

**Phylogeographic structure of the Atlantic pupfish, *Cyprinodon variegatus* (Cyprinodontidae), along the eastern coast of North America:**

Evidence from mitochondrial nucleotide sequences

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It would be disingenuous to suggest that this research is mine entirely. It is the product of the invaluable contributions of various capacities from many individuals, all of which are invaluable.

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## Table of Contents

Statement of Use and Copyright.....	ii
Acknowledgments.....	iii
Table of Contents.....	iv
List of Tables.....	v
List of Figures.....	vi
Abstract.....	1
Introduction.....	2
A. The Mitochondrial Genome.....	4
B. Hybrid Zones and Zones of Secondary Intergradation.....	8
C. Habitat and Life History.....	9
D. Additional Molecular Work.....	10
E. Geological History.....	11
Materials and Methods.....	12
A. Sampling.....	12
B. DNA Extraction.....	12
C. Polymerase Chain Reaction.....	16
D. Sequencing.....	16
E. Data Analysis.....	17
Results.....	21
Discussion.....	31
References.....	38
Appendix I. Haplotype sequences for D-loop.....	42
Appendix II. Sequences from the four haplotypes found in cytochrome- <i>b</i> .....	52
Vita.....	54

## List of Tables

I. List of populations samples.....	13
II. Models for analyses.....	19
III. Haplotype distribution.....	23
IV. Results of likelihood ratio test.....	29
V. Appendix I. Haplotype sequences for D-loop.....	42
VI. Appendix II. Sequences from the four haplotypes found in cytochrome- <i>b</i> .....	52

## List of Figures

1. Distribution of <i>Cyprinodon variegatus</i> .....	3
2. Transitions and transversions.....	6
3. Map of sampled populations.....	14
4. Higher resolution of populations samples from mid-Atlantic region.....	15
5. Example of maximum likelihood principle.....	20
6. Mitochondrial genome map with primer.....	22
7. Maximum parsimony topology.....	25
8. Distance topology.....	26
9. Parsimony/Distance topology with bootstrap values.....	27
10. Maximum likelihood topology.....	28
11. Parsimony/Distance topology with bootstrap values of cytochrome- <i>b</i> data.....	30

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**Abstract**

*Cyprinodon variegatus* is a pupfish that inhabits the Atlantic coast of North America, nearly continuously, from Massachusetts to Belize. This research attempts to resolve the phylogeography of *C. variegatus* by investigating the genetic sequence structure of the mitochondrial control region (D-loop, non-coding origin of replication) and cytochrome-*b* gene for evidence of northern and southern subspecies within the Atlantic Coast of the eastern United States. Additionally, it will be may be possible to determine if secondary hybrid zones developed as a result of the last retreat of ice from North America during the Pleistocene, about 17,000 years ago. A definitive monophyletic northern clade was found using parsimony, distance, and maximum likelihood phylogenetic methods to analyze the control region data. The cytochrome-*b* sequence data supported this monophyletic northern clade, although their utility for this analysis is marginal. Little evidence was found for the existence of a hybrid zone. More thorough sampling will be needed to make more accurate determinations regarding the existence of such a zone.

## Introduction

Isolation and subsequent dispersal are founding events of new populations that result in genetic drift and migration. Along with selection, genetic drift and migration produce variation between and within populations. This study examines the phylogeographic effects of one or more such events on the Atlantic coastal populations of *Cyprinodon variegatus*, sometimes called the sheepshead “minnow.”

Pupfishes are teleosts of the order Cyprinodontiformes, which also includes the killifishes, topminnows and mosquitofishes. The family Cyprinodontidae contains nine genera, with the genus *Cyprinodon* (pupfish) occurring only in North America; related genera are found in South America and around the Mediterranean (Parker and Kornfield 1995). Pupfishes thrive in estuaries and other habitats where salinity, temperature, and oxygenation levels can change rapidly (Moyle and Cech 1996; Jenkins and Burkhead 1994). These stressful conditions tend to reduce the number of species that might otherwise occupy the same habitat. Pupfish have even been observed burrowing into the mud to escape cold temperatures (Bennett and Beitinger 1997).

Although *C. variegatus* is a seemingly well-known species, its genetic architecture, at regional levels, is not well resolved. As part of his description of *C. v. artifrons*, from the Yucatan, Hubbs (1936) made mere mention of slight morphological variation between Atlantic coastal populations (involving slight differences in relative lateral body thickness) and used the name *C. v. ovinus* (Mitchell) for northern populations. Wildekamp (1995), in a synoptic review of *C. variegatus*, listed six nominal, and mostly poorly defined subspecies: *C. v. ovinus* ranges from Massachusetts to North Carolina, whereas *C. v. variegatus* ranges southward around the Florida peninsula and across the coast of the Gulf of Mexico, reaching the mouth of the Rio Tuxpan (24° N Lat) in the state of Veracruz. The morphologically distinct populations of the Yucatan peninsula are treated as another subspecies, *C. v. artifrons*. *C. v. hubbsi*, *C. v. riverendi*, and *C. v. baconi* are names used for populations from some interior lakes of Florida, the Caribbean and the Bahamas, respectively. The entire range of the species is shown in Figure 1. Presently, the distinctiveness and monophyly of most of these regional taxa is not clear.

Whether certain populations of *Cyprinodon variegatus* deserve subspecies or species status is under debate. For example, *C. v. hubbsi* differs in morphology and habitat preference

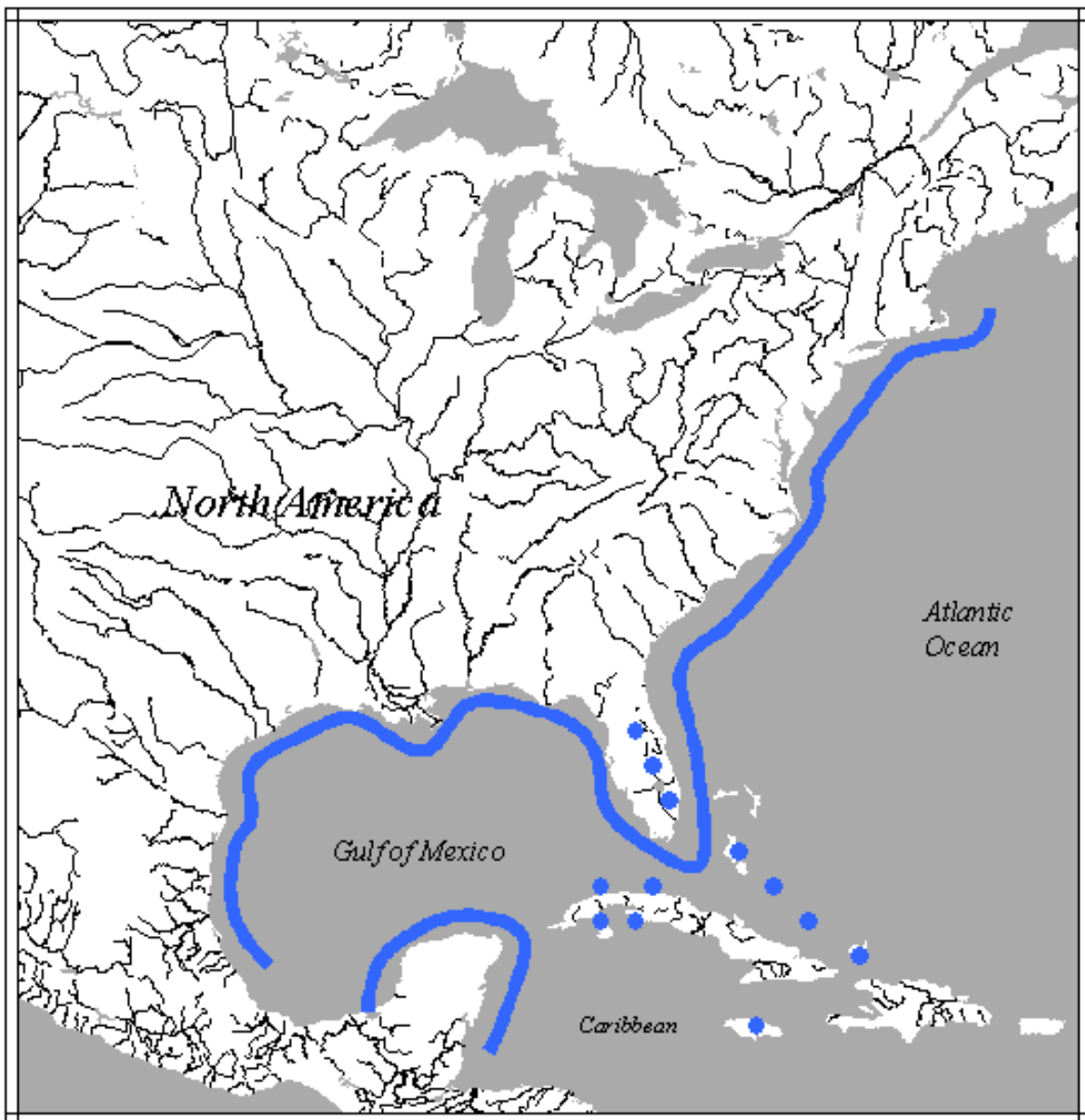


Figure 1. Map with blue areas indicating the distribution of naturally occurring populations of *Cyprinodon variegatus*. Populations are not limited to saltwater habitats and are frequently found relatively far inland.

from what is typical of congeners (Guillory and Johnson 1986). This prompted investigations concerning its species status. It has since been shown to be polyphyletic in origin, with ancestors from both the Gulf and Atlantic sides of the peninsula (Duggins et. al. 1983). Inconsistencies also exist in the literature as to whether the populations of the Florida Keys are *C. v. riverendi* or *C. v. variegatus*.

This study of *Cyprinodon variegatus* attempts to characterize regional genetic structure between *C. v. variegatus* and *C. v. ovinus* (the U.S. Atlantic coastal subspecies). Previous work showed that, despite the extremes in environmental conditions the species is capable of withstanding, *Cyprinodon variegatus* does not display an above-average amount of allelic heterozygosity (Darling 1976). However, levels of heterozygosity in the northern populations are reduced when compared to southern populations (Darling 1976). Darling also found that the average heterozygosity per individual in a population is lower in the north, with population density being strongly correlated with average homozygosity and inversely related to species diversity. Elder and Turner (1994) attempted to use satellite DNA to further investigate the regional genetic structure of *C. variegatus* but found the results uninformative on the interpopulation level.

The objective of this study was to improve resolution of the phylogeography of the subspecies *C. v. ovinus* and *C. v. variegatus* and search for evidence of a zone of secondary intergradation, if any exists. This question was explored using sequence analysis from the control region (D-loop) and cytochrome-*b* (*cyt-b*) gene of the mitochondrial genome. A wealth of literature examines the utility of the mitochondrial genome for such analyses (Brown et. al. 1979; Wilson et al. 1985; Avise 1986; Moritz et al. 1987; Saccone et al. 1987). Specifically, the *cyt-b* gene of mtDNA was used to determine the phylogeography (evolutionary and geographical history) and molecular systematics of *Peromyscus aztecus* subspecies (Sullivan et al. 1997). Death Valley pupfish relationships were most adequately resolved, to date, using mtDNA (Echelle and Dowling 1992; Duvernell and Turner 1998).

### *The Mitochondrial Genome*

Mitochondria contain high copy numbers of small circular molecules of double stranded DNA that is homoplasmic and not disrupted by introns or repetitive DNA. Mitochondrial DNA (mtDNA) length is strongly conserved in the animal kingdom, relative to other organelle

genomes. It ranges from 15.2 - 19.8 kbp in fishes with most of the smaller sizes likely to be underestimated from techniques not detecting restriction fragments smaller than 500 base pairs (bp) (Billington and Hebert 1991). It is clear that the size of fish mtDNA and gene order is highly conserved (Moritz et al. 1987). However, mitochondrial genomes of closely related species often vary in size, suggesting deletion-insertion counterbalancing (Billington and Hebert 1991).

The genes contained in the mitochondrial genome are also relatively conserved. The genes encoding for 13 proteins, 22tRNAs and 2 rRNAs (Awise 1986) appear in mtDNA genomes of all multicellular animals tested and some protozoans (Wilson et al. 1985). Some of these genes encode enzymes involved with metabolism or other equally important polypeptides that would seem to be constrained by structural requirements, the major exception is the origin of replication (displacement or conserved region, D-loop), which is non-coding. The D-loop contains a central conserved region and flanking regions that vary in sequence and length. These variable regions of the D-loop are very informative for interspecific and intraspecific studies (Lee et al. 1995).

MtDNA, as with most organelle DNA, is almost entirely maternally inherited by the offspring. Chloroplast genomes are maternally inherited in almost all angiosperms (Liu and Musial 2001).

“Nearly every species produces a substantial proportion of uniparental zygotes. Exceptions are found in the yeasts *Saccharomyces. cerevisiae* and *Schizosaccharomyces pombe* in which some crosses produce less than 10% biparental zygotes [mostly maternal inheritance]. Only in some ascomycete fungi do the progeny of all zygotes receive organelle genes from both parents” (Birky Jr. 1995).

At fertilization, the sperm contributes nuclear DNA, while the egg contributes nuclear DNA as well as organelle-containing cytoplasm. In species where leakage of the paternal cytoplasm is possible, it is estimated that less than 1 in 1000 mitochondria possess paternal DNA (Wilson et al. 1985). Maternal inheritance reduces the effective population size to 1/4 its number by nuclear counts (2 copies per mate pair = nuclear; 1 copy per mate pair = mitochondrial).

The elimination of crossing-over and recombination (with the possible exception of the D-loop) greatly simplifies systematic analyses. The mitochondrial genome evolves by mutation

alone. Single-base substitution mutations are either transitions or transversions. A transition is the substitution of a base by another base of similar structure, a purine for a purine or pyrimidine for a pyrimidine. In contrast, a transversion is the substitution of a base by a structurally dissimilar base, a purine for a pyrimidine or visa versa (see Figure 2). Transitions occur with greater frequency than do transversions and display saturation sooner than transversions. Saturation refers to the case in which mutations occur with such frequency at a single locus that signal is lost (a single base can mutate from one base to another several times, but only one mutation is read) and is no longer phylogenetically informative (Fitch 1967; Brown et al. 1982; Aquadro and Greenberg 1983; Li et al. 1984; Irwin et al. 1991; Meyer 1994; Simon et al. 1994; Kocher et al. 1995).

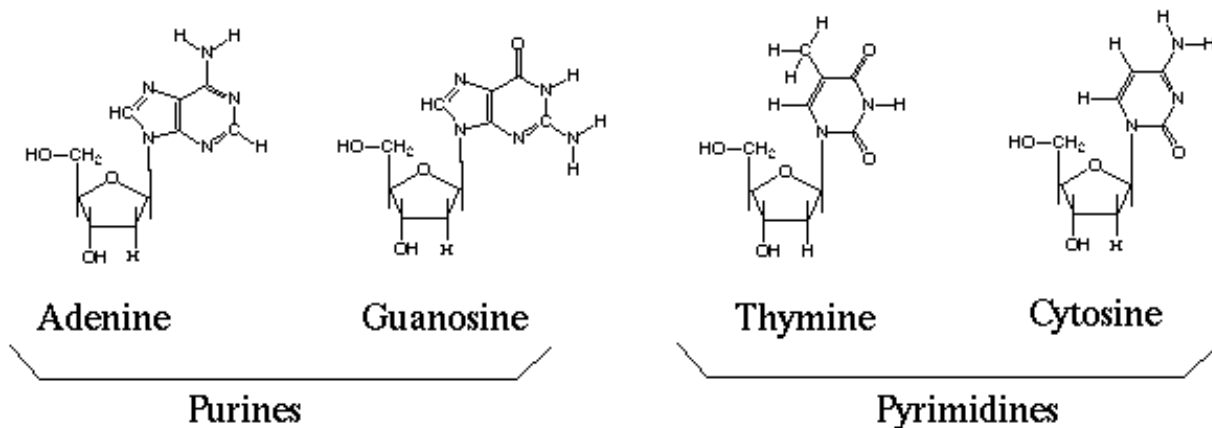


Figure 2. Transition and transversions. The substitution of a purine for a purine or a pyrimidine for a pyrimidine is a transition. The substitution of a pyrimidine for a purine or visa versa is a transversion.

Strict maternal inheritance, restrictions to mutational changes, and structural constraints suggest that mtDNA should evolve slower than its nuclear counterpart. Rather, its rapid rate of transcription, inefficient editing and apparent relaxation of structural constraints allow mutations to occur, but largely as length variation (Moritz et al. 1987) and transition mutations (Brown et al. 1979, Wilson et al. 1985). Brown et al. (1979) estimated mtDNA to evolve ten times faster than nuclear DNA. Today, it is known that different regions have different rates of evolution (Brown et al. 1979). The overall rate of evolution for the primate mitochondrial genome has been estimated at about 2% per million years (My) (Brown et al. 1979). This estimation, however, seems to be most constant among homeotherms (Brown 1983; Sheilds and Wilson 1987) and seems to decrease in other vertebrates (Billington and Hebert 1991). The subject is still under debate (Klicka and Zinc 1997; Arbogast and Slowinski 1998), and it is clear that caution should be followed when applying such molecular clock constraints to analyses.

MtDNA analysis does have limitations, however. It is important to keep in mind that lineages derived from these analyses track only matriarchal phylogenies. The small effective population sizes are more susceptible to the effects of drift, migration, selection, and bottlenecks. A single gravid female can colonize a population, greatly reducing variability with respect to the ancestral population (founder effect) and obscuring its ancestry (particularly if the female was a rare haplotype). This would confound estimations of genetic distances. Fortunately, the rapid rate of evolution helps combat these problems and thorough sampling of populations should provide accurate assessments of ancestry.

This study of the subspecies relationships of *Cyprinodon variegatus* will rely mainly on sequence analysis of the mitochondrial D-loop (non-coding origin of replication), supported by sequence analysis of the cytochrome-*b* coding region. The D-loop is the most rapidly evolving region of the mitochondrial genome (Hoelzel et al. 1991). It can be subdivided into three regions. Flanking regions contain more variation in sequence and length than does the central, conserved region (Saccone et al. 1987). The cytochrome-*b* coding region is approximately 300 base pairs in length and possesses a greater degree of functional constraint. It codes for the cytochrome-*b* protein, which is instrumental in the electron transport chain and therefore, due to structural constraint, evolves at a slower rate than the D-loop.

### *Hybrid Zones and Zones of Secondary Intergradation*

A hybrid zone (zone of intergradation) is an area where two genetically dissimilar populations interbreed. There are two types of hybrid zones: primary and secondary. Primary intergradation is the occurrence of interbreeding of two or more genetically distinct populations. Secondary intergradation occurs when a non-traversable barrier divides an original population and the subsequent populations diverge genetically as a result of drift or differing selection pressures. After a period of time, the barrier disappears (or can no longer restrict access of the populations to one another) and the populations regain contact and interbreed (Powers et al. 1986). The main assumption of these models is that the effects of random mutation outweigh other forces such as selection.

Contact zones can be found by directional changes in genetic variation such as allele frequency and sequence variation. These directional changes are referred to as clines. Determination of a contact zone as secondary depends on the knowledge of a historical barrier. The rate of change of the clines depends on the width of the contact zone, distance between populations, rate of migration, and the presence of opposing forces.

The killifishes of the *Fundulus* genus have been the subject of extensive phylogeographic analyses. Species of this genus inhabit similar ecological niches and have a similar range, often inclusive of the range of *Cyprinodon*. They are frequently collected at the same sites. A large body of evidence points to a zone of secondary intergradation (hybridization following the removal of an isolating barrier) within the distribution of the killifish, *Fundulus heteroclitus* (mummichog). It ranges along the Atlantic coast from Newfoundland to Florida, overlapping the range of *C. variegatus*. The observation of behavioral and morphological differences as well as high protein polymorphisms among populations encouraged phylogeographic analyses. The lack of sensitivity of allozyme testing methods made it difficult to assess existing variation as a relic of primary or secondary intergradation (Brown et al. 1986). These were determined to be secondary in origin by subsequent examination of gene frequencies (Ropson et al. 1990) and the more sensitive restriction fragment length polymorphisms (RFLP's) of mtDNA (Gonzalez-Villasenor 1990). The mechanisms behind the maintenance of this and smaller contact zones are discussed in Brown and Chapman (1991) and Smith et al. (1992). The occurrence and maintenance of hybrid zones of another *Fundulus* species are discussed in Duggins et al. (1995). With such genetic variation being found within species similar to *C. variegatus* in morphology,

behavior and distribution, it is reasonable to investigate the possibility of regional genetic variation in *Cyprinodon variegatus* as well.

If a hybrid zone were to be found, it would be expected to be secondary in nature. This is due to the history of the mid-Atlantic coastal region (Pleistocene) and the climate changes and water level fluctuations that occurred which might have acted as isolating barriers between southern populations and populations colonizing the north.

### *Habitat and Life History*

The main characteristics of the preferred habitat of *Cyprinodon variegatus* are shallow water with little movement, including unidirectional flow (rivers), or bi-directional wave action (coastal waters) as well as current action. With little water movement to wash away small particles, the substrate is generally sand or finer material. These pupfish are often found in, but certainly not limited to, areas with emergent vegetation, which can be used for cover (an effective method for avoiding capture by seine-wielding collectors). *C. variegatus* can inhabit both fresh and salt waters including extremely hypersaline areas with salinities measuring up to 142.2 ppt (Barton and Barton 1987) and are, therefore, very well adapted to estuarine environments where salinity fluctuation is dependent on rainfall. Pupfish species are often found inhabiting drainage ditches, backwaters, springs and swamps, which are subject to large fluctuations in water level, temperature, and oxygenation levels. Such conditions can lead to local extinctions during a drought. Generally, however, dangerous environmental fluctuations that result in high fish mortalities typically have little effect on resident pupfish populations (Hoese and Moore 1977).

Undoubtedly, this resilience and tendency to thrive under a wide range of conditions contributes to the popularity of *C. variegatus* among researchers.

It is generally thought that the tolerance of *C. variegatus* for extreme conditions either constrains its ability to compete or eliminates the need for competition outside of its own species. Intraspecific aggression between males has been observed in resource competition (which is supported by the sexual dimorphism with reference to size), but there is no evidence of interspecific aggression either with congeners or other species. Curiously, only *C. bifasciatus*, the only non-eurythermal pupfish (Darling 1976) found in the Cuatro Ciénegas complex of the

Rio Grande drainage in Mexico, thrives in a competitive environment with many other fish species.

Restrictions on dispersal and competitive abilities result in relatively isolated occurrences of the genus. *Cyprinodon diabolis*, for example, is found only in the small spring of Devil's Hole in the Death Valley complex of the southwestern United States. It has the smallest range of this genus and possibly also of all vertebrate species (Darling 1976). In contrast, *C. variegatus* has the largest range in the genus, extending from Massachusetts to the Yucatan peninsula where it is only disjunct in a small area west of the peninsula. It is also found in some interior lakes in Florida, as well as in Cuba and the Bahamas (Wildekamp 1995). Such strict allopatric occurrences and the environmental pressures that isolate these species allow for a degree of local selection that can result in outbreeding depression among hybrids (Echelle and Echelle 1994) or, in the opposite extreme, hybrid vigor (Echelle and Conner 1989, Childs et al. 1996).

#### *Analysis of Cyprinodon spp.*

Previous work with the genus *Cyprinodon* has indicated that mtDNA analysis is useful for addressing questions of phylogeography. In the interior of the Yucatan peninsula, a small flock (sister taxa, being monophyletic in origin) of pupfishes has been identified that has the closest common ancestor to the *C. v. artifrons* subspecies. This relationship was found using morphological and mtDNA data (Humphries and Miller 1981; Humphries 1984; Strecker et al. 1996). The Death Valley region of southeastern California and southwestern Nevada, with a complex of springs, marshes, and streams, has yielded a highly divergent (morphologically, physiologically, and behaviorally) assemblage of species within the *Cyprinodon* genus. Turner (1974) found little allozyme variation relative to this morphological variation in comparison with conspecifics of other vertebrate genera. Further research yielded little resolution aside from the possibility of two invasions of the area (Echelle and Dowling 1992, Echelle and Echelle 1993). Finally, sequence analysis of mitochondrial regions supported the hypothesis of two separate invasions of the region. The complexity was likely due to intermittent surface connections of the now isolated habitats (fluctuating water levels) followed by population bottlenecks causing differing degrees of divergence between populations (Duvernell and Turner 1998). It is

important that the systematic status of the *C. variegatus* nominal subspecies be resolved to give a standard for the status of the other 5 subspecies, which occur across much smaller ranges.

### *Geological History*

The Wisconsin glaciation was the fourth and final major “icing over” event of the Pleistocene epoch (Peliou 1991). It was also the largest in terms of area and volume. The Laurentide and the Cordilleran ice sheets, at the time of the Wisconsin glacial maximum (an estimated 18kbp) were approximately 15 million km<sup>2</sup> in combined area and as much as five kilometers deep at the tallest cap (over what is now the Hudson Bay). The sea level would have been as much as 120 meters lower during this time (Peliou 1991, Barendregt and Irving 1998).

The present shorelines of the Atlantic coast (and across the globe) were severely altered as a result of such glaciations and subsequent interglacial periods. Today, the northeastern coast of North America south of Newfoundland is riddled with islands and shallow banks on the continental shelf that were once a part of the coastal plain or larger islands. Bodies of water such as the Nantucket Sound, the Long Island Sound, the Delaware Bay and the Chesapeake Bay did not exist. The Susquehanna River dominated the largest scar on the Atlantic coast of the United States, the Chesapeake Bay. The Florida Keys were a part of the mainland during times of lower water levels (Baer 1998).

The range of *Cyprinodon variegatus* was, no doubt, greatly affected by the alternating advance and retreat of ice during and following the Pleistocene. The northernmost region of its present range was presumably either never colonized or repeatedly wiped out due to extreme climatic conditions mediated by the Laurentide ice sheet and fluctuating sea levels. In addition, regions as far south as New Jersey and the Delmarva Peninsula had tundra-like environments. Since *C. variegatus* does not presently inhabit such climates, it is assumed that these regions were not included in its historical distribution.

## Materials and Methods

### A. Sampling

The genetic material from *C. variegatus* was acquired through several different avenues. Many populations were sampled prior to the beginning of this research, and preserved tissue, as well as some previously extracted DNA, were supplied to the lab by collectors and researchers acknowledged below. Other samples were acquired to fill in the gaps of the collections, with respect to location (for example, there were no prior samples from the Chesapeake Bay). The dates, locations and collectors of each population are listed in Table I. Unfortunately, no samples were taken from the Virginia Beach area (southeast VA/ northeast NC) due to hurricanes.

The collection methods used were simple; employing seine nets, dip nets and minnow traps. Individuals were collected from areas of moderate salinity (estuarine areas), low to no flow (although no measurements were taken, it is expected that dissolved oxygen was very low), with the bottom consisting of fine sediment and organic debris. The collected specimens were preserved in 70% ethanol and placed in 90% ethanol in the lab. The populations and sites represented in this study include the mainland of Massachusetts, Nantucket, New York, New Jersey, Delaware, Maryland (five locations), North Carolina (three locations), Georgia, and Florida (three locations) (Figures 3 and 4). The outgroups used for this analysis were *C. n. amargosa*, *C. n. nevadensis*, and *C. n. mionectes*, subspecies of the *Cyprinodon nevadensis* from the Death Valley complex of California-Nevada (Duvernell and Turner 1998). Partial D-loop sequences for these organisms were available through NCBI (National Center for Biotechnology Information) Sequence Viewer.

### B. DNA Extraction

DNA was extracted according to the method of Walsh et al. (1991), which allows for the extraction of DNA for polymerase chain reaction (PCR), a technique used for amplifying specified regions of nucleic acid. For each individual specimen, 500  $\mu$ l of a 5% weight-to-volume mixture of Chelex 100 ion-exchange resin (Bio-Rad; Hercules, CA) and dH<sub>2</sub>O were placed in a microcentrifuge tube with 5 mg of muscle tissue from the caudal-peduncle region

**Table I. Locations sampled with collectors and dates.**

<b>Population</b>	<b>Location</b>	<b>Collector</b>	<b>Date</b>
1	Westport, MA	Bruce Stallsmith	9.95
2	Falmouth, MA	Bruce Stallsmith	7.96
3	Nantucket Is., MA	Bruce Stallsmith	
4	Hereford, NJ		
5	Bombay Hook, Delaware Bay		8.98
6	Honga River, MD (eastern shore)	Katherine Finne Tom Bunt	6.99
7	Horsehead Park, MD (eastern shore)	Katherine Finne	8.98
8	Magothy River, MD (western shore)	Katherine Finne Tom Bunt	6.99
9	Lusby, MD		2.92
10	Potomac River, MD (western shore)	Katherine Finne Tom Bunt	6.99
11	Oregon Inlet, NC		7.98
12	Laurel Pt., NC (Albemarle Sound)		6.98
13	Sage Pt., NC		
14	Sapelo Is., GA	John Elder Tom Laughlin	
15	Jacksonville, FL		10.88
16	Key West, FL		9.99
17	Big Sabine Pt., FL (Gulf of Mexico)		

Population numbers coincide with the numbers shown in Figures 2 and 3 and follow a north to south pattern along the coastline.

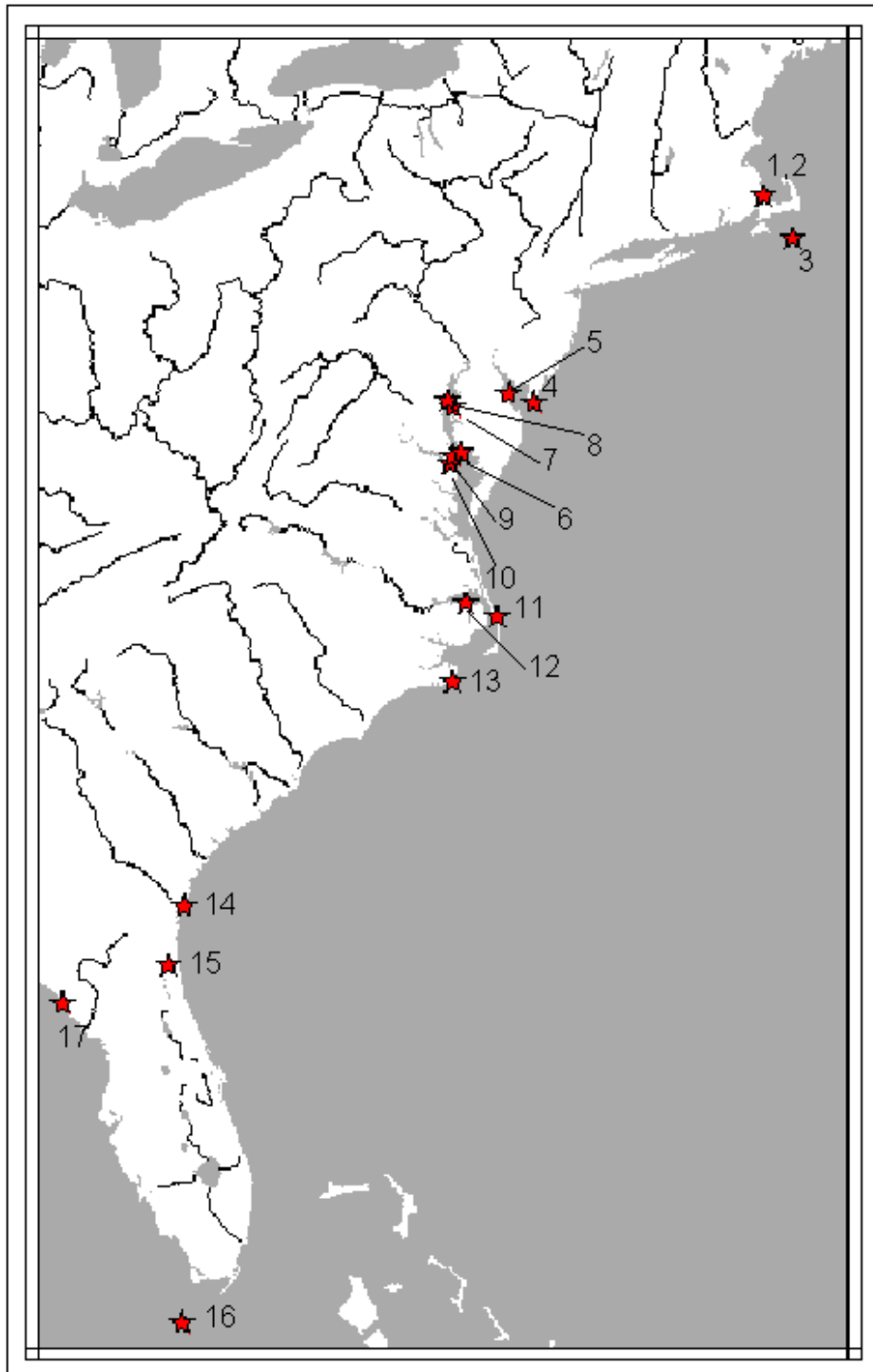


Figure 3. Locations of sampled populations. The sites include populations from the two northernmost nominal subspecies. The numbers for each population coincide with more detailed information given in Table 1.

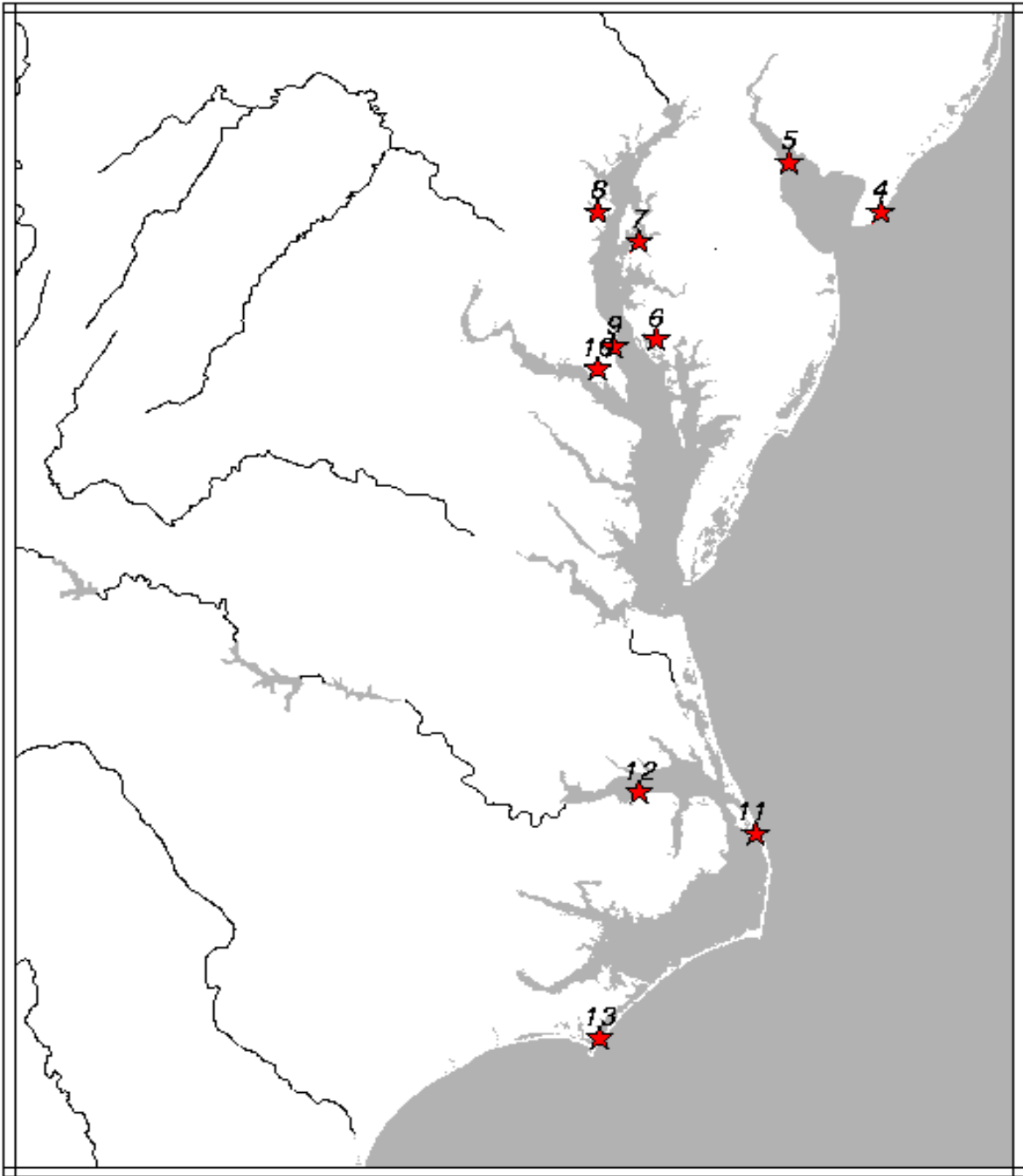


Figure 4. Higher resolution map of the populations sampled from the Chesapeake Bay and the Albemarle and Pamlico Sounds, the area thought most likely to contain a hybrid zone.

immediately anterior to the tail, or caudal fin. The tissue was ground into the mixture using Kontes\* Pellet Pestles\* (Fisher Scientific; Pittsburg, PA) and incubated for 15 min at 95°C. The samples were then centrifuged for 5 min at 14,000 rpm. The top 100 µl of the supernatant was drawn off and aliquots used directly for PCR.

### C. Polymerase Chain Reaction

PCR was used to amplify the D-loop and *cyt-b* regions prior to sequencing. The primers E and K were used to amplify a portion (~500 bp) of the D-loop region and L14841 and H15149 amplified the *cyt-b* region (~300 bp) (Kocher et al. 1989, Lee et al. 1995). Each reaction contained 2.5 µl of 10 X buffer (100mM Tris·Cl and 100 mM Mg Cl<sub>2</sub>), 2.5 µl of MgCl<sub>2</sub> (25 mM), 2.0 µl of dNTPs (360 µl dH<sub>2</sub>O and 10 µl each of 100mM dGTP, dATP, dTTP and dCTP), 2.5 µl of each primer (2 pmol/µl), 0.2 µl of *taq* polymerase (Promega; Madison, WI) (5units/µl), 10.3 µl of dH<sub>2</sub>O and 2.5 µl of template DNA (ranging from 0.1 to 0.4 µg/µl) for a total volume of 25 µl. The reactions were denatured at 92°C for 4 min. Then cycles of 95°C for 30 sec, 54°C / 50°C (D-loop / *cyt-b*) for 45 sec, and 72°C for 3.5 min were used, which denatured, annealed, and extended the fragments respectively. Following PCR, the entire 25 µl reaction was electrophoresed on a 1.0% agarose gel to ensure that the reactions were successful. The bands were then cut from the gels and the DNA cleaned (agarose, salts, and ddNTP's were removed leaving the template) using a QIAquick PCR purification kit (QIAGEN).

### D. Sequencing

Automated sequencing was performed using the dideoxy chain-termination method (discussed in Voet and Voet 1995). Reactions contained 4.0 µl of reaction mix (ddNTPs labeled with 4 different dyes for A, T, G, and C, and polymerase; Big Dye Terminator Sequencing Mix by ABI/Applied Biosystems), 1.0 µl of primer (2 pmol/µl), 5 ng of template for every 100 base pairs in the product, and dH<sub>2</sub>O to achieve a total volume of 10 µl.

The reaction mixes were then placed in the thermal cycler under the following conditions: denaturation at 95°C, annealing at 50°C, and extension at 60°C for 25 cycles. The products were precipitated using 75% isopropanol to remove unincorporated dye terminators, heated at 90°C for 3 min to evaporate any remaining alcohol, and resuspended in 12 µl of TSR (template suppression reagent, Perkin Elmer Applied Biosystems) and denatured before placed in the 310 ABI Prism sequencer (Perkin Elmer; Boston, MA).

Some sequences were obtained using  $^{33}\text{P}$  labeled dNTP's during manual sequencing (Sanger method) (Voet and Voet 1995) using a polyacrylamide gel. Prior to the final analysis, all sequences from both methods were double-checked for any anomalies that might occur (haplotype occurring with one method and not in the other), which would have a negative effect on the results.

#### E. Data Analysis

Sequences of all individuals were compared both within and between populations. Any unique sequence (varying by as little as a single base) was deemed a haplotype and given a label (Example: haplotype A). The individual haplotypes were used for the analyses rather than the complete set of sequences to eliminate redundancy.

The analysis of variation between and within pupfish populations employed the tree building capabilities of *PAUP* (version 4.0b3a, written by D. L. Swofford 2000) combining the results of maximum parsimony, distance parameters (Duvernell and Turner 1998), and maximum likelihood to determine phylogenetic topologies using the D-loop data. Due to the presence of less variation in the cytochrome-*b* region, only maximum parsimony and distance methods were used with these data.

The maximum parsimony methods used with the D-loop data were limited to heuristic searches due to the large number of taxa (26 ingroup) being analyzed, which precluded any exhaustive methods that would end with one topology. This criterion-based method (adhering to "Occam's Razor") finds a set number of phylogenies (set at 100) and computes a consensus tree that shows minimum evolution (Swofford et al. 1996). In instances where there is a small amount of variation within a given set of data, this can sometimes be an inadequate method (Felsenstein 1981). Also, in the instance of differing evolutionary rates within a data set, the results can be misleading. Since we examined within-species populations we would not expect the latter to be the case. Still, additional frameworks for analysis were employed for support.

Distance methods of phylogenetic inference use a purely quantitative framework as opposed to the criterion-based parsimony methods. The result of the analysis is a single topology with the absolute best score. The algorithm used for this research was HKY85 (Hasegawa et al. 1985). This model, like Felsenstein's (1984) model (F84) does not assume equal base frequencies and weights transitions ( $\text{T}\leftrightarrow\text{C}$ ,  $\text{A}\leftrightarrow\text{G}$ ) less than transversions ( $\text{A}$ ,  $\text{G}\leftrightarrow\text{T}$ ,  $\text{C}$ ). Table II shows all models considered in distance and maximum likelihood frameworks.

Transversions were weighted more heavily than transitions. This is due to their lower frequency and lack of saturation (a higher mutation rate for transitions results in multiple base pair changes in a short period of time, causing it to become “noisy” and therefore, uninformative, more rapidly than transversions).

Both parsimony and distance phylogenies were tested using a method of “pseudoresampling” called bootstrapping. The frequency of a lineage among the replicates (set for 500) is the given statistic (Felsenstein 1985).

To add further support to the findings of the maximum parsimony and distance methods, a maximum likelihood framework was used. The purpose of the likelihood analysis was to determine the most appropriate test model (Table II), via likelihood score (which can be converted into a likelihood ratio test), as well as the appropriate tree topology. Maximum likelihood resolves the probability of a specified tree, given the data and the model (Felsenstein 1988). This framework operates backwards from parsimony and distance methods, beginning with the terminal taxa and working inward to the ancestral nodes. In Figure 5, for example, this analysis would determine the probability of ancestor D having character state, *s*, given the character states of taxa A and B.

Using this method, a basic tree generated from various models can be tested for significance. The models were nested (the null hypothesis is a subset or special case of the alternative hypothesis) and were tested by Monte Carlo simulation (Felsenstein 1988) under the  $\chi^2$  distribution with *q* degrees of freedom, where *q* is the change in the number of free parameters between the null and alternative hypotheses. Each hypothesis was assigned a likelihood score (Sokal and Rohlf 1997), the negative of the natural log (-Ln) of the probability of the topology. The score of the next model (the alternative hypothesis, with additional parameters) was compared using  $\chi^2$  critical values with a 3% rejection level. The null hypothesis (the simpler model with fewer parameters) was

**Table II. Possible models for analysis**

<b>Model</b>	<b>Parameters</b>	<b>Degrees of Freedom</b>
JC	equal base freq., single substitution type	0
K2P	base frequencies are equal 2 substitution types (transition/transversion)	1
HKY85	unequal base frequencies 2 substitution types	4
GTR	unequal base frequencies 3 substitution types (2 transitions/transversions)	8
I	a proportion of sites estimated to be invariable equal rates assumed at variable sites	adds 1 to model
gamma	distribution of rates at variable sites	adds 1 to model
molecular clock	evolution occurs at a constant rate across lineage	number of taxa - 2

All models are nested (except the molecular clock parameter) and arranged from simplest to most parameter-laden: Jukes and Cantor (1969), Kimura 2-Parameter (Kimura 1980), Hasegawa, Kishino, and Yano (1985), General Time Reversible (Swofford et al. 1996).

accepted or rejected based on these results. This process continued until all models and parameters were tested to find the optimum framework for constructing the topology.

The models were tested in order from simplest to most parameter-laden. In the order of testing, these models were Jukes-Cantor (JC; Jukes and Cantor 1969), Kimura 2-parameter (Kimura 1980), HKY85 (Hasegawa et al. 1985) and the general time-reversible model (GTR; Swofford et al. 1996). After finding the appropriate model the additional parameters of  $I$  (estimation of proportion of invariable sites) and  $\Gamma$  (estimation of rates at variable sites) were tested as well as the presence of a molecular clock (consistent rate of evolution through entire lineage) (Sullivan et al 1997).

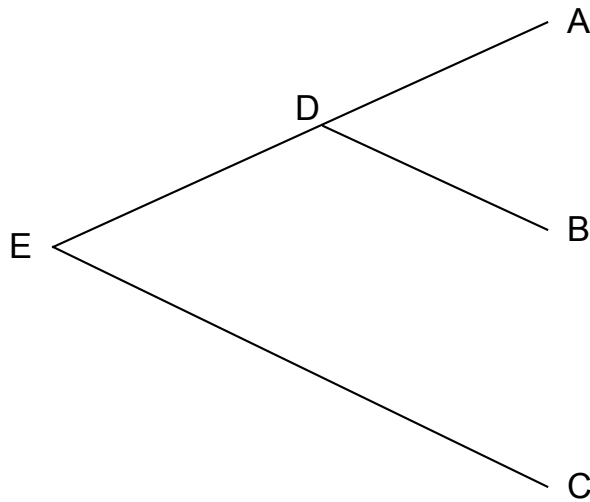


Figure 5. An example of the principle of maximum parsimony. Maximum likelihood begins at terminal taxa A, B and C and finds the probability of the ancestors, D and E, given the data and the model.

## Results

In order to resolve the phylogeography of the subspecies *C. v. ovinus* and *C. v. variegatus*, 121 direct DNA sequences from the D-loop region of the mitochondria were analyzed. These sequences yielded between 300 and 500 base pairs each, using both primers E = light chain, and K = heavy chain) (Figure 6). After editing and aligning, conserved (and therefore, uninformative) regions flanking the sequences were not analyzed further leaving a fragment of 224 base pairs per individual. This fragment, containing 12 variable regions, included a total of 26 unique haplotypes (unique sequences). The northern populations (north of the Chesapeake Bay) contained considerably fewer haplotypes. The number of haplotypes per population increased further south. Four haplotypes were found within the Big Sabine Point sample, a gulf coastal Floridian site. The haplotypes found at each site are listed in Table III.

Other interesting and informative components of the sequences are the base frequencies and types of substitutions that can be seen (transitions vs. transversions). Analysis of the sequences identified a transition / transversion ratio of 1.54 and base frequencies of 0.32349, 0.09409, 0.20450 and 0.37792 for A, C, G and T respectively, using primer E. These data were determining factors in selecting the HKY85 model (refer back to Table I) for building phylogenies.

The phylogeny generated using the maximum parsimony framework required heuristic methods (the high number of taxa precluded exhaustive methods) and contained many unresolved lineages. However an obvious northern clade was present, as illustrated



**Table III. Haplotype distribution.**

<b>Site</b>	<b>Population #</b>	<b>number collected per site</b>	<b>Haplotypes</b>
Horseneck Beach, Westport, MA (1)	1	8	A,B
Oyster Pond, Falmouth, MA (2)	2	2	A
Nantucket Is., MA (3)	3	6	A,B
Hereford, NJ (4)	4	5	A,B,C
Bombay Hook, Delaware Bay (5)	5	10	A
Where 335 and 336 meet, north of Honga R., MD (6)	6	10	D
Horsehead Park, East shore of Chesapeake Bay (7)	7	10	D
Backwater beside Manhattan Beach Marina on the Magothy R., MD (8)	8	4	D
Lusby, MD (9)	9	10	D,E,F
Breton Bay on the Potomac R., where rt.243 ends (10)	10	8	D,G,H
Oregon Inlet (South Point), NC (11)	11	9	I,J,K,L
Albemarle Sound, NC (12)	12	6	I
Sage Point (Southern Pamlico Sound) (13)	13	8	I,M,N,O,P
West side of Sapelo Is, Georgia (14)	14	10	Q,R
Jacksonville, FL (15)	15	9	Q,S,T,U,V
Key West, FL (16)	16	5	I
Big Sabine Point, FL (Gulf Coastal) (17)	17	4	W,X,Y,Z

Parentetical numbers correspond to the location numbers in Figures 3 and 4. Note that the haplotypes in populations 1-10 are not found in the “southern” populations, nor are the haplotypes in populations 11-17 found in the “northern” populations. The haplotype labels (A, B, C, etc.) were designated generally in a north-south progression.

in Figure 7, with haplotype K from the mouth of the Albemarle Sound of North Carolina (haplotype K) being the southernmost haplotype to be included.

Distance algorithms also supported the presence of a northern clade. Figure 8 shows the topology resulting from a neighbor joining method employing the HKY85 model. Since this is a strictly mathematically based method, the single tree with the best score, even if only by a small fraction, is the topology shown. Therefore, there are no consensus values and resolution is improved. The same haplotypes that make up the northern clade of the parsimony tree comprise the northern clade of the distance tree as well.

Bootstrapping (Felsenstein 1985; Hillis and Bull 1993; Felsenstein and Kishino 1993) decreased the resolution of the phylogenies, as expected. However, the northern clade persisted and haplotype K remained within the northern grouping, albeit with lower support than anticipated. Five hundred replicates (pseudosamples) were created from the data for each method (parsimony and distance) and the results are shown in Figure 9.

The final analysis performed on the D-loop data was maximum likelihood. The topology created by the parsimony method was used to estimate the various parameters ( $I$ ,  $\Gamma$ , transition/transversion ratio, etc.) and begin the process. Using the base frequencies determined earlier, and given the data and the model used to create the parsimony topology, PAUP estimated all other parameters and calculated a likelihood score ( $-\ln$  likelihood = 661.05794). The HKY85 model with both  $I$  and  $\Gamma$  parameters as well as a molecular clock were used to find the topology given in Figure 10. Table IV shows the models and parameters tested as well as likelihood scores and significance. The  $-\ln$  likelihood for the new topology was 681.74041.

Data from the cytochrome-*b* region provides a look from a different scale. Since the cytochrome-*b* gene is more slowly evolving than the D-loop, it showed less saturation than the D-loop. The initial sequence data showed much less variance between and among populations. For this reason fewer individuals were analyzed with the focus being on individuals from the extreme north and south and 2 locations in between (Jacksonville, FL; Sapelo Island, GA; Lusby, MD; and Westport, MA). A total of 28 sequences were generated, comprising 4 haplotypes (a, b, c, and d). Six variable sites were found within the 150 base pairs used for analysis. The small number of taxa allowed for simple exhaustive methods and high bootstrap values to support a northern and southern clade again using maximum parsimony and distance methods (Figure 11).

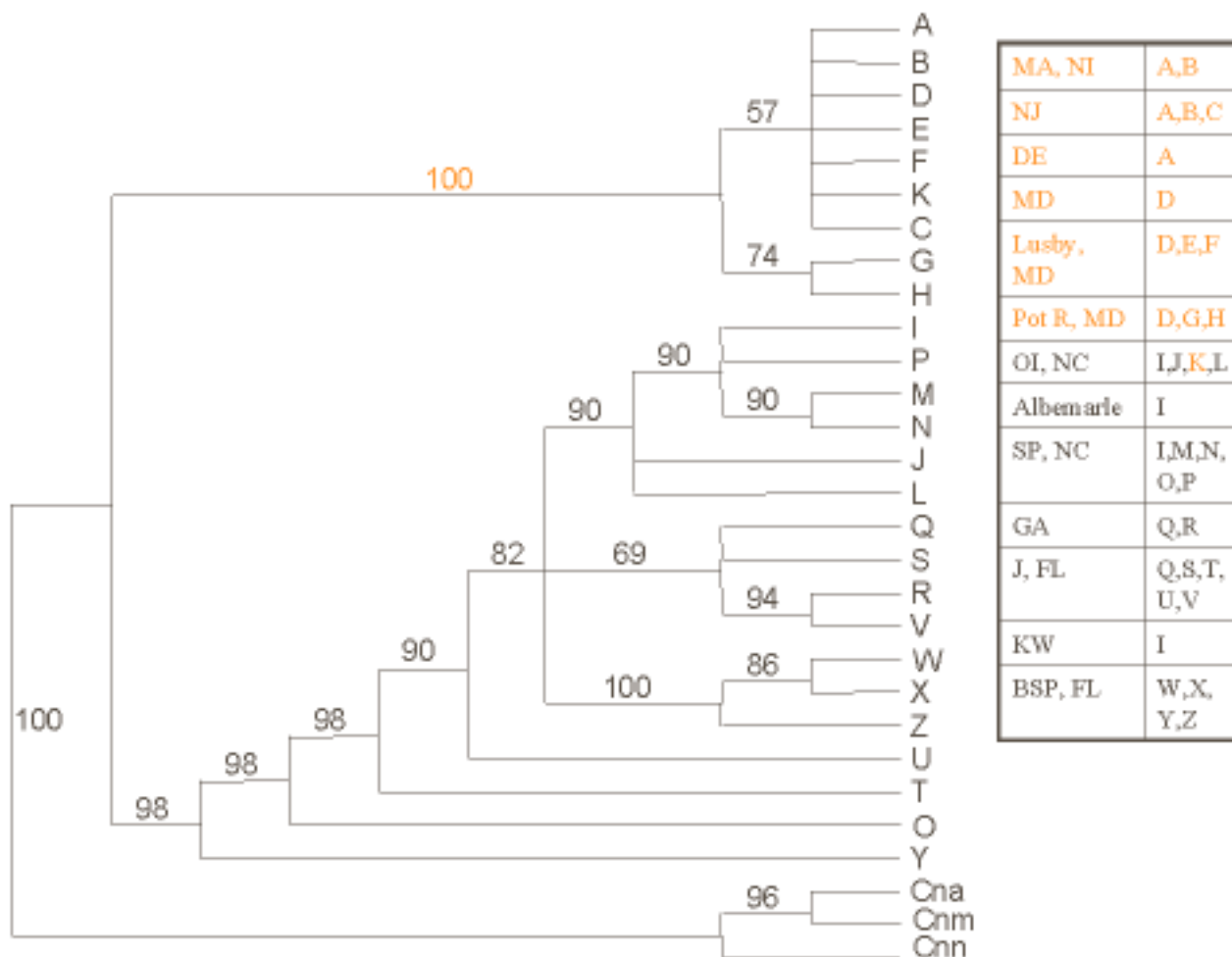


Figure 7. Results of a heuristic search under the maximum parsimony framework. The numbers are representative of the consensus of 100 trees using 50% majority rule. A well-supported northern clade is present. It is also important to note the inclusion of haplotype K, from Oregon Inlet, NC with the northern haplotypes.



Figure 8. Distance tree generated by neighbor-joining and the HKY85 algorithm. Only one tree is generated. The northern clade remains with haplotype K still being included. The entire tree is more resolved than the parsimony tree due to strict reliance on algorithms and absolute best score.

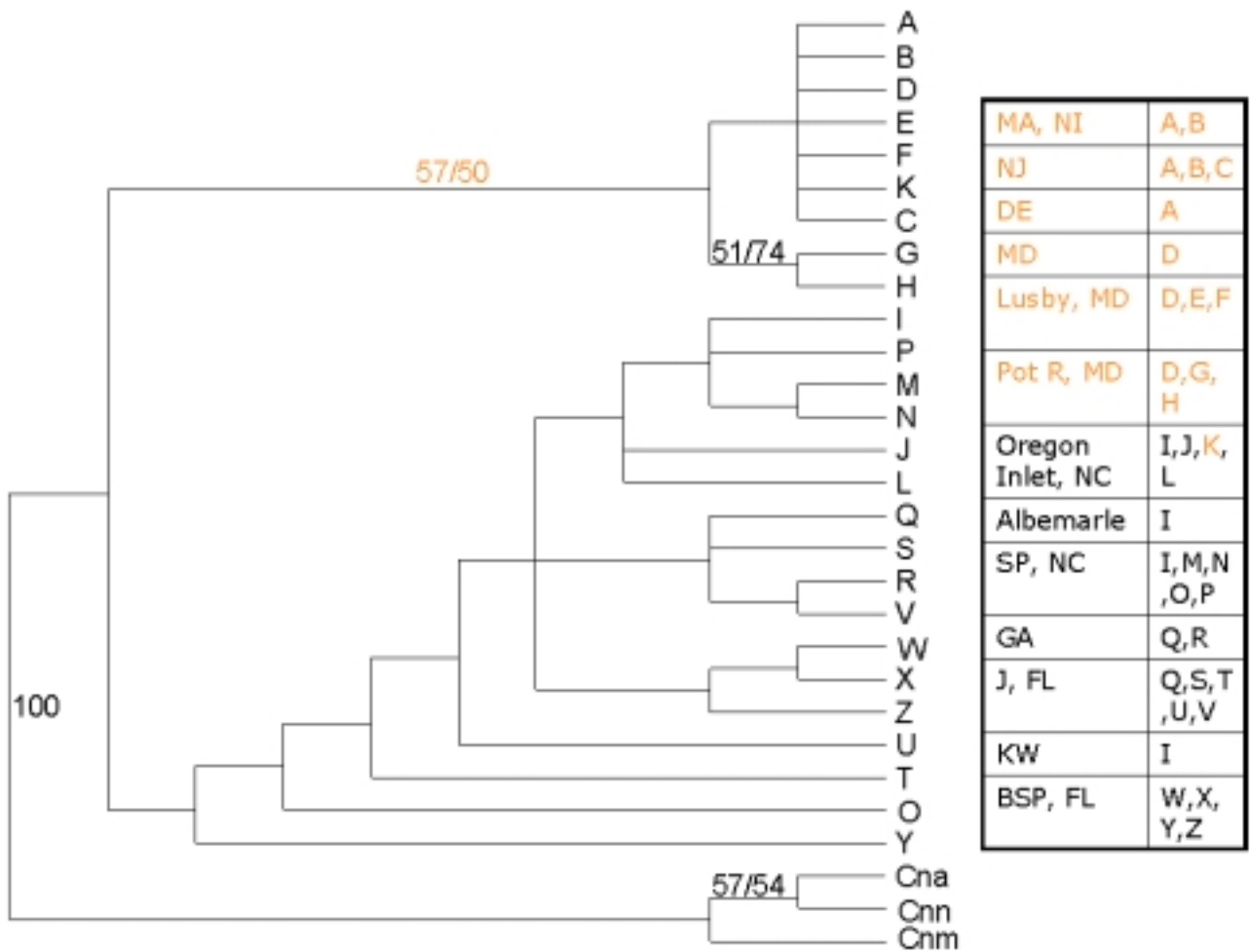


Figure 9. Topology showing bootstrap values for parsimony/distance methods. Although support isn't high, this is likely due to saturation of variation within the D-loop.

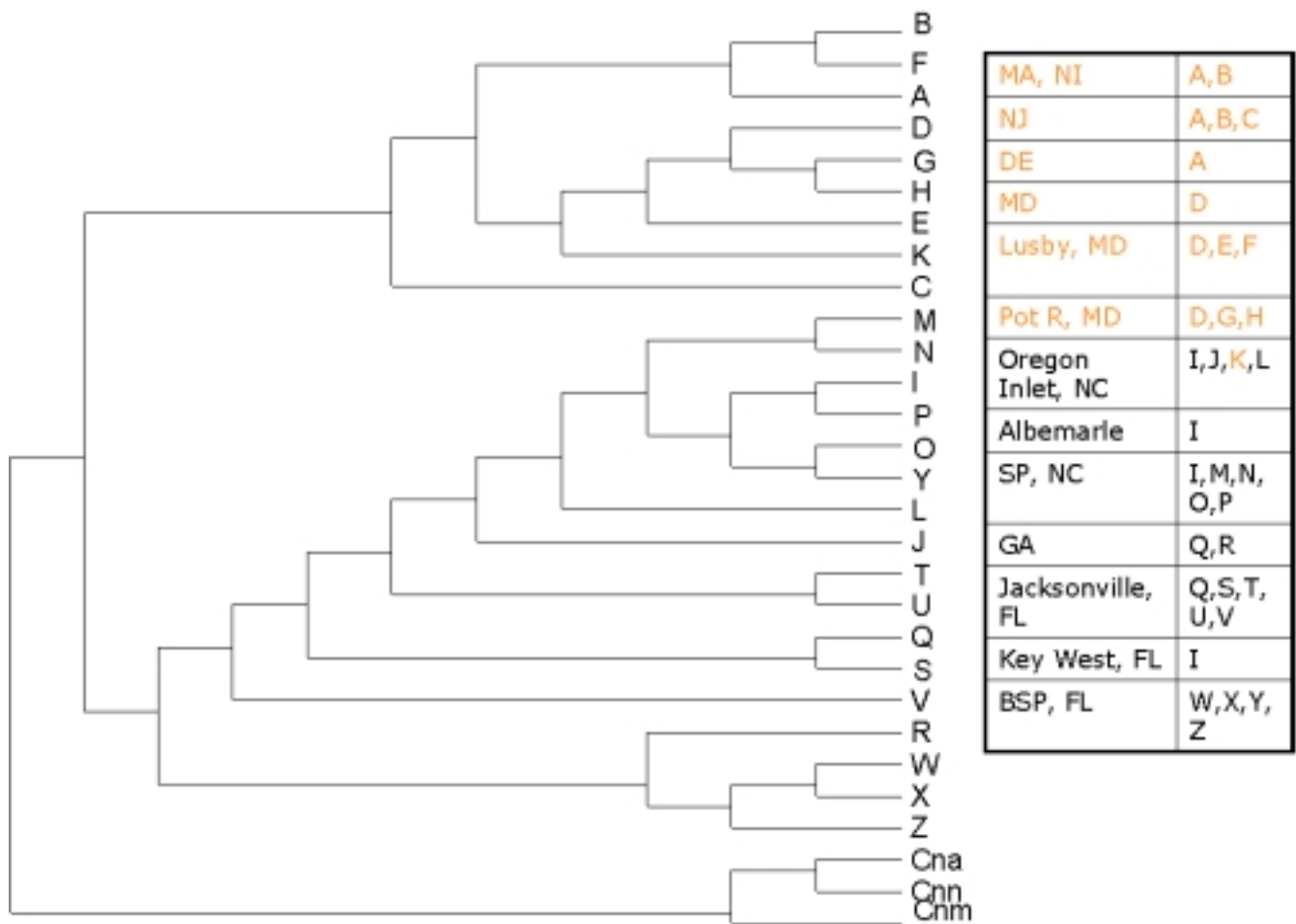


Figure 10. Topology given using maximum likelihood with the HKY85+I+ $\Gamma$ + a molecular clock. This further supports the existence of the monophyletic north and the inclusion of haplotype K within it.

**Table IV. Likelihood ratio test results**

<b>Model</b>	<b>Likelihood</b>	<b><math>\Delta</math> Degrees of Freedom (<math>q</math>)</b>	<b><math>\chi^2</math> critical value (97%)</b>	<b>Accept or Reject</b>
JC vs K2P	2 (733.14 - 726.59) = 13.12	1 - 0 = 1	5.02	reject JC accept K2P
K2P vs HKY85	2 (726.59 - 698.61) = 55.55	4 - 1 = 3	9.35	reject K2P accept HKY85
HKY85 vs GTR	2 (698.61 - 696.6) = 4.03	8 - 4 = 4	11.1	reject GTR accept HKY85
HKY85 vs HKY85+I	2 (698.61 - 670.13) = 56.97	5 - 4 = 1	5.02	accept I
HKY85+I vs HKY85+I+ $\Gamma$	2 (670.13 - 660.61) = 19.04	6 - 5 = 1	5.02	accept G
HKY85+I+ $\Gamma$ vs HKY85+I+ $\Gamma$ +molecular clock*	2 (681.74 - 660.61) = 42.26	number of taxa - 2 = 27	43.2	accept molecular clock
* Enforcing a molecular clock requires rooting the normally unrooted framework of maximum likelihood.				

All models are nested with the exception of the molecular clock therefore the simpler model is the null hypothesis. The result finds the model HKY85+I+ $\Gamma$ +molecular clock most appropriate.

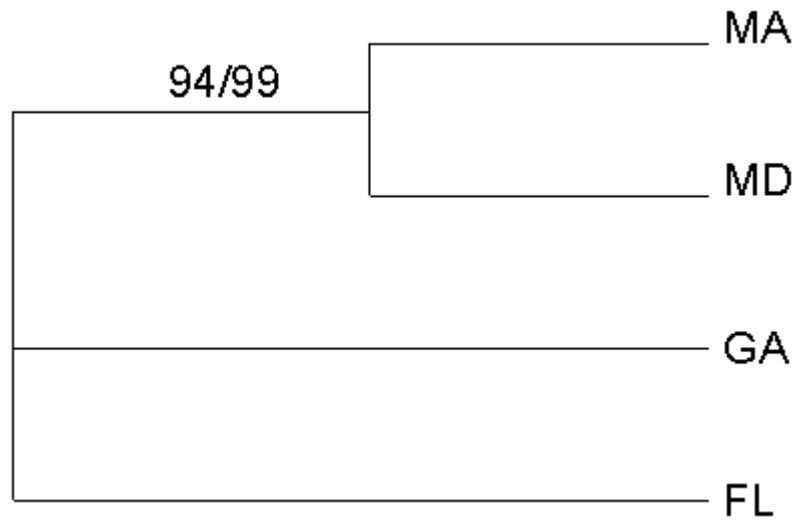


Figure 11. Topology of cytochrome-*b* analysis using maximum parsimony and distance (HKY85) methods. The values are bootstraps for parsimony/distance. The populations from Westport, Massachusetts and Lusby, Maryland are grouped together in a northern clade separate from the Georgian and Jacksonville, FL populations. The data was not informative enough to stand alone, but does lend support to D-loop analyses.

## Discussion

The distinction between the nominal subspecies, *Cyprinodon variegatus ovinus* and *Cyprinodon variegatus variegatus*, appears unequivocal using the analyses presented herein. Although the bootstrap values for the maximum parsimony and distance analyses were lower than desired for support, they are likely a result of a high degree of saturation of variation. The process of pseudoresampling data (stochastically generated “new” sequences) that defines bootstrapping only attempts to show confidence in analyses and does not generate variation where none existed. It is the persistence of the northern clade across parsimony, distance and likelihood methods of analysis that strongly supports the existence of the northern clade.

Although the molecular clock parameter added to the appropriate model (HKY85 in this case, based on the likelihood ratio test) had a likelihood score further from that of the parsimony base used to begin the likelihood search than did the other models, it is still preferred. Due to the time frame necessary to enforce the molecular clock parameter and the required rooting it inflicts, the resulting constraints would be expected to drive the likelihood score in the opposite direction somewhat. However, testing this parameter using the  $\chi^2$  critical value revealed that it was indeed appropriate and the resulting topology the most likely. In addition, the maximum likelihood topology was a consensus of the only two trees constructed by PAUP under this framework. The fact that only two trees were generated as opposed to the 100 that the analysis was allowed can also be interpreted as persuasive support for the northern clade.

The lack of variability that keeps the bootstrap values low under parsimony and distance frameworks can also be seen from a perspective that supports the existence of a monophyletic northern clade. The northern populations, in most instances, have only one haplotype per population. The southern populations have as many as five. This disparity between northern and southern populations could be a result of stronger selective pressures or more recently established populations. The disparity also matches the assumption that these northern populations were either bottlenecked as a result of fluctuating temperatures and water levels of the Pleistocene or were denied establishment of northern latitudes altogether.

Another interesting finding is the deficiency of variability within the Chesapeake Bay and Albemarle Sound populations in comparison with coastal populations. The only other population in which this occurs is the Key West population, which is commonly classified as

*Cyprinodon variegatus riverendi*. This also suggests the presence of a bottleneck in the ancestry or younger populations than are found outside of the bays. Further research focusing on Caribbean populations could provide a more definitive comparison between these populations.

Although no hybrid zone could be defined between northern and southern subspecies of *Cyprinodon variegatus*, it is still possible that a hybrid zone exists. Sites were selected based on existing literature of *Fundulus* species (killifishes) that showed a hybrid zone within the Chesapeake Bay. Due to the relatedness of these species, similarity in habitat, and well-defined hybrid zones and subspecies of *Fundulus heteroclitus*, it was reasonable that a hybrid zone for *Cyprinodon variegatus* would resemble a hybrid zone between killifish populations. This, however, will require finer sampling to detect. It is possible that a hybrid zone may be located on the eastern shore of the Delmarva Peninsula or in southeast Virginia/northwest North Carolina, areas that were not sampled due to weather. It is also possible that a hybrid zone did exist in this region but has dissipated through time as a result of continued interbreeding of populations.

Given the inclusion of haplotype K (from Cape Hatteras, North Carolina) consistently within the northern clade, it can be anticipated that individuals from the eastern coast of the Delmarva Peninsula would be included in the northern lineage. The presence of haplotype K strongly suggests the existence of a hybrid zone nearby. It is definitely interesting in terms of its potential for possessing a hybrid zone. In either case, it is certain that this research has paved the way for further interesting studies of the phylogeography and gene flow of this species.

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Appendix I. Haplotype sequences for D-loop. No longer informative beyond 166 bp.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Hap A	T	T	T	A	A	G	A	T	A	T	A	T	T	T	T	A	G	T	T	C	A	G	T	A	G
Hap B	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap S	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap U	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap W	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap X	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap Z	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
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cnn	.	.	.	.	.	.	.	.	A	.	.	A	.	.	T	.	.	C	.	.	A	.	.	.	.

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Hap A	G	G	A	T	T	A	A	T	G	G	T	T	T	A	T	G	T	A	C	G	T	C	T	T	C
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap R	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
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Hap Z	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	G	.	C	.	.
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Hap A	A	T	A	A	T	A	T	G	T	A	T	T	A	G	T	T	C	T	A	T	G	T	T	T	A
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap A	A	G	A	A	C	A	T	T	A	T	T	A	T	G	G	A	A	T	T	T	T	C	A	C	T	
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap S	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
Hap T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.
Hap U	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
Hap V	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.
Hap W	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
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Hap Z	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
cna	A	.	.	A	C	A	.	G	A	.	.	G	T	.	G	.	A	.	C	.	C	.	T	C	A	.
cnm	A	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	C	.	.	.	.	.	T	C
cnn	A	.	.	A	C	A	.	G	A	.	.	G	T	.	G	.	A	.	C	.	C	.	T	C	A	.

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	
Hap A	T	A	T	G	T	A	G	G	G	T	T	A	C	A	C	T	A	C	T	T	A	A	A	A	T	
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Z	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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cnm	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
cnn	C	.	A	T	G	T	A	.	.	G	.	T	A	C	A	C	T	A	C	.	T	.	.	G	A	

	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	
Hap A	G	T	T	T	G	A	T	A	A	A	T	A	T	T	A	A	T	G	G	G	G	A	T	A	A	
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap C	.	.	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap P	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap Z	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cna	T	G	.	A	.	.	.	T	.	.	.	.	A	.	T	G	.	.	A	T	.	A	T	.	.	.
cnm	.	.	A	A	T	A	T	.	T	A	T	A	A	T	G	G	.	A	T	A	.	.	.	.	C	.
cnn	T	G	.	A	.	.	.	T	.	.	.	.	A	.	T	G	.	.	A	T	.	A	T	.	.	.

	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
Hap A	T	A	C	A	T	A	T	A	T	G	T	A	C	T	A	T	G	G	G	G	C	C	A	A	G
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Z	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cna	C	.	T	.	.	.	.	G	.	A	C	T	A	.	G	G	-	-	-	-	-	-	-	-	-
cnm	A	T	A	T	A	T	G	T	A	C	.	.	T	G	G	-	-	-	-	-	-	-	-	-	-
cnn	C	.	T	.	.	.	.	G	.	A	C	T	A	.	G	G	-	-	-	-	-	-	-	-	-

	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	
Hap A	T	C	C	G	G	C	A	A	A	G	A	A	T	A	G	T	T	T	A	A	T	T	T	A	G	
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap O	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap U	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Z	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cna	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
Hap A	A	A	T	C	C	T	A	G	C	T	T	T	G	G	G	A	G	T	T	A	G	G	G	G
Hap B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap U	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap Z	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cna	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cnm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cnn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Appendix II. Sequences from the four haplotypes found in cytochrome-*b*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
<b>MA</b>	A	A	T	A	T	G	G	A	G	G	T	A	A	A	T	A	C	A	G	A	T	A	A	A	G	A	A	G	A	A	
<b>MD</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<b>FL</b>	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
<b>GA</b>	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	
<b>MA</b>	A	G	A	T	G	C	T	C	C	A	T	T	A	G	C	A	T	G	T	A	T	G	T	T	T	C	G	G	A	T	
<b>MD</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<b>FL</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
<b>GA</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
<b>MA</b>	G	A	G	T	C	A	G	C	C	G	T	A	A	T	T	G	A	C	A	T	C	A	C	G	A	C	A	A	A	T
<b>MD</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<b>FL</b>	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	
<b>GA</b>	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122
<b>MA</b>	G	T	G	A	G	C	A	A	C	G	G	A	T	G	A	G	A	A	A	G	C	A	G	T	A	G	A	A	A	T	A	T
<b>MD</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
<b>FL</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
<b>GA</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.

## VITA

**Katherine L. Finne**  
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### Objective

*To obtain a dynamic, interactive position using genetics and other skills in fisheries science*

### Summary of Skills

- Extensive experience with principles and techniques in population genetics
- Broad experience base with fisheries laboratory and field techniques
- Excellent interpersonal skills
- Innovative team leader

### Education

Master of Science, Population Genetics and Evolutionary Biology (focusing on fishes)  
Virginia Polytechnic Institute and State University, Blacksburg, VA, completed Feb. 2001, Thesis: Phylogeographic structure of the Atlantic pupfish, *Cyprinodon variegates* (Cyprinodontidae), along the eastern coast of North America: evidence from mitochondrial nucleotide sequences. Advisor: Dr. Bruce J. Turner

Bachelor of Science, Major-Biology, Minor-Chemistry  
Radford University, Radford, VA; completed Dec. '94

### Professional Experience

**Field and Laboratory Technician**, February 2001 to present  
Virginia Department of Game and Inland Fisheries, Blacksburg, VA  
Boat shocking walleye (*Stizostedion vitreum*) for brood stock of unique New River strain. Collecting genetic data and determining origin of individuals (native vs. stocked strain) via microsatellite DNA marker analysis (2 loci). Supervisor: Joe Williams

**Laboratory Technician**, January 2001 to present  
Virginia Tech Fisheries and Wildlife Sciences Dept., Blacksburg, VA  
Use various molecular and genetic techniques for summer flounder (*Paralichthys dentatus*) brood stock assay. PIT-tagged summer founder and collected tissue for microsatellite DNA marker analysis (10 loci). Supervisor: Eric Hallerman

**Laboratory and Field Technician**, May 1999 to present

Virginia Tech Fisheries and Wildlife Sciences Dept., Blacksburg, VA

Habitat condition and availability assessment, collection (mainly with backpack shockers) and identification of fishes in area watersheds, aged fishes using scales and otoliths, fish autopsies, data entry and analysis (BVET), PIT-tagging and recapture, Elastomer-tag recognition, snorkeling and SCUBA work in the field under extreme conditions, microsatellite work (7 loci) for phylogenetic (evolution) analysis with endangered Roanoke logperch (*Percina rex*). Immediate Supervisor: Amanda Rosenberger  
Project Leader: Paul Angermeier

**Graduate Teaching Assistant**, Jan. 1998-May 1999

Virginia Tech Biology Dept., Blacksburg, VA

Lecture and conduct biology laboratory sessions for General Biology and Principles of Biology classes (sections I and II of both classes)

**Laboratory Technician**, May 1996-Aug. 1997

Virginia Tech Biology Dept., Blacksburg, VA

Perform various molecular techniques including DNA extraction, polymerase chain reaction (PCR), direct sequencing with radio-labeled nucleotides, automated sequencing with dye-labeled nucleotides, etc. using regions of the mitochondrial genome to evaluate phylogenetics of sheepshead minnow (*Cyprinodon variegates*). Supervisor: Bruce Turner

**Catering Assistant Manager**, Jan. 1994-Aug. 1997

Blacksburg Marriott/Four Points by Sheraton, Blacksburg, VA

Manage a staff of about 20, great importance in with customer service/public relations, in charge of daily and weekly financial reports for the catering department. Supervisor: Sara Herbst

### **Hobbies and Interests**

- PADI certified scuba diver (AOW), hiking, camping, anything in the field, horseback riding, cooking, would love to work with sharks eventually
- Member of the Virginia Tech Chapter of the American Fisheries Society

## References

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