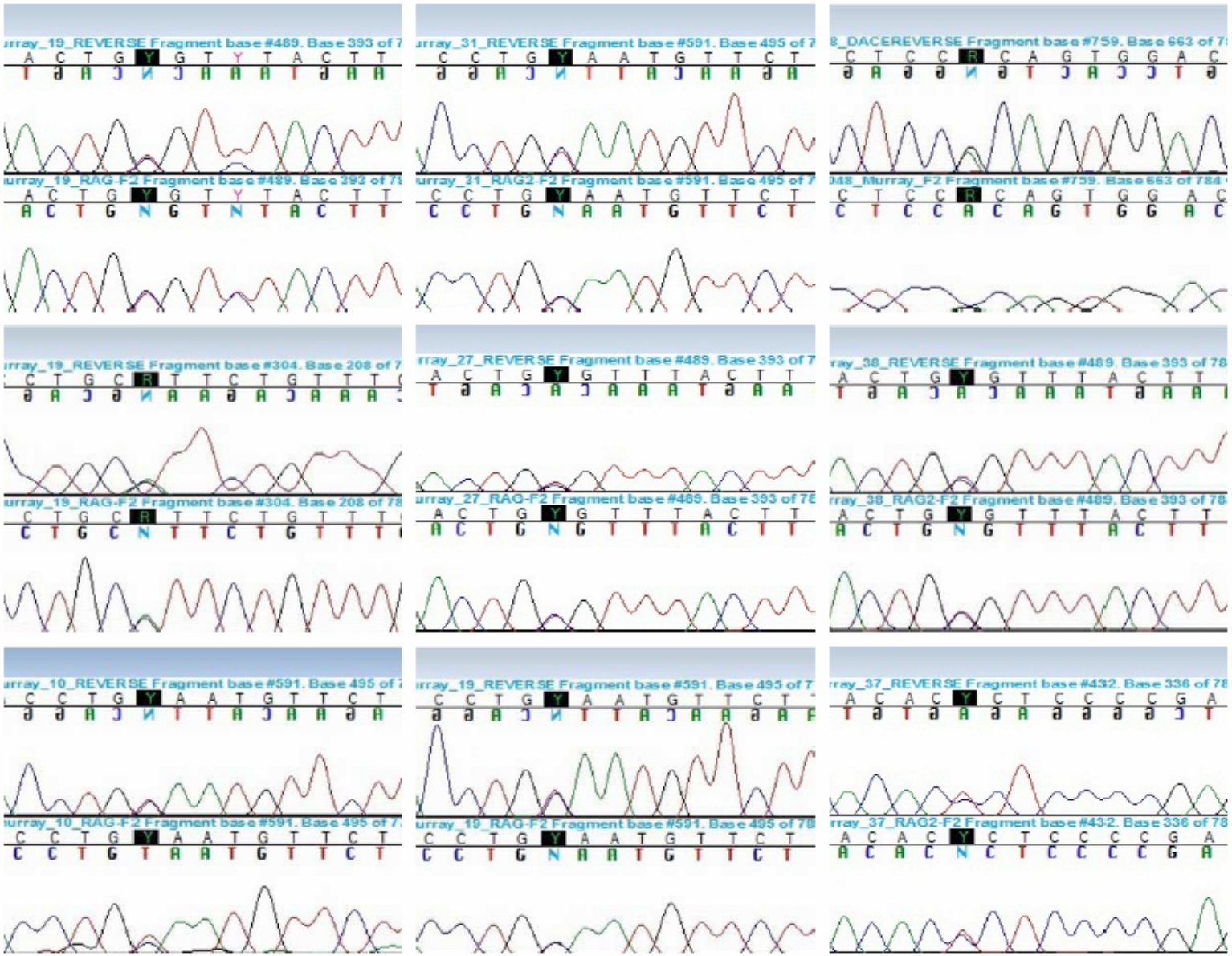
FishCode	441	466	489	492	534	540	556	591	740	759	859
BND_209											
MU_BND_140											
MU_BND_082											
MU_BND_075											
MU_BND_144	AG										
MU_BND_049											
MU_BND_141								CT			
MU_BND_070											
MU_BND_065		C									
MU_BND_073		GC									
MU_BND_071		C									
MU_BND_023											
MU_BND_028											
MU_BND_033											
MU_BND_032											
MU_BND_130											
MU_BND_031								CT			
PRUN_5								C			
PRUN_9								C			
MU_BND_087								TC			
BND_258											
MU_BND_003											
MU_BND_074											
PRUN_3								CT			
MU_BND_011											
BFORK_3									C		
BND_202											

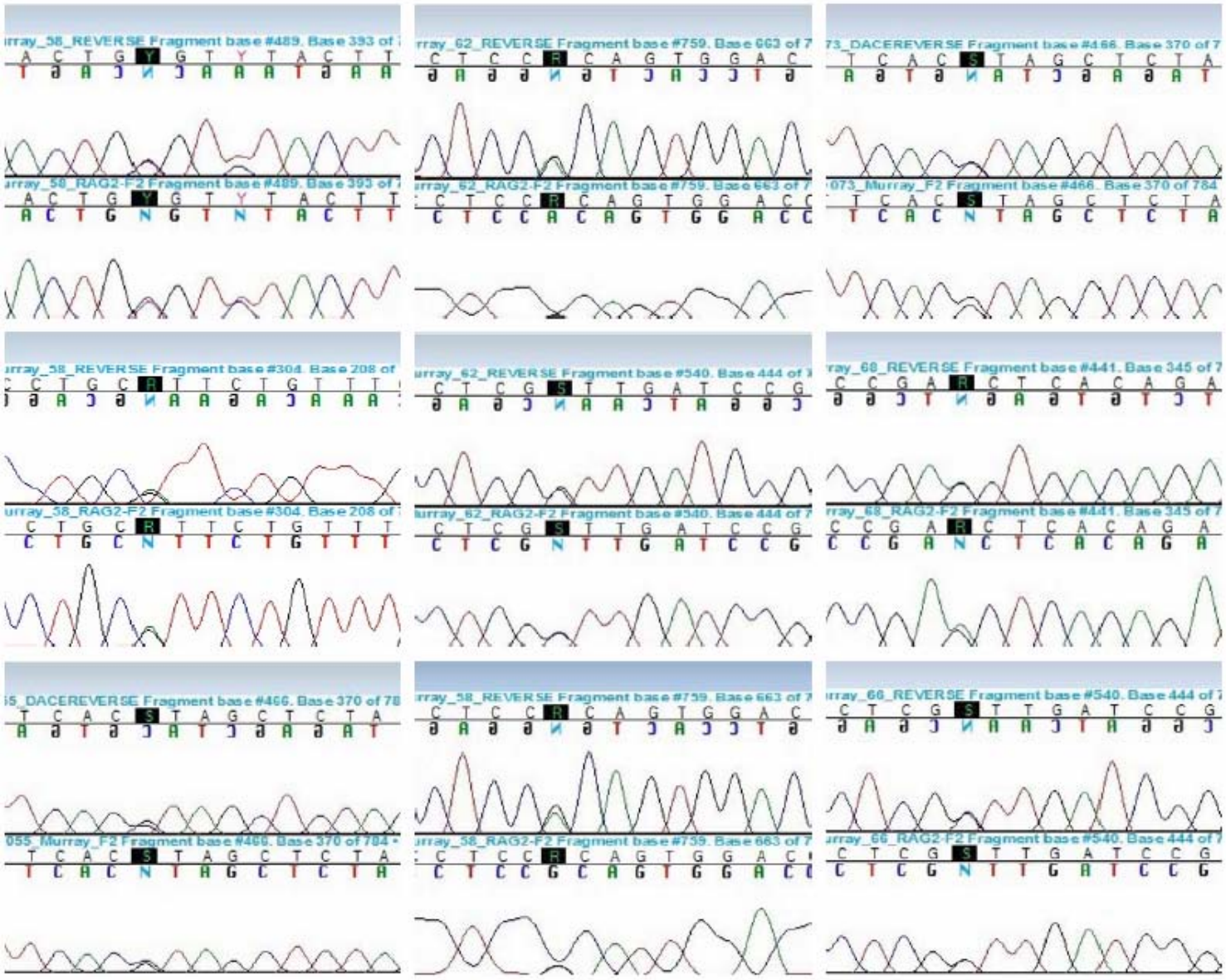
FishCode	441	466	489	492	534	540	556	591	740	759	859
MU_BND_079											
MU_BND_184											
SWCRK_9			C	T						G	
SWCRK_7			C	T						G	
SWCRK_19			C	T						G	
SWCRK_16			C	T						G	
SWCRK_12			C	T						G	
MU_BND_037			C	T						G	
SWCRK_17			C	T						G	
SWCRK_10			C	T						G	
SWCRK_8			C	T						G	
SWCRK_20			C	T						G	
MU_BND_057			C	T						G	
SWCRK_6			C	T						G	
MU_BND_174			C	T							
SWCRK_13			C	T						G	
MU_BND_066			C	T		CG				G	
BFORK_1			CT	CT						GA	
BND_040			C	T						G	
MU_BND_091			C	T						G	
MU_BND_030			C	T						G	
MU_BND_062			C	T		G				G	
MU_BND_038			CT	T						G	
MU_BND_048			C	T						GA	
MU_BND_175			CT	T						G	
MU_BND_067			C	T						G	
MU_BND_083			C	T		CG				G	

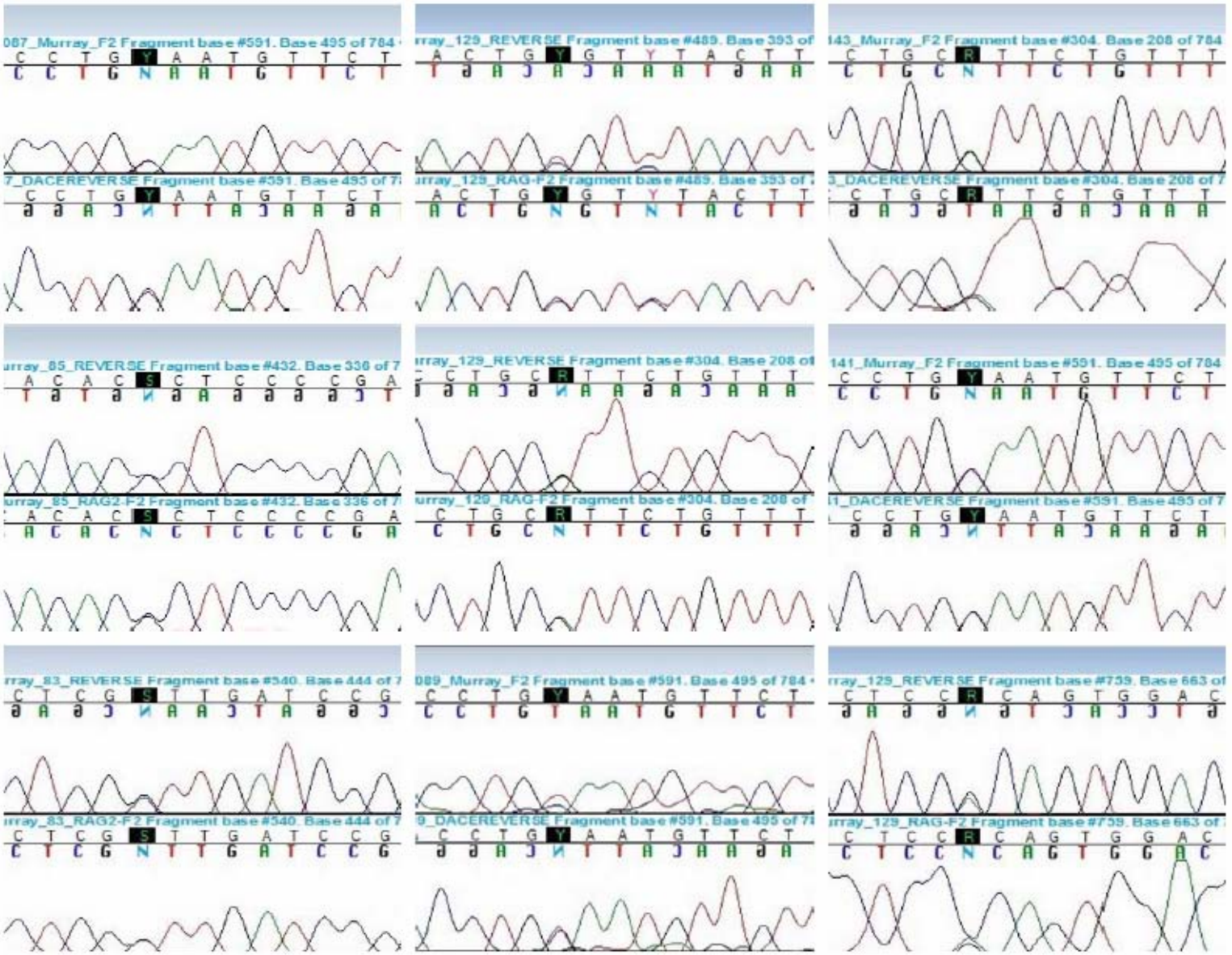
FishCode	441	466	489	492	534	540	556	591	740	759	859
MU_BND_039			C	T						G	
MU_BND_009			C	T						AG	
MU_BND_027			CT	T						G	
MU_BND_131			C	T						G	
SWCRK_14			C	T						G	
MU_BND_185											
MU_BND_046			C	T						G	
SWCRK_11			C	T						G	
MU_BND_026			C	T						G	
SWCRK_4	CG		CT	T						G	
SWCRK_15			C	T						G	
SWCRK_18			C	T						G	
MU_BND_047			C	T						G	
PRUN_8					A		A			G	

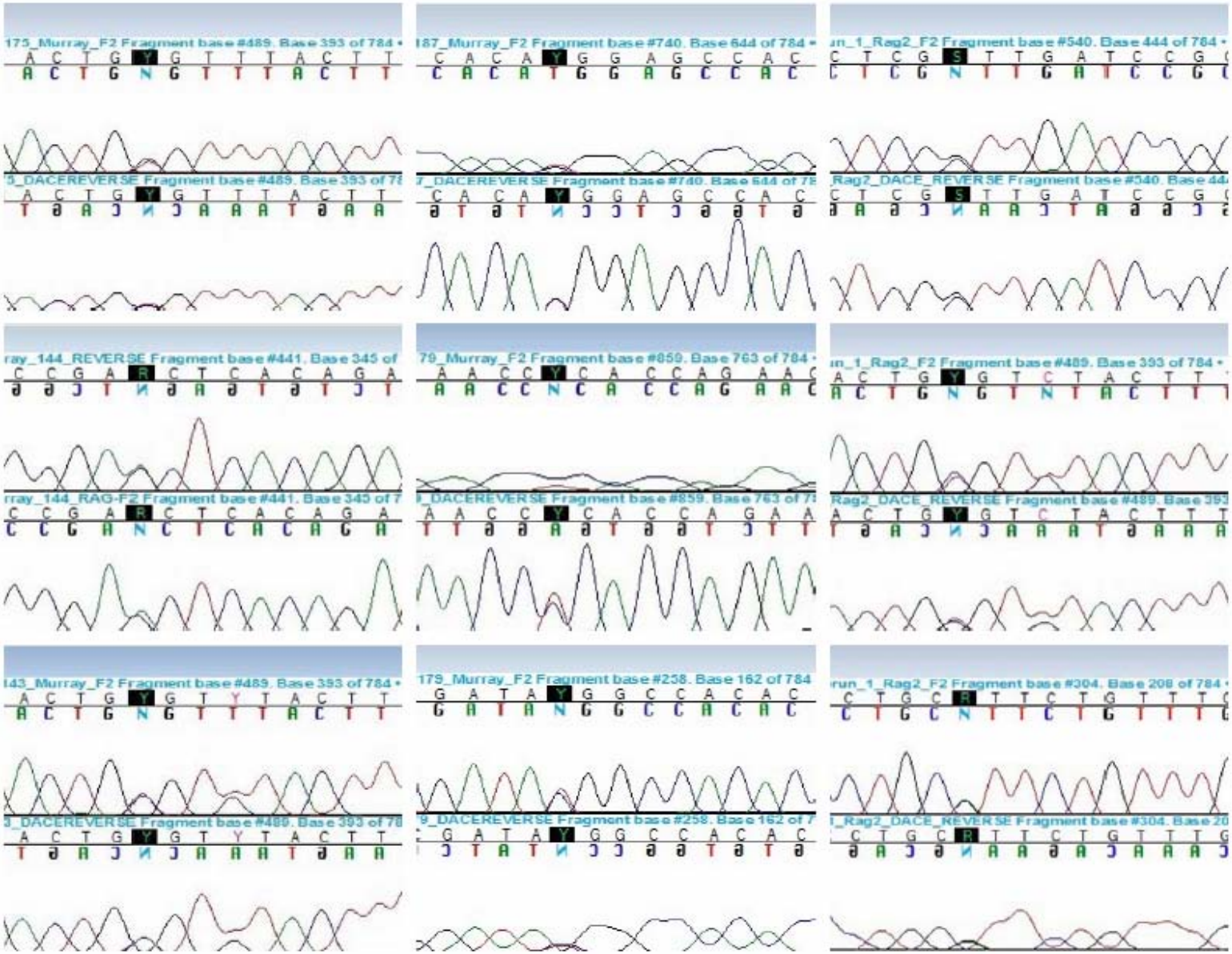
Appendix F: Images of all heterogeneity changes within the sequencing

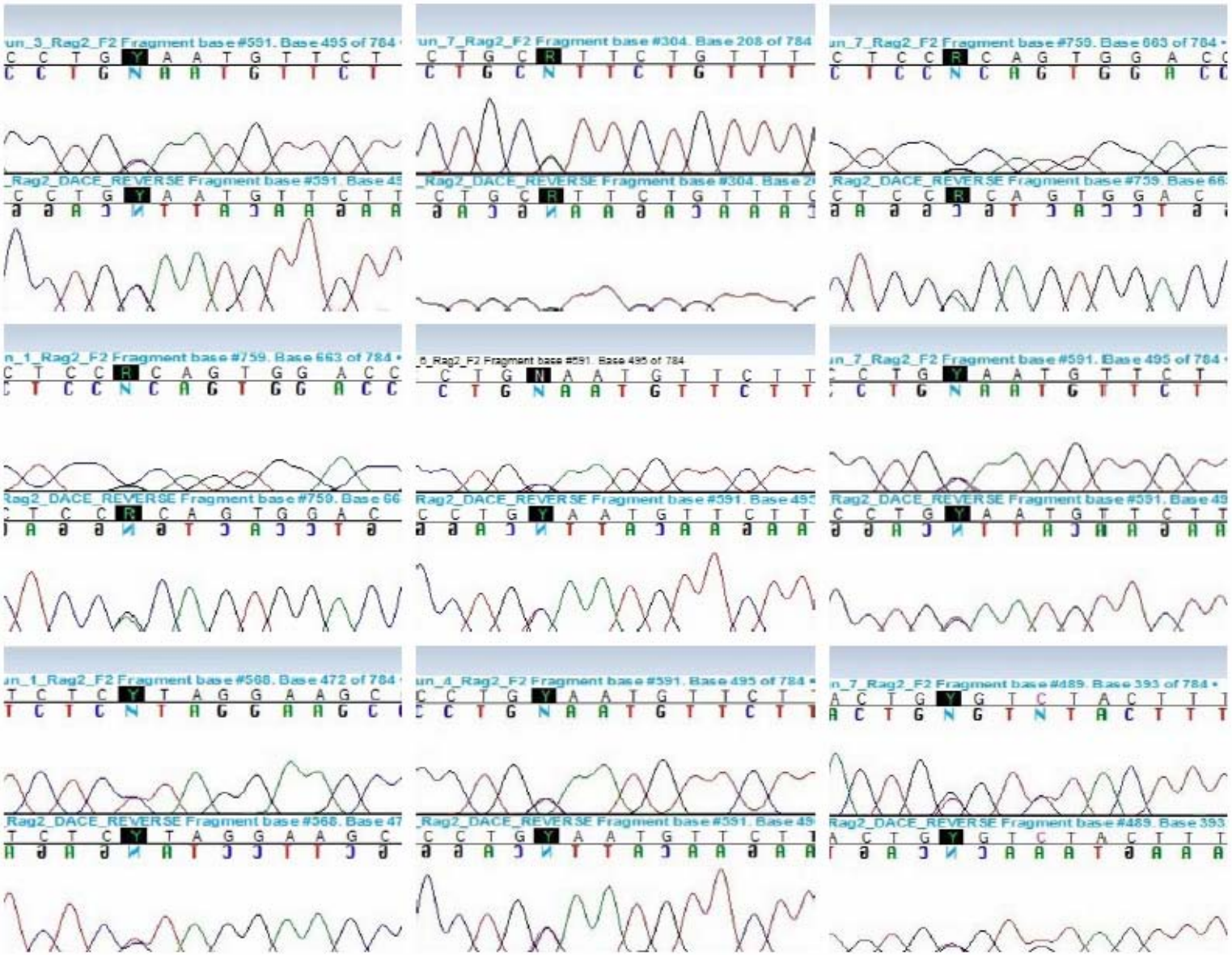


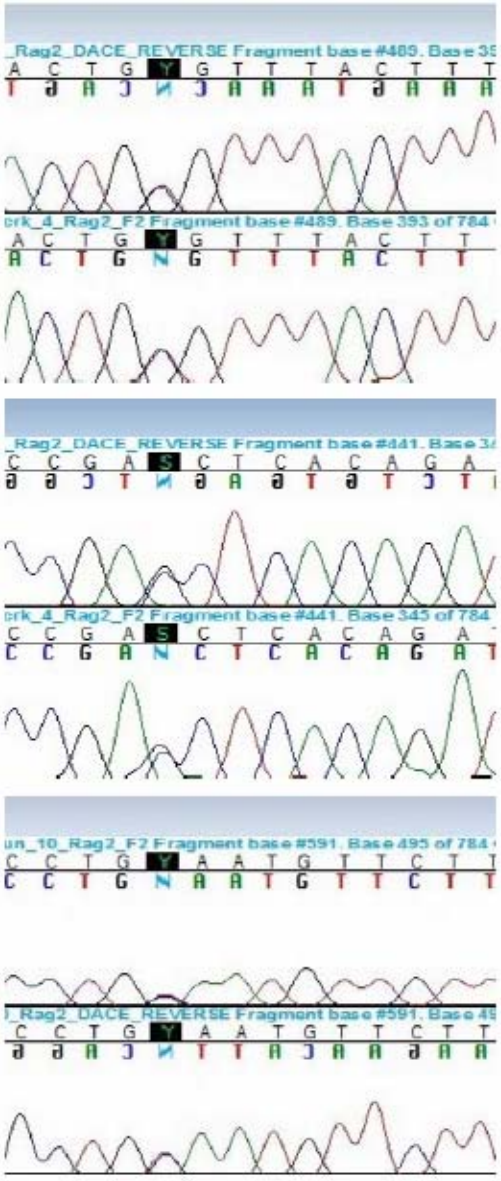











_Rag2_DACE_REVERSE Fragment base #489, Base 39
A C T G Y G T T T A C T T T
crk_4_Rag2_F2 Fragment base #489, Base 393 of 784
A C T G Y G T T T A C T T
_Rag2_DACE_REVERSE Fragment base #441, Base 34
C C G A S C T C A C A G A
crk_4_Rag2_F2 Fragment base #441, Base 345 of 784
C C G A S C T C A C A G A
un_10_Rag2_F2 Fragment base #591, Base 495 of 784
C C T G Y A A T G T T C T T
I_Rag2_DACE_REVERSE Fragment base #591, Base 49
C C T G Y A A T G T T C T T

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WORK EXPERIENCE:

Center for Bioengineering and Biomanufacturing Commercialization Marshall University
Huntington, WV

1/2009-Present

Salary: $10.00 per hour
Hours per week: 20

Lab Technician

Accomplishments:
*Supervised high school students during summer out reaches.
*Worked along side mentor to determine why amplification of PCR product was poor.
*Solved problem of poor amplification with use of on-line software resources. This solution pushed the sample data from 44 fish to more than 130 fish samples that DNA was purified and sequenced for the mitochondria DNA section of project.
*Researched and incorporated the RAG2 genomic gene into the Blacknose dace complex, adding 130 genomic samples to the project.
*Have present research at local conferences: West Virginia Academy of Science (oral), STaR Symposium (poster).
*Have presented research at nation conference: Evolution 2010 (poster), 2011 Northeastern Natural History Conference (oral).

Duties/Requires skills:
*DNA purification techniques
*Optimizing PCR amplification techniques
*Able to analyze DNA sequencing using Sequencher program
*Prepared solutions used in the laboratory
*Good organization of research space and data and up to date laboratory notebook.
*Use of Microsoft Office programs for keeping data and writing reports.
* Knowledge of how to gather scientific literature about related topics.
*Able to work independently on a problem or work together with other personnel within the lab.
*Trips to sites to collect new fish specimen using electrofishing and seining techniques. Required recording location collected and sample recognition labels. (Contact Supervisor: Yes, Supervisor's Name: Elizabeth Murray, Supervisor's Phone: 304 617-6198)

Marshall University SURE Program
Huntington, WV US

5/2007 - 8/2007

Salary: $10.00 per hour
Hours per week: 20

Lab Technician

Forward progress was made on the Project Dace. Weekly meetings with progress report of project progress. DNA purification, optimization of PCR product, agarose gel electrophoresis, analysis of fish sequences using Invetrogen contigexpress program

Marshall University
Huntington, WV US

1/2007 - 5/2007
Salary: $117.00 per month
Hours per week: 10

Lab Technician

Work focused on the purification of DNA of the Blacknose Dace complex using tissue samples. Optimized PCR samples for sequencing and analyze sequencing. Uses gel doc to take images of gels ran on agarose electrophoresis. (Contact Supervisor: Yes, Supervisor's Name: Elizabeth Murray, Supervisor's Phone: 304 617-6198)

EDUCATION

Marshall University
Huntington, WV
Master's Degree - 5/2011
Major: Geobiophysical Modeling
Relevant Coursework:

Core classes were focused on using satellite imaging to make informative maps of area of study. Programs learned were: Idrisi and ERMapper. A third program, ArcMap, was used to catalog and display visually biological information within maps using GPS coordinates and Access Datatables. Studies also included class work focusing on identification of insects and fish species, and knowledge of how land and water development projects affect fish and insects in an area. Conservation courses were also taken and the basic knowledge of wildlife laws and regulations were learned. Field work included learning how to collection samples by means such as electro fishing and seining.

Marshall University
Huntington, WV
Bachelor's Degree - 5/2009
132 Semester Hours
Major: Biotechnology
Minor: Japanese
GPA: 3.0 out of 4.0
Relevant Coursework:

Classes were focused on how to work in a laboratory setting. Early classes focused on how to use and calibrate equipment within the lab. Higher level course focused on the experimental process and how to translate results for future experiments or development of products.

PROFESSIONAL PUBLICATIONS

*Marshall Sigma Xi Research Day: Mitochondrial DNA Phylogeography of Rhinichthys Species in West Virginia
*West Virginia Academy of Science: Mitochondrial DNA Phylogeography of Rhinichthys Species in West Virginia
*Evolution 2010: Mitochondrial DNA Phylogeography of Rhinichthys Species in West Virginia
*STaR Symposium: Interspecies Dating? A Genetic Analysis of the Blacknose Dace Species Complex
*Master's Thesis: PHYLOGENETIC ANALYSIS OF BLACKNOSE DACE (RHINICHTHYS) IN WEST VIRGINIA STREAMS

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