Final Report

Investigation into the Hybridization of *Ameiurus* catfish in Presque Isle Bay, Erie, Pennsylvania

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ABSTRACT

The Brown Bullhead, *Ameiurus nebulosus* (Lesueur, 1819), is a bottom-dwelling fish native to the Great Lakes that is commonly used to determine tumor prevalence in degraded aquatic environments. Brown Bullheads are in constant contact with benthic sediments due to their feeding habitats which may naturally expose them to industrial wastes and other contaminants trapped in bottom sediments. In 1991, the United States Department of State listed Presque Isle Bay, Lake Erie, Erie, Pennsylvania, as an Area of Concern of aquatic habitat for the primary impairments of sediment contamination and high incidences of epidermal and hepatic tumors in Brown Bullheads. Studies conducted in Presque Isle Bay found skin and liver tumor rates of Brown Bullheads have decreased between 1992 and 1999. It was proposed by Eric Obert, extension director of Pennsylvania Sea Grant that the Brown Bullhead population of Presque Isle Bay may contain some hybrids within the genus *Ameiurus*. Studies of hybrid fishes have shown that hybrids and succeeding backcross generations are highly sensitive to pollutants, while the parental wild species are less susceptible. The purpose of this study was to determine morphological and genetic variation within and among populations of Brown Bullheads and Black Bullheads in Presque Isle Bay, compared to other Brown Bullheads in other sites in Lake Erie. Morphological and meristic analysis indicates the majority of Brown Bullheads from Presque Isle Bay group with the reference Brown Bullhead population and not the reference Black Bullhead collection morphologically using principal component analysis. Collections from the Lagoons and Thompson's Bay each include an individual which maybe a hybrid, but what is likely being collected as a Brown Bullhead for the tumor studies in Presque Isle Bay is morphologically a Brown Bullhead. Genetically, over half of the Bullheads sampled and examined using microsatellite DNA were identified as having all *Ameiurus nebulosus* alleles, but multi-locus nuclear genotypes suggest the presence of extensive backcrossing between *Ameiurus nebulosus* and *Ameiurus melas* in Presque Isle Bay.

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

Chapter 1

Introduction

The Brown Bullhead *Ameiurus nebulosus* (Lesueur, 1819), is a bottom-dwelling fish native to the Great Lakes that is commonly used to determine tumor prevalence in degraded aquatic environments (Baumann et al. 1996, Lesko et al. 1996, Smith et al. 1994). Brown Bullheads are in constant contact with benthic sediments due to their feeding habitats which may naturally expose them to industrial wastes and other contaminants trapped in bottom sediments (Lesko et al. 1996). In 1991, the United States Department of State listed Presque Isle Bay, Lake Erie, Erie, Pennsylvania, as an Area of Concern of aquatic habitat for the primary impairments of sediment contamination and high incidences of epidermal and hepatic tumors in Brown Bullheads. Studies conducted in Presque Isle Bay found skin and liver tumor rates of Brown Bullheads have decreased between 1992 and 1999 (Pyron et al. 2001). It was proposed by Eric C. Obert extension director of Pennsylvania Sea Grant (personal comm.) that the Brown Bullhead population imposed with tumors in Presque Isle Bay may be a hybrid within the genus *Ameiurus*. Studies of hybrid fishes have shown that hybrids and succeeding backcross generations are highly sensitive to pollutants (Setlow et al. 1989), while the parental wild species are less susceptible (Harshbarger and Clark 1990). If, in fact, the bullhead population in Presque Isle is comprised of hybrids and/or back-crossed individuals, then the tumor rate in this population may be exacerbated.

1.1 Presque Isle Bay – Area of Concern

Presque Isle Bay is located north of the city of Erie, Erie County, in the northwest corner of Pennsylvania. Presque Isle Bay is formed by a 1,295 hectare sandy, crescent peninsula reaching in a northeast direction on Lake Erie from the western portion of the city, and is Pennsylvania's only port on the Great Lakes. The bay is a relatively sheltered body of water and a closed system with a flushing time of almost 2.5 years. Presque Isle Bay is roughly 7.24 kilometers long with a maximum width of 2.41 kilometers and connects with Lake Erie through a narrow channel at the eastern end. The land use within the Presque Isle Bay watershed is approximately 80 precent urban and spans roughly 41 kilometers. Its primary tributaries are Cascade Creek and Mill Creek, which together account for two-thirds of the water flowing into the bay. Presque Isle Bay has suffered from the accumulation and degradation of contaminants discharged by point and nonpoint sources.

Presque Isle Bay was declared the Great Lakes' 43rd Area of Concern by the United States Department of State as recommended by the International Joint Commission in January of 1991. Great Lakes Areas of Concern (AOC) are severely degraded geographic areas within the Great Lakes Basin. Areas of Concern are defined by the United States-Canada Great Lakes Water Quality Agreement (Annex 2 of the 1987 Protocol) as geographic areas that fail to meet the general or specific objectives of the agreement where such failure has caused or is likely to cause impairment of beneficial use of the area's ability to support aquatic life. Currently, there are forty identified Areas

of Concern, twenty-five are located completely within the United States, ten exclusively in Canada, and five are shared by both countries along river systems.

The International Joint Commission lists fourteen beneficial use impairments to be used by Areas of Concern as criteria for the listing and delisting process. In Presque Isle Bay, the impaired beneficial uses are restrictions on dredging of sediments; and fish tumors and other deformities. Sediments in Areas of Concern are often contaminated with industrial or agricultural pollutants released in the environment long ago such as polychlorinated biphenyl (PCB), polycyclic aromatic hydrocarbons (PAH), nitrosamines, and many heavy metals including: arsenic, barium, cadmium, chromium, copper, lead, nickel, and zinc (Diz 2002). Other contaminants continue to enter the environment though the burning of fossil fuels and runoff from agricultural and urban areas (International Joint Commission 1989). By restricting dredging activities in an Area of Concern, contaminated sediments are thus less likely to be disturbed and dispersed. A fish tumor or deformity impairment occurs when incidence rates of fish tumors or other deformities exceed rates at unimpacted control sites that are locally relevant and when survey data confirm the presence of neoplastic or preneoplastic liver tumors in Brown Bullheads or White Sucker (*Catostomus commersoni*). Unimpacted sites are areas where there is a lack of industrial or municipal pollution discharges located upstream or in the immediate areas where neighboring land uses have not disrupted ecosystem function or structure.

1.2 Indicator Organism

Brown Bullheads are frequently used in environmental contaminant studies because they are a scaleless, benthic fish in constant contact with the sediments, and have a known sensitivity to environmental carcinogens (International Joint Commission 1989).

Studies on the Brown Bullhead in Presque Isle Bay (PADEP 1992, PADEP 1995, PADEP 1997) showed rates of orocutaneous tumors decreased from 64 percent to 22 percent and liver tumors decreased from 10 percent to 3percent from 1992 to 1997. It was noted in the 1997 study that the age distribution of bullheads collected in the 1992 study were markedly older than bullheads in the 1995 study, which in turn were older than bullheads in the 1997 study. The oldest population, 1992, has the highest tumor rates while the youngest study population, 1997, had the lowest tumor rates. In the 1997 study however, tumors were shown in bullheads aged fifteen years or older, including the reference population. In a study of Presque Isle Bay Brown Bullheads conducted in 1999 (Pyron et al. 2001) a decrease in skin and liver tumor rates was not associated with the losses of larger, older individuals or declining reproduction rates. Their data provide evidence that the population is not losing older individuals; therefore the decline in tumor rates cannot be attributed to a younger population. It is suspected that hybridization between Brown and Black Bullheads may be a factor in the decrease in tumor rates (Eric Obert, Personal comm.). Studies of hybrid fishes have shown that hybrids and succeeding backcross generations are highly sensitive to pollutants, while the parental wild species are less susceptible (Harshbarger and Clark 1990, Setlow et al. 1989). If the Brown

Bullhead population in Presque Isle Bay is comprised of hybrids, their quantitative value may in fact be compromised.

In December 2002, with respect to the tumor rates decrease in bullheads, Presque Isle Bay was upgraded from an Area of Concern and designated to be an Area of Concern in the Recovery Stage, as the result of significant environmental improvement in the bay since the early 1990s. It became the first Great Lake Area of Concern in the United States to be upgraded to the recovery status. However, tumors are still present on bullheads in Presque Isle Bay and it is still unclear what is causing the tumors and deformities in the fishes.

1.3 Taxonomic status

Taylor (1954), while assembling the records of fishes collected by John N. Lowe in the Upper Peninsula of Michigan, placed the generic name *Ameiurus* in synonymy with *Ictalurus* and proposed to use the name Ictaluridae for the North American catfishes and bullheads. This submission had been generally followed until Lundberg (1992) separated *Ameiurus* from *Ictalurus*.

The catfish family Ictaluridae contains about sixty living and extinct species. Modern genera of Ictaluridae share several synapomorphies, including extensive jaw adductor muscle origin from the skull roof that is known to have evolved in the early Oligocene (Lundberg 1992). In the genus *Ameiurus*, seven extant species are recognized and seven extinct species are known from their fossilized remains. The oldest of these

fossils provides a minimum age estimate for the genus of approximately thirty million years (Lundberg 1992).

Ameiurus is divisible into two morphological species groups, the *natalis* group and the *catus* group. The *catus* group is comprised of four species, including three "flat-head" bullheads not found in Pennsylvania: *A. platycephalus* (Flat Bullhead), *A. brunneus* (Snail Bullhead), and *A. serracanthus* (Spotted Bullhead). They usually have a flatted head, large eye, emarginated tail, and a large dark blotch in the basal portion of the dorsal fin. *Ameiurus catus* (White Bullhead) also has a relatively large eye, but has a more convex head, lacks dorsal fin blotch, and is somewhat intermediate between *Ictalurus* and the bullheads in having a moderately forked tail (Jerkins and Burkhead 1994). In Pennsylvania, the geographic range of *A. catus* has included the Susquehanna and Delaware river systems, and it has been introduced into parts of the Ohio River watershed.

The *natalis* group is comprised of three species: *Ameiurus melas* (Black Bullhead) *A. natalis* (Yellow Bullhead), and *A. nebulosus*. Of the three species, *A. natalis* and *A. nebulosus* commonly occur in Pennsylvania, whereas *A. melas* has an endangered status in Pennsylvania. The Black Bullhead's most eastern distribution occurs in western Pennsylvania and as a result, is rarely found. The last documented collection in Presque Isle Bay took place during the late spring of 1972 (AEA 1973) and was reported to be in a 1987 checklist from the Pennsylvania Fish Commission (PADEP 1991).

The native distribution of *Ameiurus* catfishes ranged from southern Canada, the St. Lawrence River, all the Great Lakes except Lake Superior and the Red River of the North in Ontario and Manitoba, south to the Gulf of Mexico and northern Mexico, in the

streams of the Atlantic Coast from New York to Lake Okeechobee in Florida, to their westernmost point in central Montana (Smith 1985, Page and Burr 1991, Hubbs and Lagler 2004). Introductions have extended the range west of the Rockies in isolated pockets including areas of British Columbia, Alberta, Mexico, California, Arizona, Nevada, and Idaho.

1.4 Identification

Fishes belonging to the genus *Ameiurus* are medium sized, lack scales and have a large and flattened head. The teeth of the upper and lower jaws are minute and sharp, and arranged in broad pads. The swim bladder is connected with the Weberian ossicles, and is involved in the reception and production of sound. All members possess an often elongated adipose fin free at the posterior edge, four pairs of paired barbels, and a spinous ray in the dorsal fin and in each pectoral fin (Becker 1983).

Ameiurus melas (Rafinesque, 1820) Black Bullhead: *Ameiurus* –"primitive" or "curtailed" in reference to the slight notch in the caudal fin, *melas* - black.

Black Bullheads have a robust body, rounded anteriorly, compressed posteriorly (Figure 1). Snout is bluntly pointed in lateral view and broadly rounded in dorsal view; with elongated barbels on the snout just anterior to posterior nostrils. Black Bullheads have a mouth that is short but wide, terminal and horizontal. Black Bullheads have very long barbels sweeping posteriorly from upper jaw at each corner of the mouth and four shorter barbels attached in a transverse line on the lower chin. The fish has numerous

minute needlelike teeth in broad bands on upper and lower jaws. Dorsal fin origin about midway between pectoral and pelvic fins; dorsal fin with a stout spine and 5-6 rays; dorsal adipose fin free at posterior end. Anal fin rays including rudimentaries are 15-21 (Becker 1983), sometimes 17-21 (Smith 1985, Trautman 1981). The pectoral fin has a stout spine without sharp teeth on the posterior edges that catch the finger (Trautman 1981). The caudal fin is somewhat square and slightly notched at midpoint, and the lateral line is complete (Becker 1983). Trautman (1981) notes the body of an adult Black Bullhead is usually bi-colored with a sharp demarcation between the darker lower sides and the lighter ventral sides and a light, ventral, caudal bar, usually conspicuous in large young and adults that connects with the light color of the ventral surface.

Ameiurus nebulosus (Lesueur, 1819) Brown Bullhead: *nebulosus* – clouded, in reference to mottled coloring.

The Brown Bullhead has a stout body, compressed posteriorly (Figure 2). The head of the Brown Bullhead is depressed and the profile of the dorsum is straight in juvenile to distinctly convex in some adults (Jenkins and Burkhead 1994); they have a small eye, and the mouth is slightly subterminal with jaws equal or with the upper jaw slightly longer. The caudal fin is usually slightly emarginated, sometimes straight in small young. Chin barbels are gray, black, or black-spotted by their base. The anal fin usually has 22-23 (extremes 21-24) rays, counting rudimentaries; its distal margin usually slightly rounded. The posterior edges of pectoral spines have many sharp teeth, which may become blunted in large individuals (Trautman 1981). The dorsal fin has a stout spine and 6-7 soft rays. The body of adult Brown Bullheads is often conspicuously

mottled, especially on the sides, and there is no sharp demarcation line between ventral surface of the body and lower sides (Trautman 1981).

Brown Bullheads and Black Bullheads are often difficult to distinguish but have been reported to be separable by the character of the serrae on the posterior edge of the pectoral spine: moderate serrae in Brown Bullheads (Figure 2) and weak serrae in Black Bullheads (Figure 1) (Trautman 1981, Hubbs and Lager 1991, Jenkins and Burkhead 1994). The posterior spine serrae in Black Bullheads are variable, being absent to moderately developed. Although most often weakly developed in adult Black Bullheads, the pectoral serrae are unreliable for consistently distinguishing Black Bullheads from Brown Bullheads (Burkhead et al. 1980).

Fin pigmentation differences have also been reported. Of these characters, only the depigmented "bar" at the caudal base of Black Bullhead is consistently present, and then only in larger juveniles and adults. However, it is often evident only when directly compared to specimens of Brown Bullheads (Burkhead et al. 1980). Black Bullheads are best distinguished from Brown Bullheads by higher and rarely overlapping gill raker counts. Brown Bullheads have 3 or 4 gill rakers on the epibranchial limb, and 8 or 9 gill rakers on the first ceratobranchial limb. Black bullheads will have 5 to 7 gill rakers on the epibranchial limb and 10 to 15 gill rakers on the first ceratobranchial limb.

1.5 Biology

Ameiurus spawn in late spring to early summer in Pennsylvania. Spawning takes place in open excavations in sand and gravel, and in the shelter of logs, rocks, or

vegetation (Becker 1983, Cooper 1983). Both males and females may contribute to nest construction but this is primarily the female's duty (Smith 1985). The spawning act takes place by the pair facing in opposite direction with their bodies in close contact and the female depositing from 50 to 10,000 or more eggs in the nest. Generally, the male or both parents guard the nest and protect the young for a time (Trautman 1981). When the young rise off the nest, the parents swim about them in circles to keep them in a compact school, and strays are caught in their parents' mouth and returned to the school (Becker 1983). Adult bullheads are most active at night. When they are active in daytime, it is generally in muddy, clouded water. They have poor vision and use their sense of smell and the taste buds on the skin, lips and barbels to find food. Bullheads are opportunistic feeders that eat whatever food is available, including carrion (Becker 1983).

The Black Bullhead seems to prefer silty waters and soft mud bottoms, and is highly tolerant of many types of industrial and domestic pollutants, as well as warm water temperatures (Trautman 1981). It appears incapable of invading in abundance the deeper, cooler, clearer waters, with or without some vegetation, which is the habitat of the Brown Bullhead, or the very clear, heavily vegetated habitat of the Yellow Bullhead (Trautman 1981).

1.6 Hybridization

The offspring between one full species and another full species are called F1 hybrids. The offspring between an F1 hybrid and an individual of either parent species

are called backcrosses. In fish hybrids a blending of the parent characters normally occur, and the identification of F1 hybrids can be difficult or impossible (Trautman 1981).

Brown and Black Bullheads are known to naturally hybridize and Trautman (1981) reports that there had been considerable hybridization and backcrossing between Black and Brown Bullheads in western Lake Erie. Trautman (1981) goes on to note that "when mass hybridization occurs in the small, silty, largely vegetationless impoundments, the majority of the population resembles Black Bullheads, and that a large number of "typical" Black may be present, but there may be few or no "typical" Browns. In deeper waters the situation appears to be reversed, and backcrosses usually favor the Brown rather that the Black Bullhead. Both bullheads are spring spawners and rely on thigmotactic and chemosensory clues to modify their spawning behaviors and recognize individuals in a population (Cooper 1983, Page and Burr 1991).

Other freshwater fishes, such as sunfish in the genus *Lepomis* readily hybridize in polluted waters, where conditions hinder species recognition (Page and Burr 1991). Stauffer et al. (1979) attributed natural hybridization to the overcrowding of spawning fishes, abiotic stress, and cohabitation of rare and abundant fishes.

Studies of hybrid fishes have shown that hybrids and succeeding backcross generations are highly sensitive to pollutants, while the parental wild species are less susceptible. Certain hybrids such as the Platyfish-Swordtail hybrid (*Xiphophorus maculates* x *Xiphophorus helleri*) and succeeding backcross generations are highly sensitive to carcinogens, while the parental wild species are not susceptible to neoplasia (Setlow et al. 1989). Also, the hybrid of European Carp (*Cyprinus carpio*) x Goldfish hybrid (*Carassius auratus*) is believed to have a genetic predisposition to neoplasia,

unlike its parental species (Harshbarger and Clark 1990). A better understanding of the bullhead population in Presque Isle Bay is essential for the continued use of this population as an indicator species for the Great Lakes.

1.7 Purpose

The purpose of this study is to determine morphological and genetic variation within and among populations of Brown Bullheads and Black Bullheads in Presque Isle Bay, compared to other Brown Bullheads in other sites in Lake Erie. Further study maybe warranted to determine if hybridization of the Lake Erie or Presque Isle Bay Brown Bullheads promote higher tumor rates than areas outside the Great Lakes' basin.

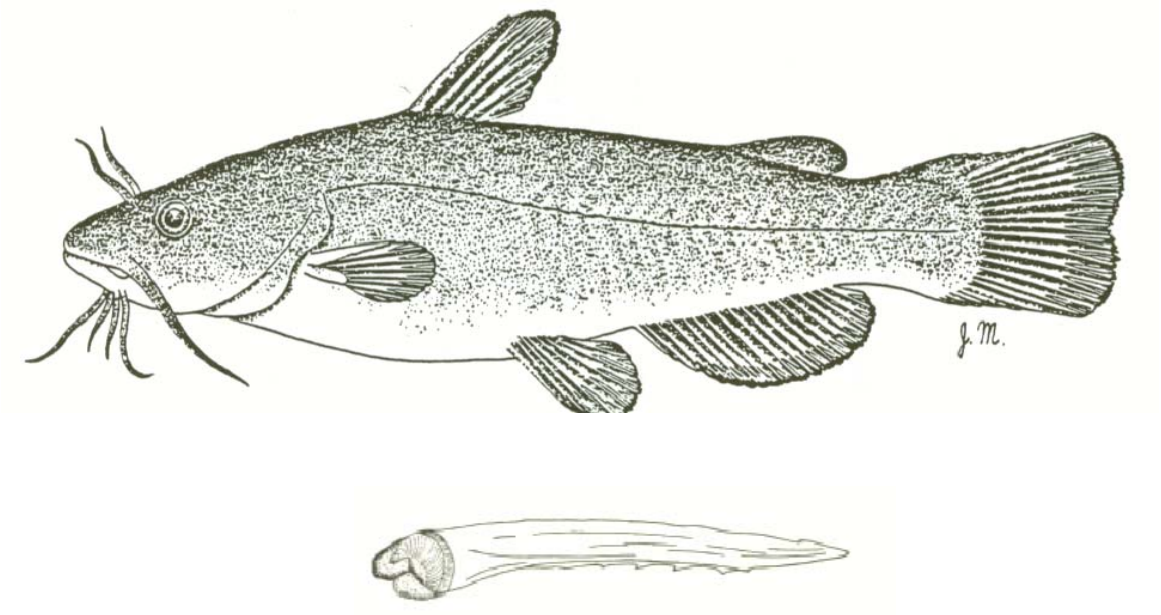

Figure 1. Lateral view of *Ameiurus melas* and pectoral spine serrae (Cooper 1983)

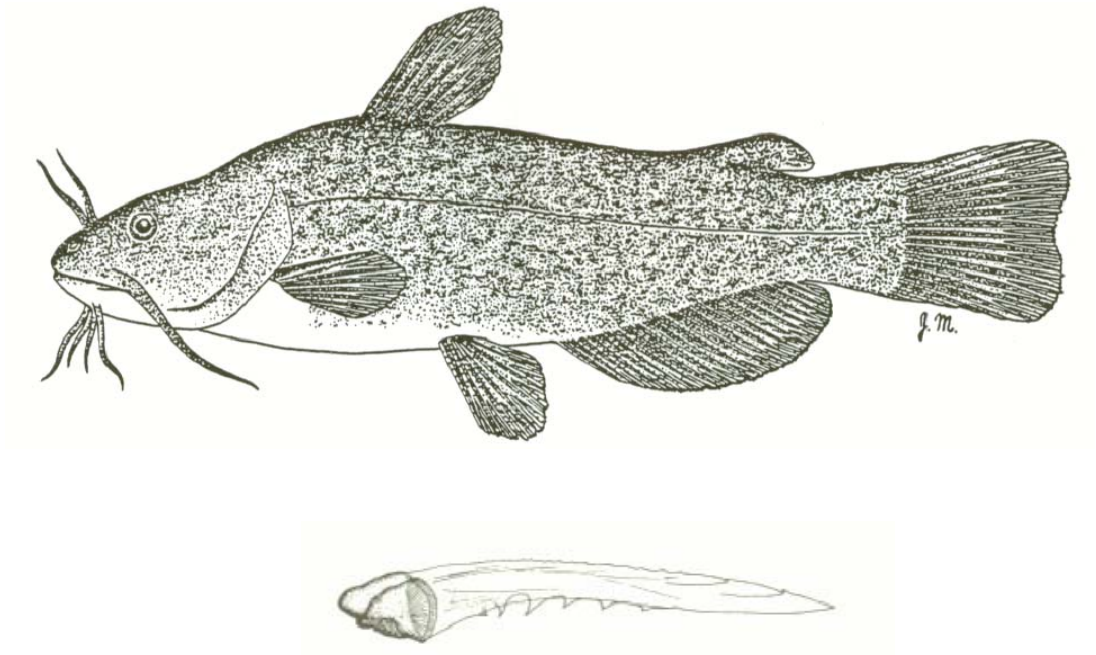

Figure 2. Lateral view of *Ameiurus nebulosus* and pectoral spine serrae (Cooper 1983)

Chapter 2

Methods and Materials

2.1 Samples and Collections

Ameiurus nebulosus specimens for this study from Presque Isle Bay, Lake Erie, Pennsylvania *(Latitude = 42°09'N; Longitude = 80°04'W)* (Figure 3); Dunkirk Harbor, Dunkirk, New York *(Latitude = 42°49'N; Longitude = 79°34'W)*; and Old Woman Creek, Ohio *(Latitude = 41°21'N; Longitude = 82°30'W)* (Figure 4) were collected by electrofishing. After capture, all individuals were anesthetized with sodium benzocaine (MS-222). A piece of the right pectoral fin was removed and placed in a numbered 1.5 ml microcentrifuge tube containing 95% ethanol for later genetic analysis. All individuals were tagged with a number corresponding to the genetic analysis, preserved in 10% formalin for one week, washed in water for two days, and later stored in 70% ethanol. Additionally, the Pennsylvania Department of Environmental Protection provided thirty *A. nebulosus* specimens from Long Point Bay, Ontario, Canada *(Latitude = 42°58'N; Longitude = 82°36'W)* (Figure 3); and the Pennsylvania Fish and Boat Commission collected thirty *A. nebulosus* in trap nets from Tamarack Lake, Crawford County, Pennsylvania *(Latitude = 41°35'N; Longitude = 80°05'W)*. Twenty-eight *A. nebulosus* specimens were collected by rod and reel from a reservoir in Petersburg, Huntingdon County, Pennsylvania *(Latitude = 40°57' N; Longitude = 78°05' W)* and served as reference specimens for the Brown Bullhead. These individuals were collected from

outside the historic range of the Black Bullhead in Pennsylvania. All collections at a particular site were obtained in one sampling trip.

Thirty specimens of *Ameiurus melas* were obtained from Clear Lake in Clear Lake, Iowa *(Latitude = 42°56' N; Longitude = 93°63'W)* by the Iowa Department of Natural Resources. Eleven samples of *A. melas* were provided by the Wisconsin Department of Natural Resources but were used only for comparisons to the Iowa samples. The Iowa specimens served as reference specimens for Black Bullheads. The *A. melas* specimens were shipped frozen and later thawed, when the fish were tagged and the right pectoral fin was removed, placed in 1.5 ml microcentrifuge tubes containing 95% ethanol, and stored in the laboratory prior to DNA preparation leaving the left side of the fish intact for morphological analysis.

All specimens were assessed for the presence of external lesions and gross deformities using the specifications of the Pennsylvania Fish and Boat Commission's Deformities, Erosions, Lesions, and Tumors (DELTs) index. Bullheads used in previous tumor studies from Long Point Bay, Ontario were a reference population located on Lake Erie with no known point-source of contaminants and Old Woman Creek, Ohio is a reference site having only low-level PAH contamination at railway and highway bridges (Baumann et al. 1996).

Twenty-eight specimens from each site were included in this study with the exception of the Lagoons, Presque Isle Bay (thirty specimens) and Dunkirk, New York, which consisted of twenty-two specimens.

All specimens were deposited in the permanent collections of the Pennsylvania State University Fish Museum.

2.2 Mensural and Meristic Characters Examined

A total of six meristic (count) and twenty-five mensural (measurement) characters (Table 1) was examined on each individual from all nine sites totaling 7,688 separate measurements or counts. All measurements were made with Fowler Promax 150 mm digital calipers and recorded to the nearest .01 mm.

Counts followed methods as outlined in Hubbs and Lager (1958). Morphometric distances were measured also as described by Hubbs and Lager (1958) with the exception noted. All measurements were taken point to point except head depth, which was measured from the point equidistant and dorsal to the midline of each eye to a point directly vertical on the base of the fish. All counts and measurements were made on the left side of the fish, except gill raker counts which required incisions to the dorsal and ventral junction of the operculum to expose the gill rakers on the right side of the fish. All gill rakers on the right arch were counted including rudiments on the first ceratobranchial limb with the exception of the raker straddling the angle of the arch. Counting was aided by the use of a variable magnification stereo dissecting microscope.

2.3 Mensural and Meristic Character Analysis

Meristic differences were analyzed using principal component analysis in which the correlation matrix was factored. Head, body, and fin shape variation were assessed by analyzing the mensural data using sheared principal component analysis, in which the

covariance matrix was factored. This procedure restricts size variation to the first principal component; and subsequent components are strictly shape related (Humphries et al. 1981). Comparisons among species were made by plotting the first principal component (PC1) from the meristic variation and the sheared second principal component (SPC2) from the mensural variation. Minimum polygon clusters were drawn to encompass the points of a species or a population on the principal components plots. A multivariate analysis of variance (MANOVA) was used to test differences among the minimum polygon clusters formed by each species in the plots.

Principal component analysis (PCA) was chosen for this study over discriminant analysis (DA) to uncover unknown trends in the data. Principal component analysis does not attempt to *a priori* group data by user-specified criteria or presume multiple groups and thus allow for their discovery (Humphries et al. 1981). Principal component analysis is a way of identifying patterns in data, and expressing the data in such a way as to emphasize their similarities and differences. The use of PCA allows the number of variables in a multivariate data set to be reduced, while retaining as much as possible of the variation present in the data set (McGarigal et al. 2000). Principal component analysis organizes entities along continuous gradients defined by the principal components and seeks to describe the sources of greatest variation among the entities, where entities are generally assumed to represent a single random sample of a known of unknown number of populations. For PCA to work, the data set must consist of a single set of two or more continuous, categorical and/or count variables, and no distinctions exists between independent and dependent variables (McGarigal et al. 2000).

The main purpose of discriminat analysis is to describe the differences among two or more well-defined groups and predict the likelihood that an entity of unknown origin will belong to a particular group based on a suite of discriminating characteristics. Discriminate analysis assumes the variables are independent. Classification is a part of discriminate analysis and classifies entities into groups using a classification criterion that, in general, maximizes correct classification of entities into prespecified groups (McGargal et al. 2000). Principal component analysis was performed using the SAS® system for windows, version 8.02 and MINITAB®, release 14.

2.4 Fin Digestion and DNA Extraction

Fin clips were blotted dry of ethanol, minced into small pieces using a clean razor blade and placed into 1.5 ml microcentrifuge tubes. 500µL lysis buffer (0.1M Tris, 4M urea, 0.2M NaCl, 0.01M CDTA, 0.5% lauroyl sarcosine) with 5µL proteinase K solution (0.1mg/ml concentration) was added, and the samples were incubated overnight at 55°C.

500µL equilibrated Phenol:Chroloform:Isoamyl alcohol (25:24:1) was added and inverted seven times, and spun in a microfuge for 10 minutes. The top layer was transferred to a new tube and 500µL Chloroform:Isoamly alcohol (24:1) was added, inverted and centrifuged for two minutes. The top layer was removed to a new tube and 1000µL of cold 95% EtOH was added and inverted and centrifuged for 20 minutes at 4°C. The ethanol was removed by decanting and the DNA pellet was washed with 200µL cold 70% EtOH. The ethanol was again removed by decanting and the pellet was dried

over night. The extracted DNA was resuspended in 100µL HPLC grade water and stored at 4°C.

2.5 Polymerase Chain Reaction Recovery of Enriched DNA

Eight samples of extracted *Ameiurus nebulosus* DNA were initially sent to Dr. Travis Glenn (Savannah River Ecological Laboratory, Aiken, South Carolina) for construction of a genomic library enriched for microsatellite loci (Glenn and Schable 2005). Extracted DNA was enriched for $(AAAG)^6$, $(ACAG)^6$, $(AGAT)^8$, $(ATCC)^5$ and $(ACAT)^8$ following a protocol available from Travis Glenn (glenn@srel.edu). In brief, the DNA was digested with *Rsa*I, ligated to Super- SNX linkers, hybridized to biotinylated microsatellite oligonucleotides, captured on Dynabeads (Dynal Biotech Inc.) and unwanted DNA was washed away.

The enriched DNA fragments were amplified using polymerase chain reaction (PCR) using, 1.67µM SuperSNX-f (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′), 10X PCR buffer, 250µg/ml BSA (Bovine Serum Albumin), 0.3125 mM of each dNTP, 4.17 mM $MgCl_2$, 0.5 units/µL *Taq* DNA polymerase (Fisher Brand), and HPCL H_20 in a total volume of 12 µL. PCR was conducted in a DNA Dyad Thermalcycler (MJ Technologies) with the following profiles: 2 minute hot start at 95°C, followed by 25 cycles of 20 seconds at 95ºC, 20 seconds at 60°C, and 1.5 minutes at 72ºC, with a final extension step of 30 minutes at 72°C. Electrophoresis was conducted with 5µL of PCR product using a 3% SB agarose gel containing ethidium bromide and SB buffer (Brody et al. 2004) at 300 V for 10 minutes for verification of successful enrichment and DNA recovery.

2.6 Ligating Enriched DNA into Plasmids and Sequencing of MiniPrep Clones

The enriched DNA library was ligated into the PCR 4-TOPO cloning vector by TA cloning using Invitrogen's TOPO-TA cloning kit and following the manufacture's protocol. The ligated cloning vectors were transformed into One-Shot TOP10 chemically competent *E. coli* cells (Invitrogen) following the manufacture's protocol. Ampicillin (*amp*) sensitive bacteria and a vector that carries a gene conferring *amp* resistance were used to incorporate the enriched/recovered DNA + cloning vector into a bacterial host. Colonies were plated on LB plates containing *amp* antibiotic, to permit screening of successful transformants. One hundred-twenty clones were picked and swabbed into 3 mL tubes of LB medium with *amp* antibiotic and incubated overnight at 37°C. Plasmid DNAs were purified using a S.N.A.P. MiniPrep Kit (Invitrogen). Colonies were screened for inserts by PCR as following: each 10µL reaction contained miniprepped 1.5 µL plasmid DNA as template, along with 250µg/mL BSA (Bovine Serum Albumin), 10X PCR reaction buffer, 10 mM each T3 and T7 primers, 4.17mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 units/µL *Taq* DNA polymerase (Fisher Brand), and dH_20. PCR was conducted in a DNA Dyad Thermalcycler (MJ Technologies) with the following profiles: 2 minute hot start at 95°C, followed by 35 cycles of 20 seconds at 95ºC, 20 seconds at 50°C, and 1.5 minutes at 72ºC, with a final extension step of 10 minutes at 72°C.

Electrophoresis was conducted with 5µL aliquots of PCR product using a 3% SB agarose gel containing ethidium bromide and SB buffer at 300 V for 10 minutes. PCR

products containing an insert were cleaned by column centrifugation using Princeton separation columns with Sephadex® G-50 (Sigma). The clean PCR products were quantified by spectrophotometer and saved for cycle sequencing. Sequencing reactions were conducted using ¼ reactions with BigDye® v 3.1 cycle sequencing kit (ABI). Reactions consisted of 2µL BigDye® master mix, 2µL 10 µM T7 sequencing primer, 6µL 2.5x sequencing buffer, and ~40-80ng of clean PCR product + HPLC water to make a total volume of 20µLs. Samples were cycled 55-75 times in a DNA Dyad Thermalcycler (MJ Technologies) according to manufacturer's suggestion.

Following the cycle sequencing reaction, products were again cleaned by Sephadex® G-50 column centrifugation and placed into a DNA SpeedVac on medium heat for about 30 minutes or until dry. The dry samples were reconstituted with 10µL of DI formamide, transferred to a 96 well plate, denatured for 2 minutes at 95°C, and snap cooled on ice. Sequences were analyzed on an ABI PRISM® 3100-Avant Genetic Analyzer following the manufacturer's settings.

2.7 Primer design and selection

Twenty microsatellite primers were designed using Oligo 6.6 (Molecular Biology Insights, Cascade, CO) and ordered from the Penn State Nucleic Acid Facility (Penn State University, University Park, PA). Each microsatellite locus was screened in six *A. nebulosus* and three *A. melas* "pure" parental type specimens by PCR and electrophoresis. PCR conditions were optimized by altering $MgCl_2$ concentrations and/or annealing temperatures. To check for amplification, 5 µL of PCR product was loaded

onto a 2% SB agarose gel and electrophoresed in 1X SB buffer for 45 minutes at 150 V. Primers were then chosen to be fluorescently labeled for genotyping based on non-overlapping allele sizes between the parental type specimens.

2.8 Fluorescent Primer optimization, selection, multi-plexing, and genotyping

Nine fluorescently labeled microsatellite primer sets for *A. nebulosus* were designed (Table A2). The alleles ranged in size from 160-300 base pairs in length.

PCR conditions were optimized by altering $MgCl_2$ concentrations and/or annealing temperatures. The optimized PCR conditions for each individual locus can be found in the appendix. Each PCR reaction used 12 ng of DNA. To check for amplification, 6 µL of PCR product was loaded onto a 2% SB agarose gel and electrophoresed in 1X SB buffer for 15 minutes at 300 V.

Of the nine loci, five were selected to be the primary markers for this study (Aneb16, Aneb37, Aneb61, Aneb63, and Aneb64). These diagnostic loci were then applied to the remaining reference, Presque Isle Bay, Lake Erie, Tamarack Lake, and Wisconsin specimens. Multiplexing was performed with the Aneb37 and Aneb64 primer pairs in one reaction and Aneb61 and Aneb63 in another (Table 2 A-B). Aneb16 was performed separately (Table 2 C). The optimized multiplex and single PCR reactions were used to genotype a total of 248 bullheads.

Fluorescently labeled PCR product was then prepared for fragment analysis on the ABI PRISM® 3100-Avant Genetic Analyzer. A size standard of GeneScan-500 LIZ (Applied Biosystems) was run with each sample. Samples for fragment analysis

consisted of 0.5 µL of LIZ size standard, 9.5 µL of formamide and 0.5 µL of fluorescently labeled PCR product and were loaded into each well of a 96-well plate. Once all PCR products were added, the plate was denatured at 95^{o} C for 2 minutes and snap cooled on ice. The plate was mounted on the ABI PRISM® 3100-Avant Genetic Analyzer and programmed for fragment analysis according to the manufacturer. The data were analyzed using GENESCAN, and genotypes were recorded. Genotypic data of the five loci were run through Hardy-Weinberg exact tests, linkage disequilibrium tests, allele frequency tests and the F-statistics F_{IS} and F_{ST} using the population genetics software GENEPOP (Raymond and Rousset 2004).

Figure 3. Presque Isle Bay collection sites; 1) Sara's Cove located at the head of the bay, 2) the lagoons, a series of connected ponds, and 3) Thompson's Bay located in the outer harbor.

Figure 4. Lake Erie collection sites: 4) Old Woman Creek, Ohio, 5) Long Point Bay, Ontario, 6) Dunkirk Harbor, New York, and 7) Presque Isle Bay, Pennsylvania.

Table 1. Morphological characters recorded from specimens of *Ameiurus nebulosus* and *Ameiurus melas.*

Mensural variable	**Mnemonic**	**Corrected by ***
Standard length	SL	
Head length	HL	A
Head width	HW	B
Postorbital head length	POHL	B
Interorbital width	HED	B
Interorbital height	VED	B
Preorbital length	PRE	B
Cheek depth	CD	B
Lower jaw length	LJL	B
Head depth	HD	B
Body depth	BD	A
Distance from snout to dorsal fin insertion	SNDOR	A
Distance from snout to pelvic fin insertion	SNPEL	A
Dorsal fin base length	DFBL	A
Distance from anterior dorsal fin to anterior anal fin	ADAA	A
Distance from anterior dorsal fin to posterior anal fin	ADPA	A
Distance from posterior dorsal fin to anterior anal fin	PDAA	A
Distance from posterior dorsal fin to posterior anal fin	PDPA	A
Distance from posterior dorsal fin to ventral point of least caudal peduncle	PDVC	A
Distance from posterior anal fin to dorsal point of least caudal peduncle	PADC	A
Distance from anterior dorsal fin to insertion of pelvic fin	ADP2	A
Distance from posterior dorsal fin to insertion of pelvic fin	PDP2	A
Caudal peduncle length	CPL	A
Least caudal peduncle length	LCPD	A
Anal fin base length	AFBL	A
Meristic variable	**Mnemonic**	
Dorsal fin rays	drays	
Anal fin rays	arays	
Pectoral fin rays	P1rays	
Pelvic fin rays	P2rays	
Epibranchial gill raker	EGR	
Ceratobranchial gill raker	CGR	

* All measurements except SL were corrected for the size of the fish by either dividing by SL denoted as A or by dividing by HL denoted as B.

Table 2. Optimized Multiplexing Conditions for Selected Loci

A. Aneb37 and Aneb64 primer sets multiplex

multiplex	1 rxn	Thermocycling	conditions	
10X buffer B	1.92 µl	Step	Temp °C	Time
dNTP [1.25 mM]	2.0 µl	Denature	95	30 s
$MgCl_2$ [1.50 mM]	0.792 µl	Annealing	57	30 s
Aneb37F-PET [0.01mM]	0.3 µl	Extension	72	1 m
Aneb37R [0.01 mM]	0.3 µl	Cycles	32	
Aneb64F-FAM [0.01mM]	0.3 µl	Final Extension	72	2 m
Aneb64R [0.01 mM]	0.3 µl	Incubate	15	forever
Taq [5U/µl]	0.11 µl			
HPLC water	3.998 µl			
DNA template	2 µl			
Total	12 µl			

B. Aneb61 and Aneb63 primer sets multiplex

mulitplex	1 rxn	Thermocycling	conditions	
10X buffer B	1.92 µl	Step	Temp °C	Time
dNTP [1.25 mM]	2.0 µl	Denature	95	30 s
$MgCl_2$ [1.50 mM]	0.792 µl	Annealing	57	30 s
Aneb61F-FAM [0.01mM]	0.3 µl	Extension	72	1 m
Aneb61R [0.01 mM]	0.3 µl	Cycles	32	
Aneb63F-NED [0.01mM]	0.3 µl	Final Extension	72	2 m
Aneb63R [0.01 mM]	0.3 µl	Incubate	15	forever
Taq [5U/µl]	0.11 µl			
HPLC water	3.998 µl			
DNA template	2 µl			
Total	12 µl			

C. Aneb16 primer

	1 rxn	Thermocycling	conditions	
10X buffer B	1.20 µl	Step	Temp °C	Time
dNTP [1.25 mM]	2.0 µl	Denature	95	30 s
$MgCl_2$ [2.25 mM]	1.19 µl	Annealing	50	30 s
Aneb16F-FAM [0.01 mM]	0.3 µl	Extension	72	1 m
Aneb16R [0.01 mM]	0.3 µl	Cycles	32	
Taq [5 U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	4.004 µl	Incubate	15	forever
DNA template	2 µl			
Total	12 µl			

Chapter 3

Meristics and Morphometrics

3.1 Meristics - Principal Component Analysis

Meristic differences were analyzed using principal component analysis (PCA). Principal component analysis is a multivariate ordination technique commonly used for examining morphological variables and to differentiate closely related species (Stauffer et al. 1997). Principal component analysis identifies patterns in a data set and eliminates redundancy in univariate analysis when mulitcollinear data are involved (Iezzoni and Pritts 1991). The main purpose of PCA is to convert a number of correlated variables into a smaller set of components of the original variables called principal components with minimum loss of information. Each set is uncorrelated with any other set, but components with in the set are related. This is done by creating linear combinations of the original variables, which are oriented in directions along continuous gradients defined by the principal components and seeks to describe the sources of greatest variation among entities (McGarigal et al. 2000). The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible (McGarigal et al. 2000). Principal component analysis compares the sources of greatest variation in the data set and

produces scores for each individual. Morphological relationships are determined by comparing these scores to the scores of other individuals also contained in the data set.

3.2 Morphometrics - Sheared Principal Components Analysis

Sheared principal component analysis (SPCA) is effective in identifying shape differences among the populations independent of size (Reyment et al. 1984) and was used to assess the head, body and fin variation. Sheared principal component analysis ordinates morphometric data independently of a main ordination, allowing for the mensural variables to be analyzed independent of size (Reyment et al. 1984). The first principal component identifies size differences while succeeding sheared principal components, being independent of size, detect shape (Brookstein et al. 1985, Humphries et al. 1981). Sheared principal component analysis was used by Stauffer (1991) to distinguish among *Pseudotropheus pursus* Stauffer, *P.lanisticola* Burgess, and *P. livingstonii* (Boulenger) from Lake Malawi and by Stauffer et al. (1997) to describe a new genus of North American minnows *Pararhinicthhys*, which arose from intergentic hybridization events between *Rhinichthys cataractae* and *Nocomis micropogon*.

3.3 Reference Specimens

Principal component analysis was conducted on the meristic data. The clusters were formed by plotting the second principal component of the meristic data, PC2, against the first principal component of the meristic data, PC1 (Figure 5). The non-

overlapping of the two minimum polygon clusters, generated from the principal components plots, illustrates the difference in the meristic data that distinguish *A. nebulosus* and *A. melas*. Variable loadings on these two factors are listed in Table 3. The three factors accounting for 2.3 %, 1.16 % and 0.99 % of the variability follow respectively. Gill rakers on first epibranchial, gill rakers on first ceratobranchial, and anal fin rays, account for almost all of the variability in PC1, while pectoral fin (p1) rays and dorsal fin rays account for the majority in PC2.

Sheared principal component analysis was then conducted on the mensural variables of the reference specimens. The first principal component of the SPCA (SPC2) of the mensural data was plotted against the second sheared principle component of the SPCA (SPC3) of the mensural data (Figure 6) to assess the ability of the mensural data in detecting shape differences. Minimum polygon clusters were then made for the reference specimens to determine the differences for *Ameiurus nebulosus* and *A. melas*. A minimum polygon cluster is a closed figure on the two dimensional plot that includes the spatial data points of all individuals belonging to a particular sample. The non-overlapping of the two minimum polygon clusters, generated from the principal components plots demonstrates the difference in the mensural data that distinguish *A. nebulosus* and *A. melas*. The variable loadings SPC2 and SPC3 are listed in Table 4. The first principal component of the morphometric data, which is a size component, accounted for 89.9 % of the total variance while first and second sheared principal components accounted for 2.3% and 1.6% of the remaining 10.1 % variance, respectively. Variables that had highest loadings on the first sheared principal component were cheek depth (0.258), preorbital distance (0.244) and lower jaw length (0.241). Snout

to pelvic fin distance accounted for the most variability on the second sheared principal component.

Minimum polygon clusters were formed by plotting the first principal component (PC1) of the meristic data against the first sheared principal component (SPC2) of the morphometric data (Figure 7). This plot yields no overlap between the minimum polygon clusters of the reference specimens of *A. nebulosus* and the reference specimens of *A. melas* and show differences between the two taxa. This technique was used for the remaining collections including the fifty-six reference specimens and either one collection or as all collections in Presque Isle Bay, Lake Erie, or Tamarack Lake.

3.4 Presque Isle Bay Collections

A plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from a data set including reference specimens of *A. nebulosus* and *A. melas* and the collections from Presque Isle Bay were plotted individually and pooled against the reference specimens.

Collections from Sara's Cove contained individuals very similar morphologically to the reference specimens of *A. nebulosus* along both the PC1 (meristic data) and SPC2 (mensural data) axes (Figure 8). Almost all individuals from these collections fell in close proximity to the cluster or in the minimum polygon cluster for reference specimens of *A. nebulosus*. An individual is considered as falling within a minimum polygon cluster if a data point is within the boundaries of the polygon or touching any side of the polygon. Those individuals falling above and outside the range of PC1 and SPC2 for *A. nebulosus*

may suggest heterosis in Sara's Cove. Collections from the lagoons (Figure 9) and Thompson's Bay (Figure 10) each include one individual whose PC1 value fall outside of the range for *A. nebulosus* and within the range of *A. melas*. When all the Presque Isle Bay specimens are pooled together and plotted (Figure 11), most all the individuals fall in close proximity to the cluster or in the minimum polygon cluster for the reference specimens of *A. nebulosus*, but does contain an individual whose PC1 value falls outside of the range for *A. nebulosus* and within the range of *A. melas*.

3.5 Lake Erie Collections

A plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data form a data set including reference specimens of *A. nebulosus* and *A. melas* and all of the collections from Lake Erie were plotted individually and pooled against the reference specimens. Collections from Old Woman Creek, Ohio, contain a few individuals whose PC1 value falls outside of the range for *A. nebulosus* and within the range of *A. melas*. Most individuals fell in close proximity to the cluster or in the minimum polygon cluster for reference specimens of *A. nebulosus* (Figure 12). Collections from Long Point Bay, Ontario Canada have individuals very similar morphologically to the reference specimens of *A. nebulosus* along both the PC1 and SPC2 axes (Figure 13). Collections from Dunkirk Harbor, New York includes one individual whose PC1 values fall outside of the range for *A. nebulosus* and within the range of *A. melas*. Most individuals fell in close proximity to the cluster or in the minimum polygon cluster for reference specimens of *A. nebulosus* (Figure 14).

When all the Lake Erie specimens are pooled and plotted along with the reference specimens (Figure 15), there are many more individuals whose PC1 values fall outside of the range for *A. nebulosus* and within the range of *A. melas*.

3.6 Tamarack Lake – Inland Brown Bullhead Collection

A plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data form a data set including reference specimens of *A. nebulosus* and *A. melas* and the collection from Tamarack Lake is shown in Figure 16. Almost all individuals from these collections fell in close proximity to the cluster or in the minimum polygon cluster for reference specimens of *A. nebulosus* along both the PC1 and SPC2 axes.

3.7 Wisconsin – Black Bullhead Population

A plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data form a data set including reference specimens of *A. nebulosus* and *A. melas* and the collections of *A. nebulosus* from Tamarack Lake and *A. melas* from Wisconsin is shown in Figure 17. Almost all brown bullheads from Tamarack Lake fell in close proximity to the cluster or in the minimum polygon cluster for reference specimens of *A. nebulosus*. In contrary, all Black Bullheads but one from the Wisconsin population has SPC2 value that fell outside of the range for *A. melas* and within the range of *A. nebulosus*.

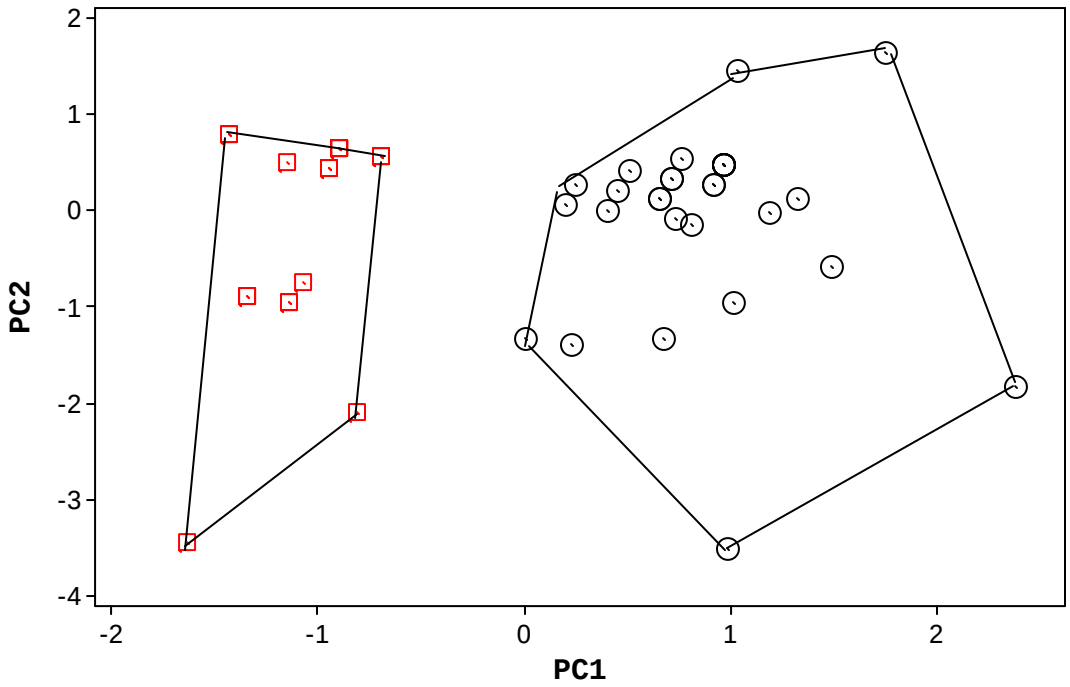

Figure 5. Plot of the first and second principal components, PC1 and PC2 respectively, derived from the principal component analysis of the reference specimens of *Ameiurus nebulosus* (squares) and *A. melas* (circles) using the meristic data.

Table 3. Variance loadings of the meristic characters on the first two principal components describing variation in fin ray counts and gill rakers of the reference specimens of *Ameiurus nebulosus* and *A. melas*.

Character	PC1	PC2
Dorsal fin rays	0.32465	0.56980
Anal fin rays	-0.76364	-0.20202
Pelvic fin rays	-0.32276	0.26438
Pectoral fin rays	0.20522	0.77254
Gill raker count on first epibranchial	0.87536	-0.33094
Gill raker count on first ceratobranchial	0.83773	-0.14656

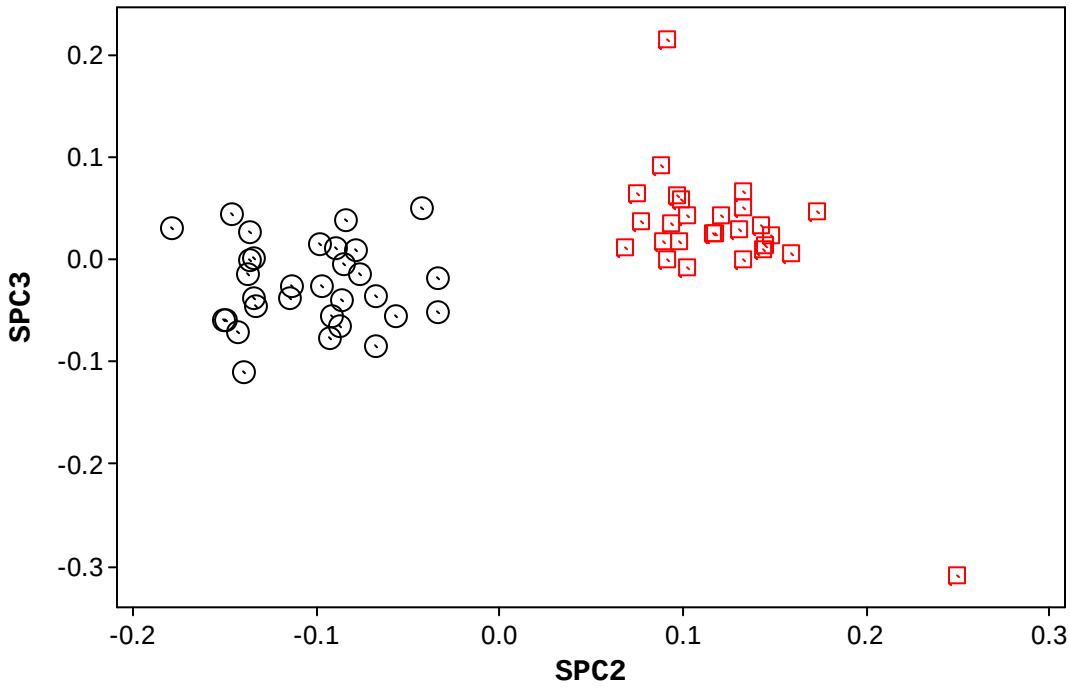

Figure 6. Plot of the first and second sheared principal component, SPC2 and SPC3 respectively, derived from the sheared second principal component analysis of the reference specimens of *Ameiurus nebulosus* (squares) and *A. melas* (circles) using the mensural data.

Table 4. Variance loadings of the mensural characters on the first two sheared principal components describing variation in shape of the reference specimens of *Ameiurus nebulosus* and *A. melas.*

Character	**SPC2**	**SCP3**
SL	0.04750	-0.12354
HL	-0.04957	-0.05888
HW	-0.13415	-0.05297
POHL	-0.04070	-0.21031
HED	0.01585	0.38523
VED	0.01700	0.19233
PRE	-0.22750	0.70155
CD	-0.37289	0.04866
LJL	-0.27757	-0.27279
HD	0.04163	0.06032
BD	0.19364	0.07696
SNDOR	-0.07245	-0.08943
SNPEL	0.67590	0.12456
DFBL	0.09837	-0.23889
ADAA	0.08872	0.01544
ADPA	0.08603	-0.12868
PDAA	0.09118	0.03520
PDPA	0.11655	-0.11772
PDVC	0.07988	-0.06405
PADC	0.12900	0.02364
ADP2	0.20695	0.04013
PDP2	0.21529	0.11873
CPL	0.04502	-0.10634
LCPD	0.17280	0.04409
AFBL	0.12244	-0.15568

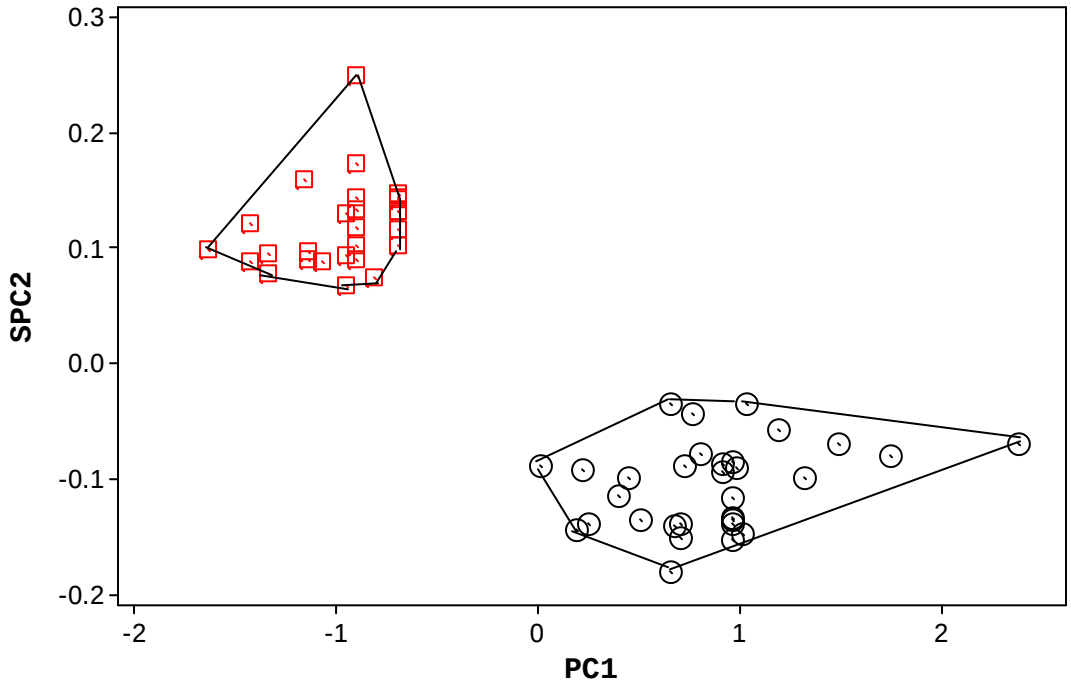

Figure 7. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares) and *A. melas* (circles).

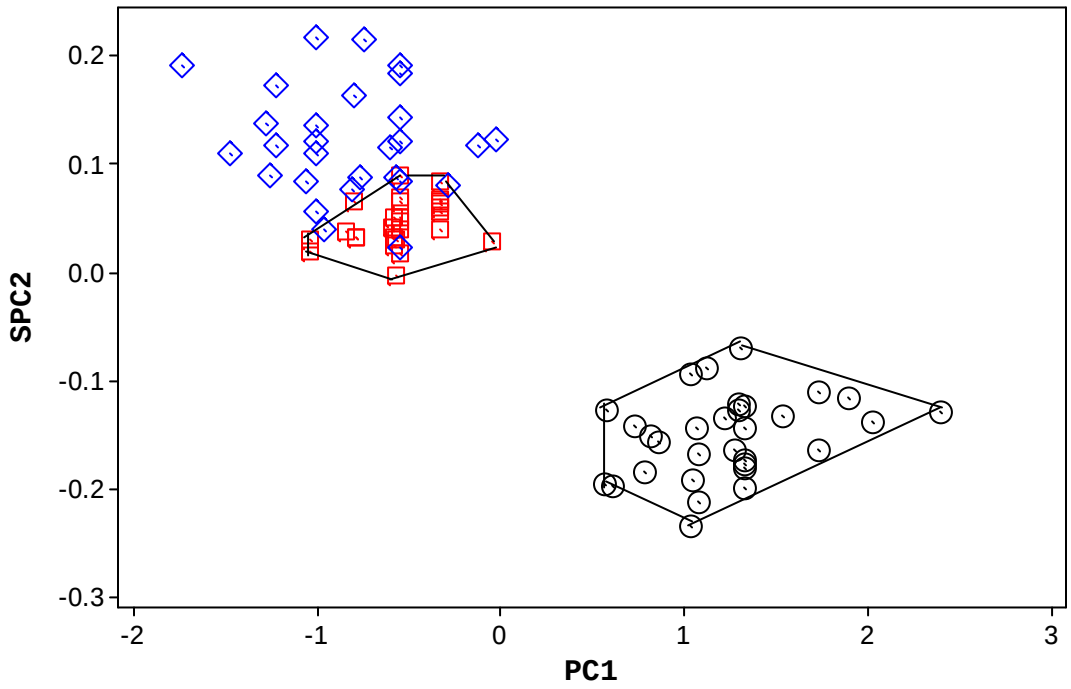

Figure 8. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles) and Sara's Cove (diamonds).

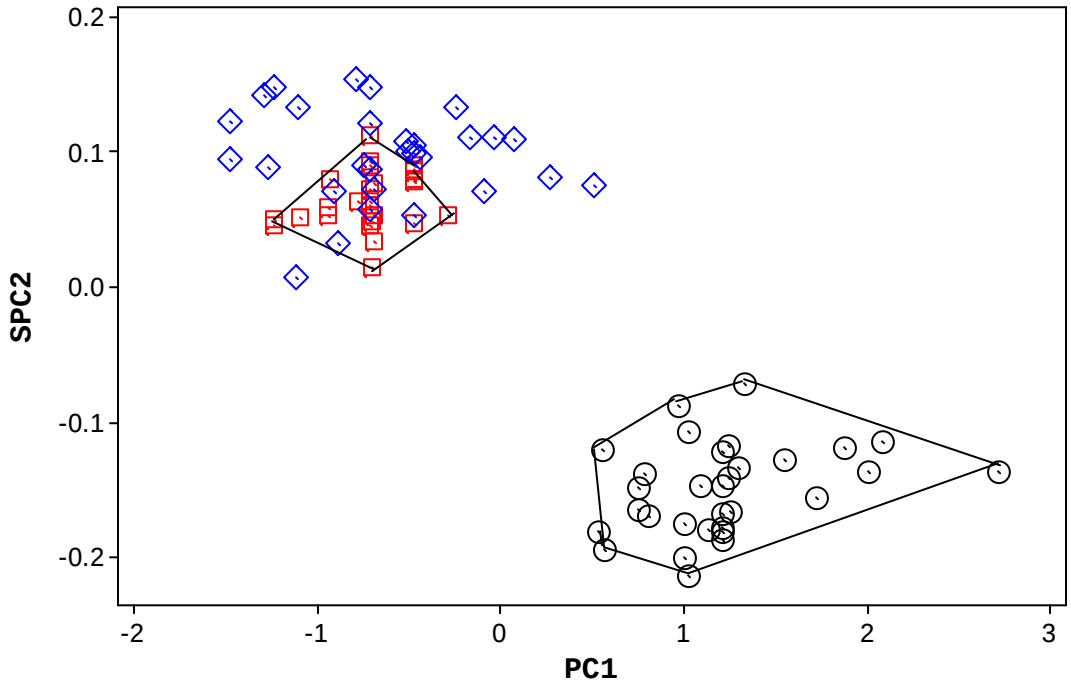

Figure 9. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles) and lagoons (diamonds).

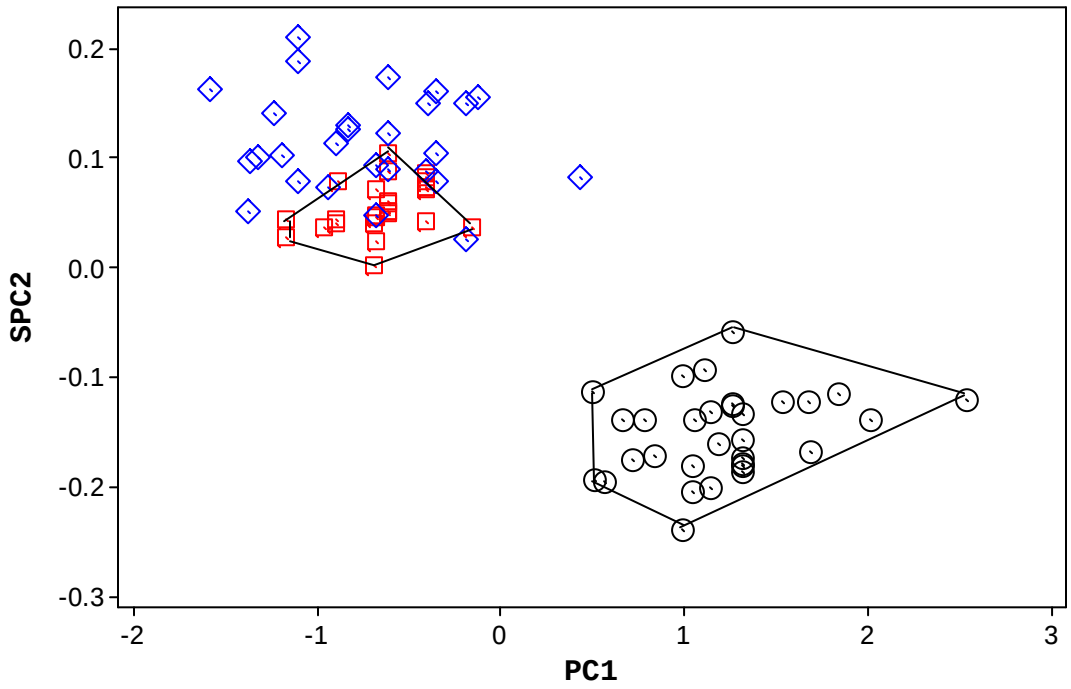

Figure 10. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles) and Thompson's Bay (diamonds).

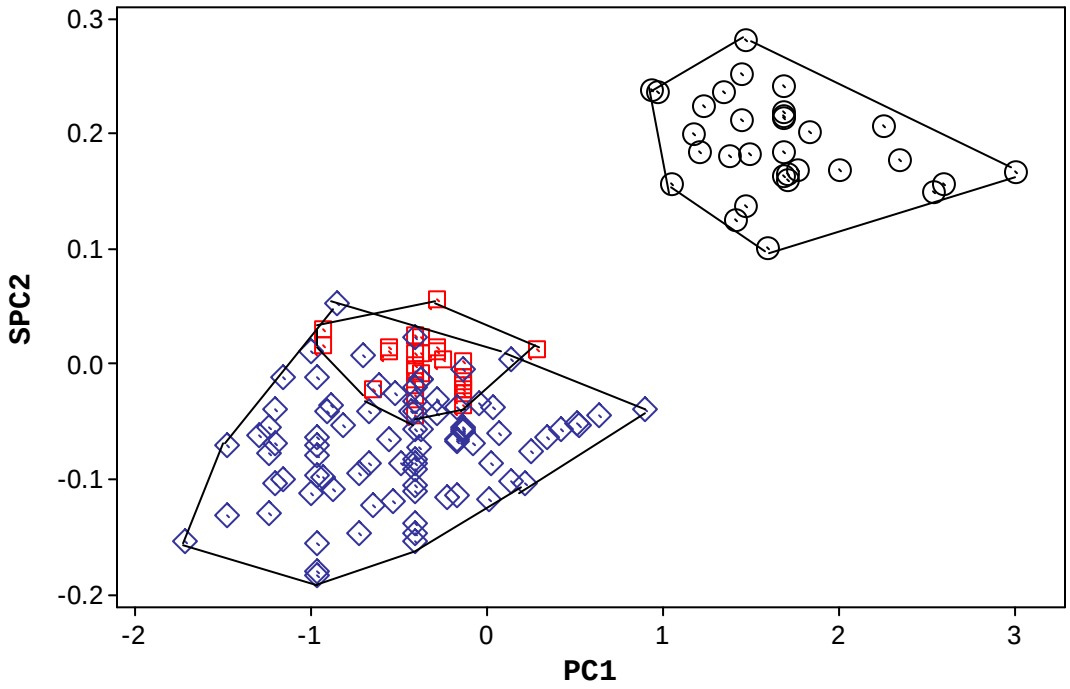

Figure 11. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Presque Isle Bay collections (diamonds).

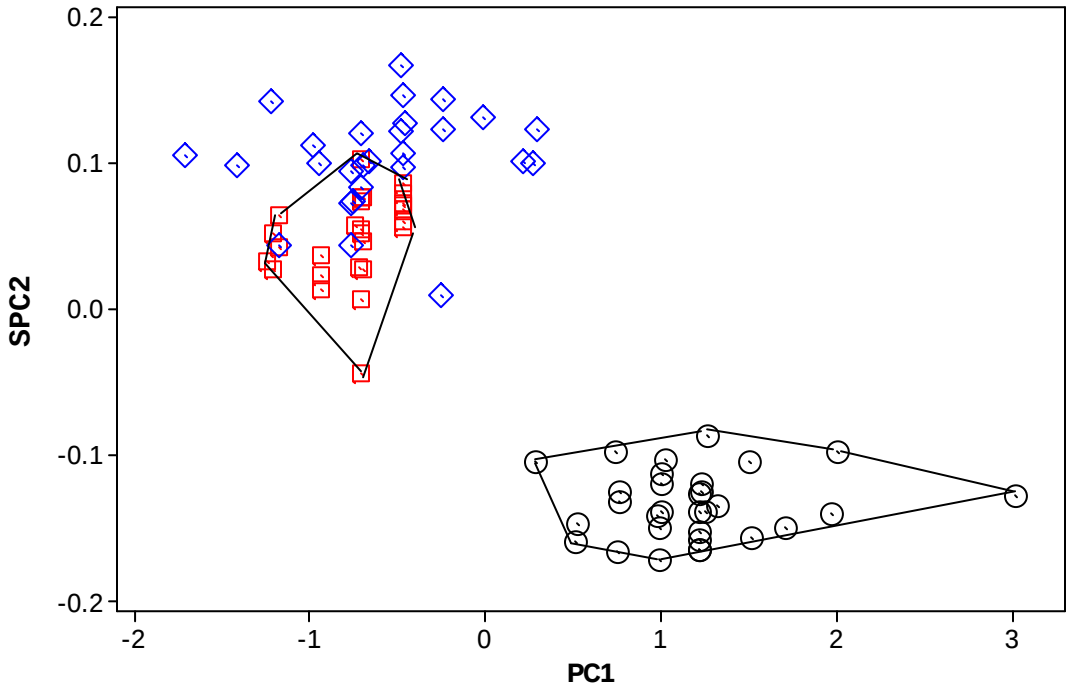

Figure 12. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Old Woman Creek, Ohio collections (diamonds).

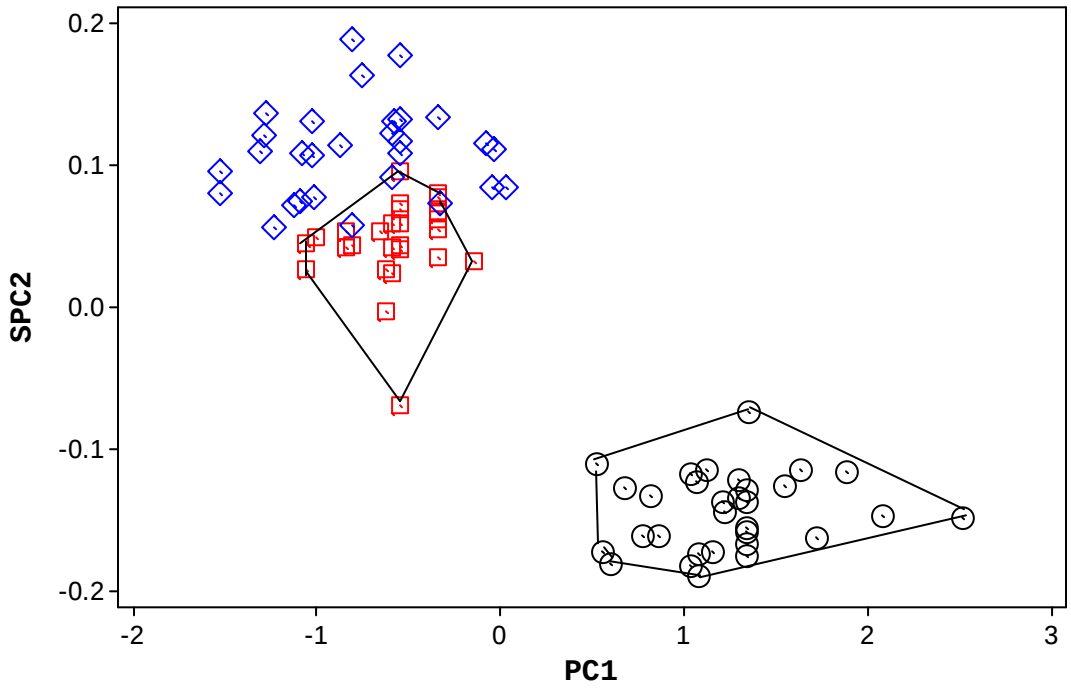

Figure 13. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Long Point Bay, Ontario, Canada collections (diamonds).

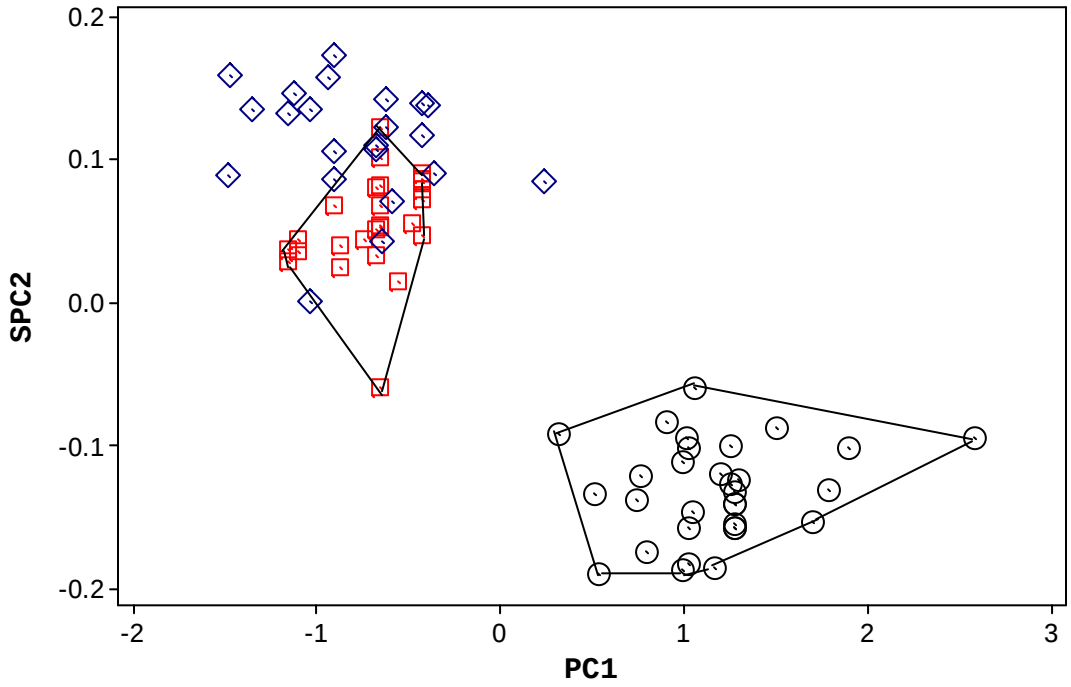

Figure 14. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Dunkirk Harbor, New York collections (diamonds).

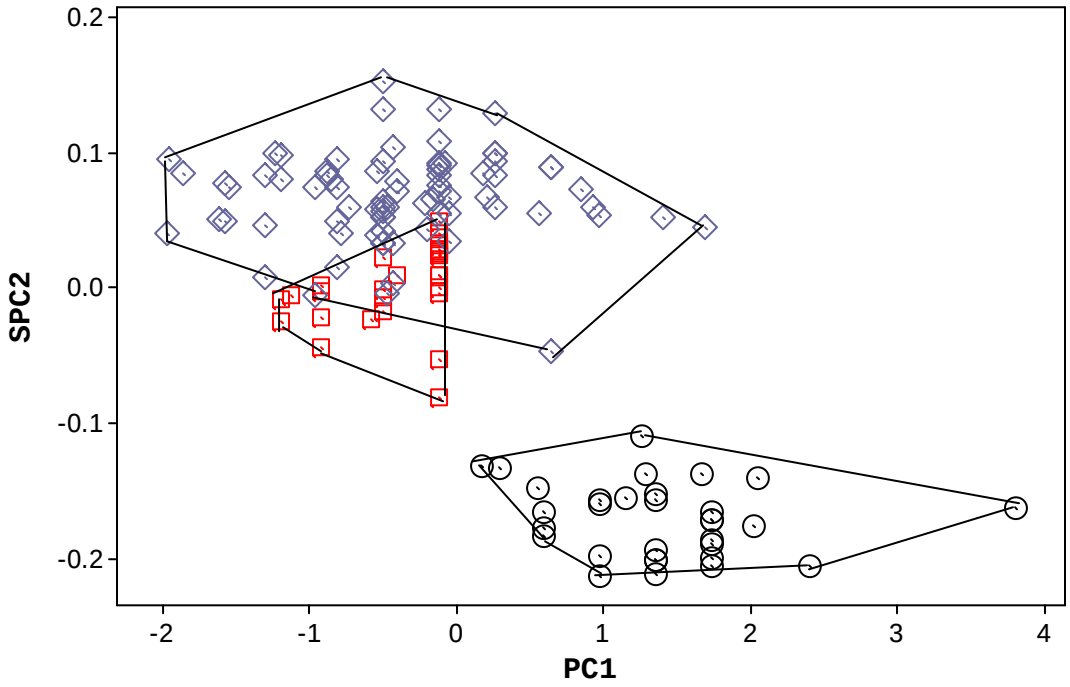

Figure 15. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Lake Erie collections (diamonds).

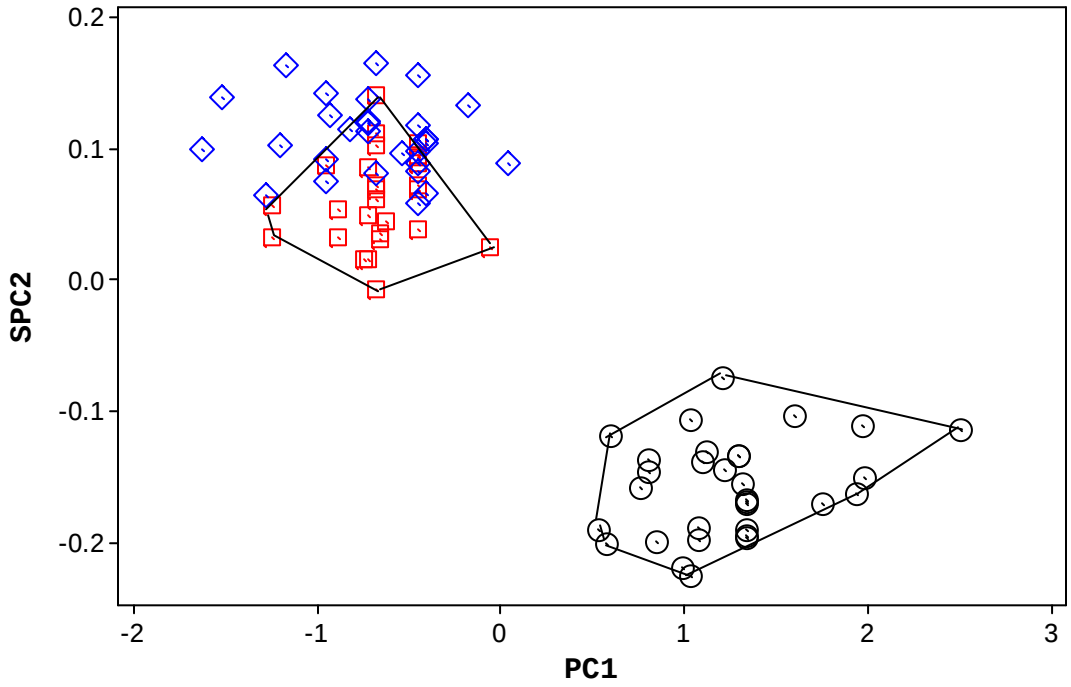

Figure 16. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), and Tamarack Lake (diamonds).

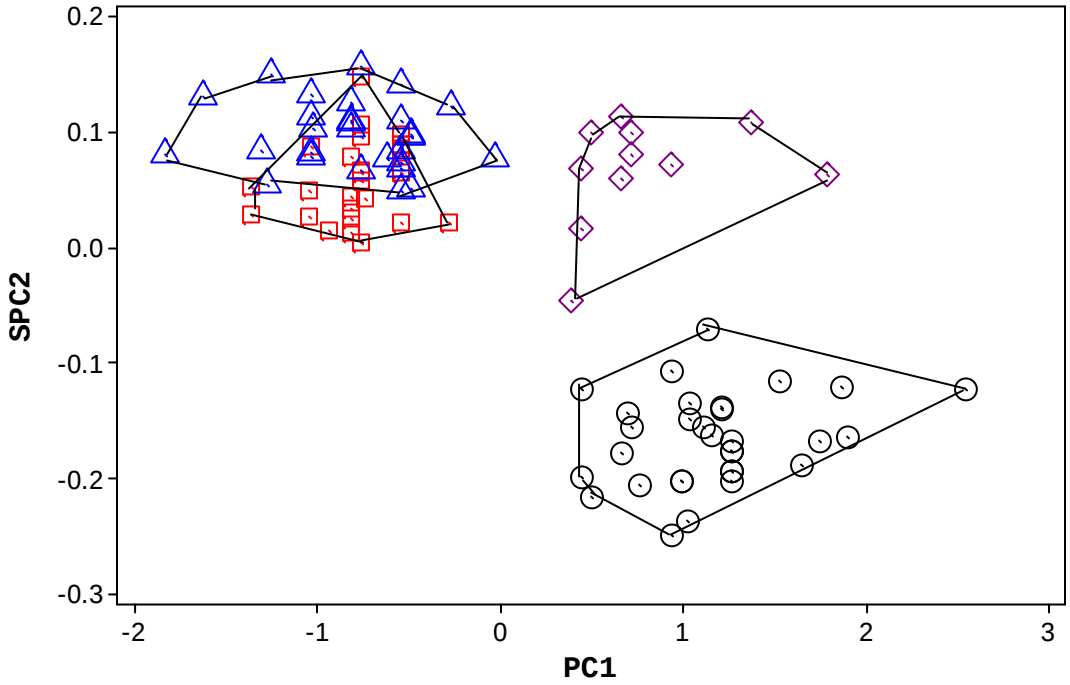

Figure 17. Plot of the first principal component (PC1) of the meristic data and the first sheared principal component (SPC2) of the mensural data from the reference specimens of *Ameiurus nebulosus* (squares), *A. melas* (circles), Tamarack Lake (triangles) and Wisconsin (diamonds).

Chapter 4

Microsatellites

4.1 Introduction to Microsatellites

Microsatellites were used to estimate the genetic structure of the two bullhead species and characterized the extent of potential hybrid populations. Microsatellites are extremely important markers for revealing genetic variation at population levels and between closely related species because of their high polymorphism, distribution across the genome, abundance, co-dominant inheritance pattern, and their short length, which facilitates genotyping by polymerase chain reaction (Lui et al. 1999a). Alleles are distinguished by size through electrophoresis. Prior studies have used microsatellites as genetic markers to estimate gene flow, effective population size, and inbreeding, as well as in parentage determination (Lui et al. 1999b, and Waldibieser and Bosworth 1997).

Microsatellites are tandem repeats of 1-6 nucleotides found at high frequency in the nuclear genomes of most taxa (Lai and Sun 2004). A microsatellite locus usually varies in length between 5 and 40 repeat units long, but longer strings of repeats are possible. Dinucleotide, trinucleotide, and tetranucleotide repeat motifs are the most common choices for molecular genetic studies. The differing numbers of repeats observed at polymorphic loci between two homologous chromosomes within an individual and between different individuals represent microsatellite alleles.

The DNA adjacent to a microsatellite locus is termed the flanking region. Because the sequence of flanking regions for a given locus are generally conserved (i.e. identical) across individuals of the same species and sometimes different species, a particular microsatellite locus can often be identified by its unique flanking sequences. Short stretches of synthesized DNA, called oligonucleotides or primers, can be designed to bind to specific flanking regions and guide the amplification of a microsatellite locus with the polymerase chain reaction (PCR). These ideal markers allows for the use of small tissue samples and can be amplified with PCR despite some DNA degradation (Selkoe and Toonen 2006).

Microsatellites DNA loci are highly unstable and mutate at high rates compared to other genetic markers (Goldstein and Pollock 1997). While the exact mechanism of mutation at such loci is still not well characterized at a molecular level, it is generally believed that the process and patterns of mutation at different loci may differ from locus to locus, depending on the motif as well as the size of alleles at each locus (Xu and Fu 2004). The instability of these DNA regions may result from DNA polymerase slippage as well as unequal recombination. During replication, dissociation, and subsequent reannealing of the DNA strands, one or more of the repeats is unpaired and forms a single stranded loop; a process called slippage. This can result in either the addition or deletion of a repeat unit, depending on whether the looped strand is located on the template or replicating strand. If this mistake is not corrected by the proof reading mechanism of DNA polymerase, it will remain as a mutation at that locus, and the alleles will differ in size by having different numbers of repeat units. It is not uncommon for an individual to have two different-sized microsatellite alleles between its two homologous chromosomes,

making it a heterozygote for that locus. One strength of microsatellite markers is that they are co-dominant, such that both alleles of a heterozygote are visualized under normal conditions.

Experimental and theoretical studies indicate that for most microsatellite loci, mutations lead to stepwise changes of the repeat size of alleles through the rate of mutation leading to expansion may not be equal to that of contraction of allele size. The stepwise mutation model, originally proposed for the study of protein charge changes in a more generalized form may be more suitable for the study of most microsatellite loci (Xu and Fu 2004). The stepwise mutational model adds or subtracts one or more repeat units from the string of repeats at some constant rate to mimic the process of errors during DNA replication that generates mutations, creating a Gaussian-shaped allele frequency. However, non-stepwise mutation processes are also known to occur, including point mutation and recombination events such as unequal crossing over and gene conversion. While debate continues about the prevalence of non-stepwise mutations for microsatellites, the current consensus is that the frequency and effects are usually low, and the stepwise mutation appears to be the dominant force creating new alleles in the few model organisms studied to date (Selkoe and Toonen 2006).

When analyzing microsatellite data it is important to determine if microsatellite allele frequencies fall within Hardy-Weinberg equilibrium and do not violate the assumptions of random mating, no genetic drift, no mutation, no migration, and no natural selection. Non-random mating tends to reduce genetic variation. Random mating means that alleles as carried by the gametes come together strictly in proportion to their frequencies in the population as a whole. Situations where the random mating assumption

does not hold include: inbreeding, geographic structures, assortative mating, rare allele advantages, and mating system effects (Graur and Li 2000). Random genetic drift removes genetic variation from the population at a rate inversely proportional to population size. Mutation is the process that produces a new allele that is different from the ancestral allele. Mutation restores genetic variation to a population by producing novel alleles. Mutation is difficult to measure or observe directly, and rates of mutation can vary between loci. Genetic migration (gene flow) is the permanent movement of genes from one population into another. Migration can restore genetic variation into isolated and differentiated populations or homogenize allele frequencies between populations when it occurs frequently (Graur and Li 2000). Selection is the differential survival and reproduction of phenotypes that are better suited to the environment or to obtaining mating success and is the evolutionary force responsible for adaptation to the environment. Microsatellites are not usually considered to be under positive or negative selection, since they are non-coding regions and different sized alleles are believed to be effectively neutral.

Deviations from Hardy-Weinberg equilibrium may also be caused by the presence of null alleles. One method to detect such deviations is to compare the expected levels of heterozygosity to the observed levels of heterozygosity of alleles at a locus within a population. A null allele is any allele at a locus that consistently fails to amplify to detected levels through PCR (Dakin and Avise 2004). When null alleles occur, any genotype observed as a homozygote may contain one observable allele and one null allele and the genotypes observed may therefore be scored as a homozygote when in effect it is a heterozygote. This can lead to observed heterozygosity that is lower than expected by

Hardy-Weinberg equilibrium. One may either choose to ignore the problem, drop the affected loci from consideration, or redesign and optimize the primers to eliminate null alleles (Dakin and Avise 2004). Although null alleles lead to underestimated heterozygosity within samples, it is a minor source of error in estimating heterozygosity excess (Dakin and Avise 2004). The occurrence of null alleles is widely acknowledged and many papers report the results of diagnostic tests for the presence of null alleles (Dakin & Avise 2004), but options for dealing with null alleles are limited.

The program GENEPOP (Raymond and Rousset 1995) was used to calculate observed (H_o) and expected (H_e) heterozygosity, linkage disequilibrium, and p-values for the exact Hardy-Weinberg test associated with H_o and Hardy-Weinberg equilibrium. The program ML-Relate was used to test for the presence and frequency of null alleles (Kalinowski and Taper 2006).

4.2 Genetic Characteristics

When selecting microsatellite loci for a hybridization study, it is often possible and desirable to identify specific loci that have alleles that do not overlap in size between pure reference populations of the two species under study. It is therefore possible to come up with a set of alleles that are unique to each species. It is possible to distinguish between the types of hybridization present in Presque Isle Bay and the Lake Erie sites because each type will leave a characteristic genetic signature. Because microsatellite loci with non-overlapping allele size ranges were selected for this study (Figures 18 – 22), each genotyped individual can be scored for the frequency and pattern of "*melas*" alleles

and "*nebulosus*" alleles. If an individual is the F1 progeny of *A. nebulosus* and *A. melas*, all loci will be heterozygous; with one *nebulosus* allele and one *melas* allele, with the net frequency of 0.5 "*nebulosus*" alleles and 0.5 "*melas*" alleles.

If the F1 hybrids are breeding with themselves to produce F2 progeny, their offspring will be a random combination of the two species' alleles, and therefore some loci may be homozygous for "*nebulosus*" alleles, while others will be homozygous for "*melas*" alleles, and still others will be heterozygous for the two species. These three allelic categories should be likely with 0.25 of the loci being all *nebulosu*s, 0.25 being all *melas*, and 0.50 being heterozygous for the two species. F2 individuals created by two F1 hybrids will have an overall frequency across all loci of 0.5, because they are a random recombination of F1 individuals that have overall frequency of 0.5 for their pooled alleles across all loci. F2 individuals are distinguished form F1 individuals by having some loci that are homozygous for one or both parental species, but an overall frequency of approximately 0.5

If the hybrids have backcrossed with the pure parental species, they will be skewed in the direction of the species in which they backcrossed. For example, if an F1 hybrid backcrossed with a pure *nebulosus*, approximately half of the loci will be homozygous for "*nebulosus*" alleles but a few loci will have "*melas*" alleles in the heterozygous state. Overall, a backcrossed individual will have 75% or more of its alleles from its parental species.

4.3 Polymorphism, Heterozygosity, and Hardy-Weinberg Equilibrium- Reference specimens

Nine microsatellite markers were considered for this study. Five of these nine original loci were used (Aneb16, Aneb37, Aneb61, Aneb63, and Aneb64) by screening the fluorescent fragments generated by PCR against the two pure populations analyzed with GENESCAN software (Applied Biosystems). According to the results from GENEPOP v. 3.4 (Raymond and Rousset 1995) none of the loci showed linkage disequilibrium. Observed heterozygosity for *Ameiurus nebulosus* ranged from 0.077 (locus Aneb64) to 0.778 (Aneb16 and Aneb37), with an average of 0.483. Hardy-Weinberg equilibrium was tested with GENEPOP v. 3.4 (Raymond and Rouset 1995) with departure from Hardy-Weinberg equilibrium in two loci (Aneb64 and Aneb63). Observed heterozygosity for *Ameiurus melas* ranged from 0.143 (locus Aneb61) to 0.793 (Aneb16), with an average of 0.495. Hardy-Weinberg equilibrium was tested with GENEPOP v. 3.4 (Raymond and Rouset 1995) with departure from Hardy-Weinberg equilibrium in one locus (Aneb64).

The loci were then analyzed for the presence of null alleles using the software program ML-Relate (Kalinowski and Taper 2006) and two loci (Aneb63 and Anb64) had an estimated frequency of null alleles in the population of over 0.10. Because the null alleles had a higher frequencies in *A. nebulosus* for both of these loci, a frequency of *A. melas* alleles may have been overestimated (Table 5). Aneb63 and Aneb64 were used for the scoring, and then omitted from data set for the rescoring of specimens for the Presque Isle Bay and Lake Erie populations.

4.4 Polymorphism, Heterozygosity, and genetic distances- Presque Isle Bay and Lake Erie Collections

All five microsatellite loci were polymorphic in the Presque Isle Bay and Lake Erie populations (Table 6 and 7). The Lake Erie populations had fewer alleles per locus than the Presque Isle Bay collections although it was not significant (one-tailed test, P = 0.82). Of the sixty-eight alleles detected in the Presque Isle Bay population, forty-six were present in the Lake Erie samples, with an additional fifteen alleles from Lake Erie not being found in the Presque Isle Bay samples. The distance estimates show a small level of genetic differentiation between populations at each locus, but high levels within the populations in comparisons of F_{IS} and F_{ST} values. F_{IS} estimates ranged from 0.2198 to 0.2668 for the Presque Isle Bay specimens (Table 9) and 0.155 to 0.303 for the Lake Erie and Presque Isle Bay specimens (Table 10). Pair-wise F_{ST} estimates for Presque Isle Bay ranged from 0.0064 to 0.0319 and 0.0020 to 0.0199 for the Lake Erie and Presque Isle Bay collections.

4.5 Genotypes – Presque Isle Bay and Lake Erie Collections

A genetic hybrid index score was developed by assigning a value of 1 for each *Ameiurus nebulosus* allele and 0 for each *A. melas* allele and dividing by the total number of alleles for the specimen. This score was used to characterize the individual as *A. nebulosus* or as having some genetic material from *A. melas*. Before adjustment for suggested presence of null alleles, the data from Brown Bullheads collected in Presque

Isle Bay shows over 40 percent of the bullheads sampled from Sara's Cove and Thompson's Bay contain some genetic material from Black Bullheads. Twenty-seven percent of the Brown Bullheads in the lagoons had Black Bullhead genetic material in their DNA (Figure 23). These numbers were reduced to 22 percent of the bullheads in Thompson's Bay and 10 percent of the bullheads in the lagoons having some Black Bullhead alleles after adjustment of null alleles. Sara's Cove still had over 40 percent of the bullheads sampled containing some Black Bullhead alleles (Figure 24).

Twenty-nine percent of the Brown Bullhead specimens from Old Woman Creek, Ohio contain some black bullhead alleles, while Long Point Bay, Ontario, Canada and Dunkirk Harbor, New York had 32 and 38 percent respectively before adjustment of null alleles (Figure 25). After adjustment, Old Woman Creek had 25 percent and Long Point Bay and Dunkirk both had 29 percent (Figure 26).

With the adjustment for the suggestion for the presence of null alleles, the multi-locus nuclear genotypes suggest the presence of advanced-generation hybrids or backcrosses between *A. nebulosus* and *A. melas* in Presque Isle Bay and Lake Erie.

4.6 Polymorphism, Heterozygosity, and Genetic distances- Tamarack Lake and Wisconsin Collections

All five loci were polymorphic for the Tamarack Lake Brown Bullheads except Aneb61, which only had one allele. All loci were polymorphic for the Wisconsin Black Bullhead specimens however Loci Aneb61 and Aneb63 had only two alleles (Table 7). Tamarack Lake and Wisconsin had fewer alleles but also had fewer specimens in the

collection compared to Presque Isle Bay and Lake Erie collections. Observed heterozygosity ranged from 0.1111 (Aneb63 and Aneb64) to 0.8333 (Aneb16) in Tamarack Lake, and 0.1818 (Aneb61 and Aneb63) to 1.0 (Aneb37) in the Wisconsin specimens. The estimates of F_{IS} for Tamarack Lake ranges between -0.027 and +0.71 (Weir and Cockerham 1984). The value of F_{IS} for Wisconsin ranges between -0.202 and +0.608 (Weir and Cockerham 1984). Negative F_{IS} values indicate heterozygote excess (outbreeding) and positive values indicate heterozygote deficiency (inbreeding) compared with Hardy-Weinberg equilibrium expectations.

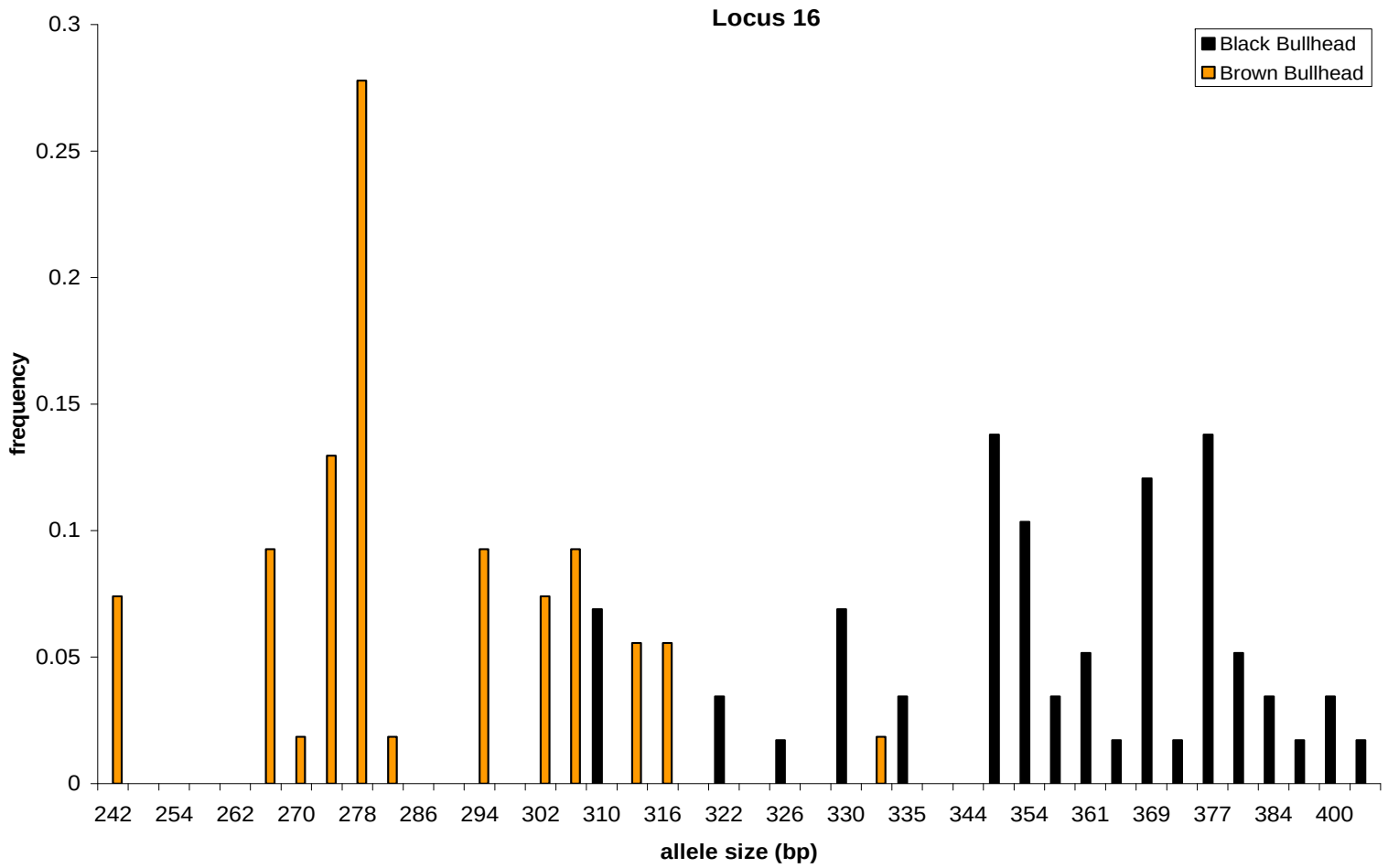

Figure 18. Allele frequencies at microsatellite locus 16 from samples of the reference specimens

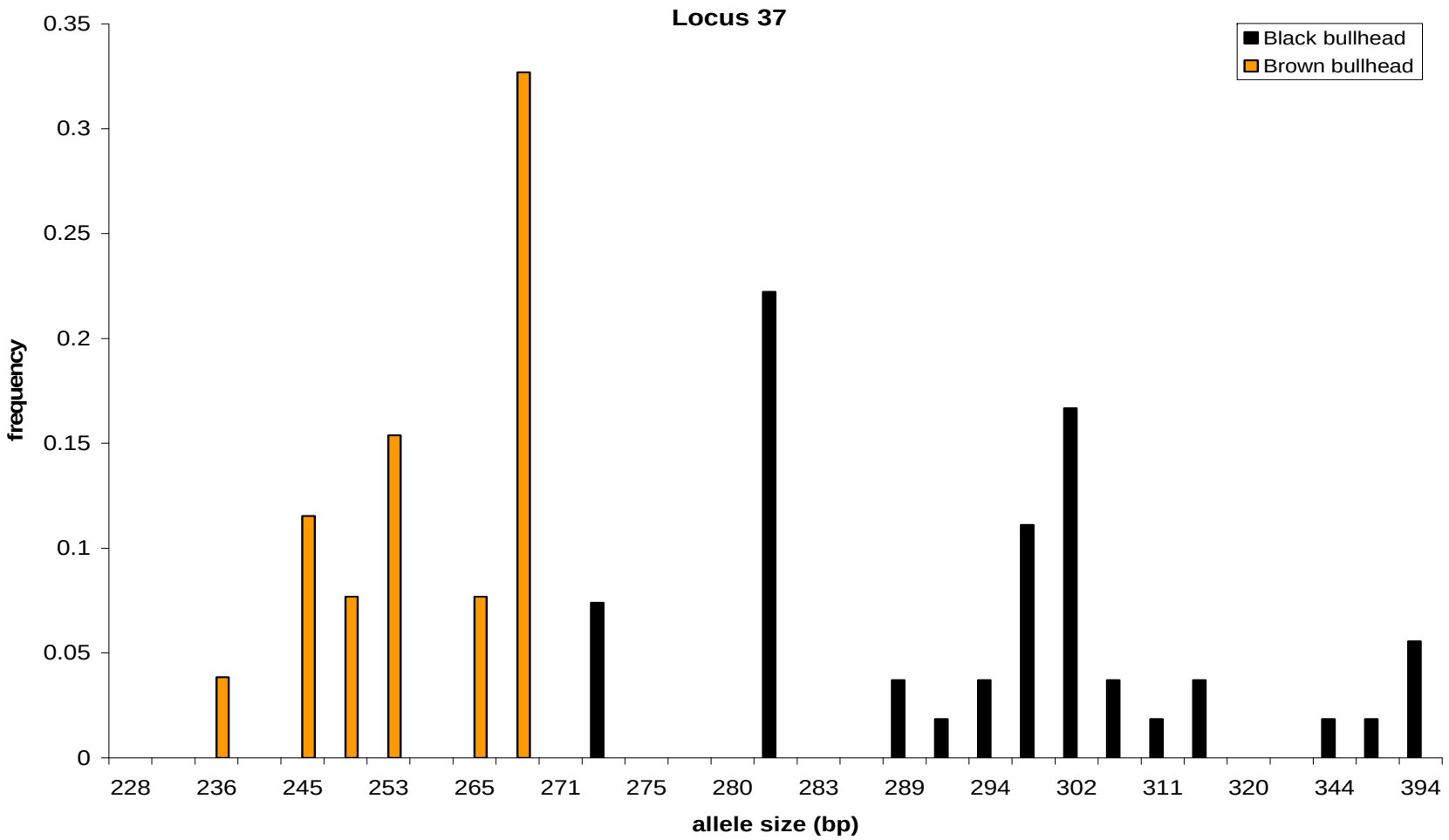

Figure 19. Allele frequencies at microsatellite locus 37 from samples of the reference specimens

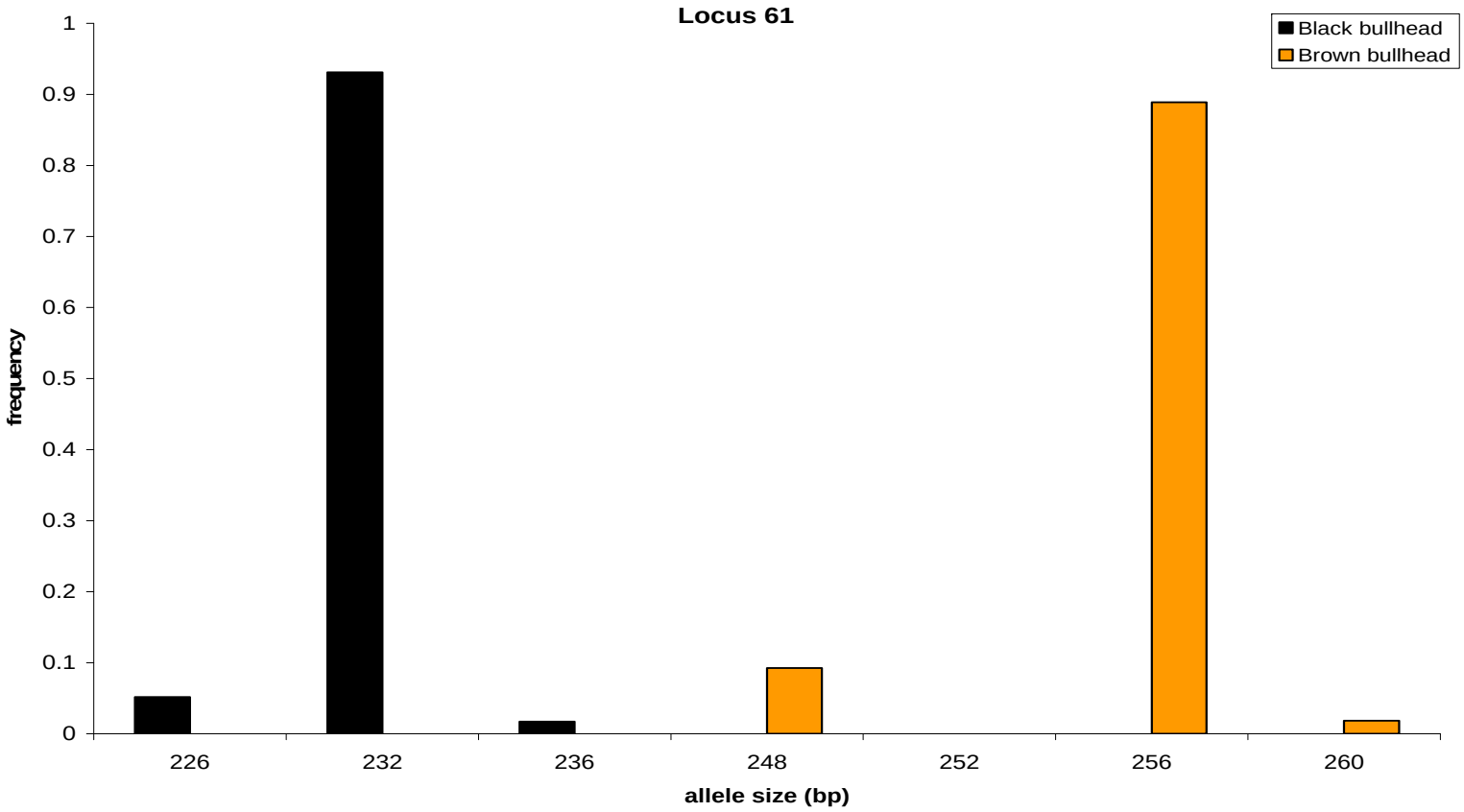

Figure 20. Allele frequencies at microsatellite locus 61 from samples of the reference specimens

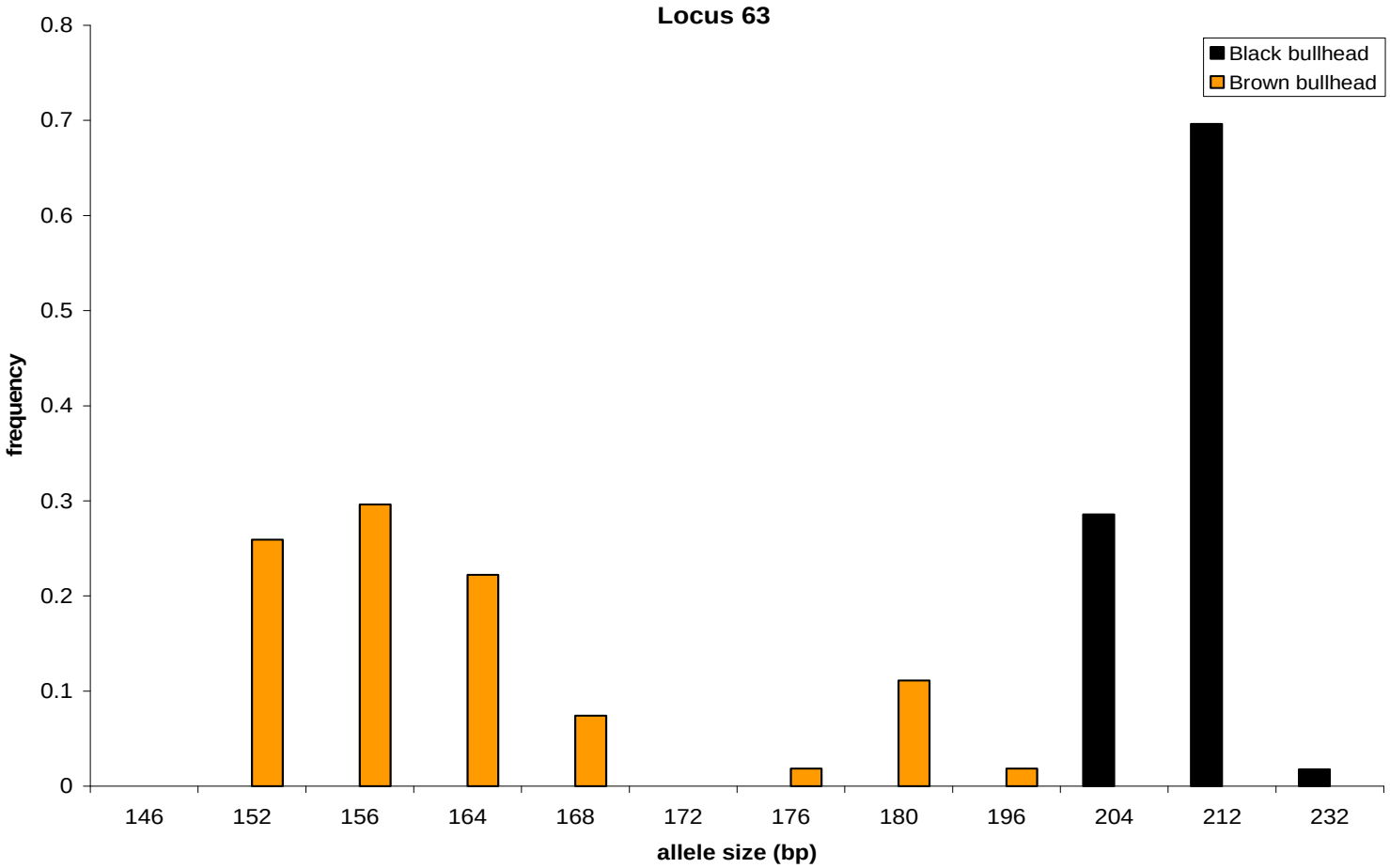

Figure 21. Allele frequencies at microsatellite locus 63 from samples of the reference specimens

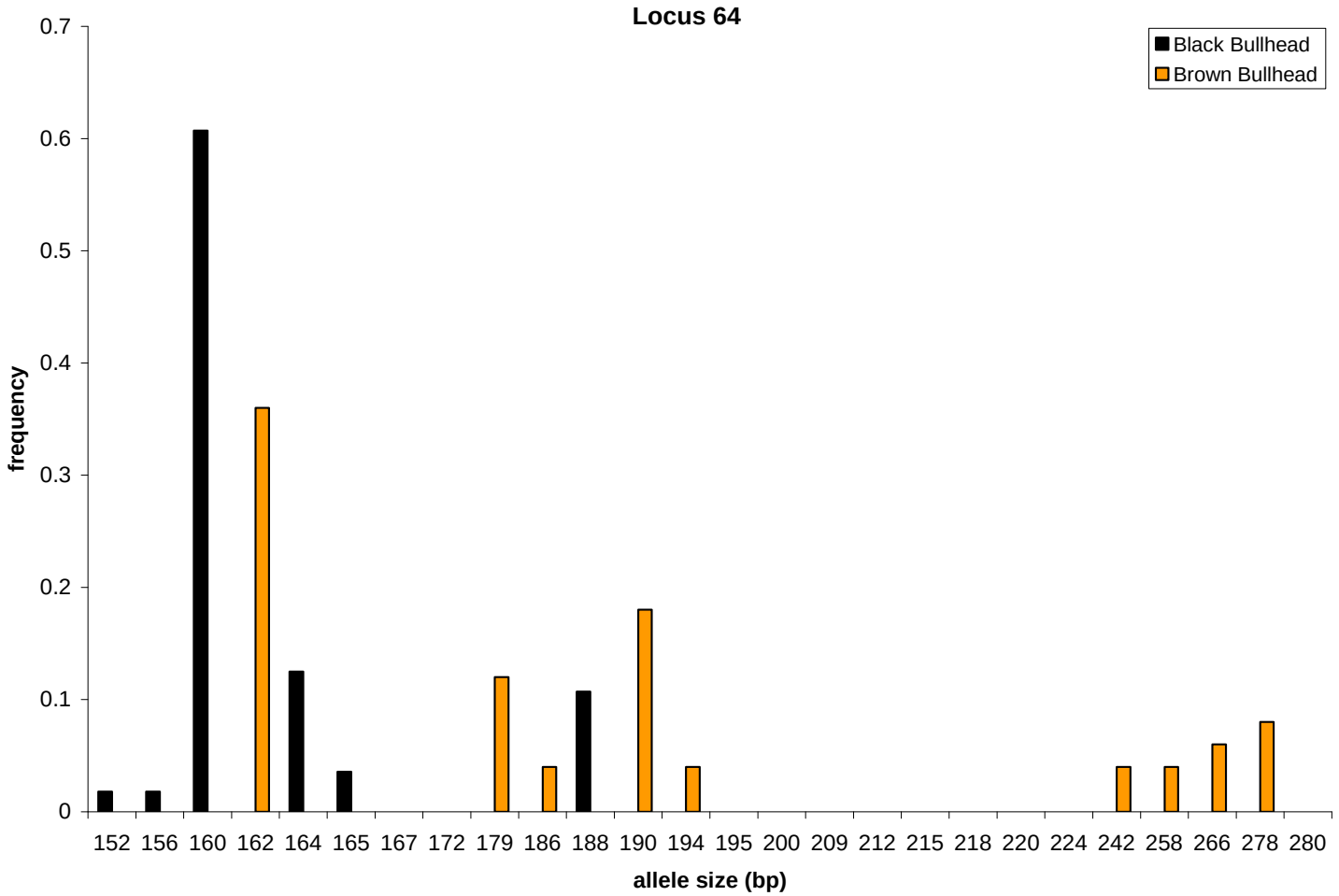

Figure 22. Allele frequencies at microsatellite locus 64 from samples of the Reference specimens.

Table 5. Multilocus variation in the reference *Ameiurus* populations. Numbers of specimens (N), number of alleles per locus (A), size of the allele, observed heterozygosity (Ho), heterozygosity as expected under Hardy-Weinberg equilibrium (He), an unbiased estimate of the P-value of the probability test for Hardy-Weinberg, as described by Raymond and Rousset (1995), and percentage of null alleles per locus (pNull).

A. nebulosus							
Locus	**N**	**A**	**size**	**Ho**	**He**	**p-value**	**pNull**
Aneb16	27	12	242-232	0.778	0.88	0.0771	0.0449
Aneb37	27	8	236-285	0.778	0.803	1	0.0368
Aneb61	27	3	248-260	0.222	0.204	0.004	0
Aneb63	25	7	152-196	0.56	0.866	0	0.1396
Aneb64	26	8	162-278	0.077	0.79	0.0132	0.4572
A. melas							
Locus	**N**	**A**	**size**	**Ho**	**He**	**p-value**	**pNull**
Aneb16	29	18	310-408	0.793	0.9304	0.0006	0.0484
Aneb37	29	15	273-394	0.69	0.839	1	0.0758
Aneb61	28	3	226-236	0.143	0.137	0.3017	0
Aneb63	27	3	204-232	0.518	0.457	0	0
Aneb64	27	6	152-196	0.333	0.0123	0.0082	0.2118

Table 6. Multilocus variation in the Presque Isle Bay *Ameiurus* populations. Numbers of specimens (N), number of alleles per locus (A), size of the allele, observed heterozygosity (Ho), heterozygosity as expected under Hardy-Weinberg equilibrium (He), and an unbiased estimate of the P-value of the probability test for Hardy-Weinberg, as described by Raymond and Rousset (1995).

Presque Isle Bay - Sara's Cove

Locus	N	A	size	Ho	He	p-value
Aneb16	27	19	242-381	1	0.9426	0.9991
Aneb37	27	13	185-283	0.814	0.919	0.0203
Aneb61	27	5	215-256	0.074	0.21	0.0012
Aneb63	27	4	152-168	0.185	0.395	0.0028
Aneb64	27	4	150-164	0.074	0.21	0.0006

Presque Isle Bay - Lagoons

Locus	N	A	size	Ho	He	p-value
Aneb16	27	18	242-230	0.963	1	0.1613
Aneb37	27	13	185-283	0.814	0.971	0.0567
Aneb61	27	2	215-256	0.111	0.177	0.0364
Aneb63	25	4	152-180	0.44	0.385	1
Aneb64	26	8	160-248	0.115	0.633	0

Presque Isle Bay - Thompson's bay

Locus	N	A	size	Ho	He	p-value
Aneb16	30	21	202-344	1	1	0
Aneb37	30	12	236-281	0.833	0.9241	0.0845
Aneb61	29	3	232-256	0.103	0.134	0.6233
Aneb63	28	6	152-180	0.357	0.359	0
Aneb64	30	9	160-224	0.2	0.7256	0.0408

Table 7. Multilocus variation in the Lake Erie *Ameiurus* populations. Numbers of specimens (N), number of alleles per locus (A), size of the allele, observed heterozygosity (Ho), heterozygosity as expected under Hardy-Weinberg equilibrium (He), and an unbiased estimate of the P-value of the probability test for Hardy-Weinberg, as described by Raymond and Rousset (1995).

Old Woman's Creek, Ohio

Locus	N	A	size	Ho	He	p-value
Aneb16	28	17	242-374	0.8925	0.935	0.6488
Aneb37	28	10	228-281	0.714	0.8471	0.1341
Aneb61	28	2	252-256	0.286	0.249	1
Aneb63	28	5	146-172	0.286	0.468	0.0025
Aneb64	27	3	162-167	0.222	0.326	0.1341

Long Point, Ontatio, Canada

Locus	N	A	size	Ho	He	p-value
Aneb16	26	17	250-344	0.923	0.923	0.3862
Aneb37	28	9	241-285	0.785	0.785	0.696
Aneb61	28	2	252-256	0.143	0.232	0.0039
Aneb63	28	4	152-172	0.429	0.544	0.442
Aneb64	26	6	162-224	0.115	0.379	0

Dunkirk Harbor, New York

Locus	N	A	size	Ho	He	p-value
Aneb16	21	17	242-356	0.715	0.928	0.0005
Aneb37	21	11	233-281	0.81	0.897	0.2603
Aneb61	21	2	252-256	0.048	0.048	
Aneb63	16	3	152-164	0.687	0.59	0.6165
Aneb64	21	8	160-280	0.048	0.605	0

Table 8. Multilocus variation in the Tamarack Lake and Wisconsin *Ameiurus* populations. Numbers of specimens (N), number of alleles per locus (A), size of the allele, observed heterozygosity (Ho), heterozygosity as expected under Hardy-Weinberg equilibrium (He), and an unbiased estimate of the P-value of the probability test for Hardy-Weinberg, as described by Raymond and Rousset (1995).

Tamarack Lake - *A. nebulosus*

Locus	N	A	size	Ho	He	p-value
Aneb16	27	13	202-316	0.8333	0.8986	0.0006
Aneb37	27	9	241-281	0.6667	0.6495	0.2268
Aneb61	27	1	256	0	0	na
Aneb63	27	4	152-176	0.1111	0.1338	0.0181
Aneb64	27	6	162-280	0.1111	0.4797	0

Wisconsin - *A. melas*

Locus	N	A	size	Ho	He	p-value
Aneb16	12	12	296-392	0.909	0.935	0.6939
Aneb37	12	8	277-315	1	0.8398	0.3383
Aneb61	12	2	232-234	0.1818	0.4502	0.0096
Aneb63	12	2	204-212	0.1818	0.3117	0.2767
Aneb64	12	3	160-196	0.4545	0.3796	1

Table 9. Average F_{IS} and F_{ST} values and the number of migrants per generation (N_M) for Presque Isle Bay specimens.

	Sara's Cove	Thompson's Bay	Lagoons
Sara's Cove	$F_{IS} = 0.2668$	$N_M = 7.59$	$N_M = 15.78$
Thompson's Bay	$F_{ST} = 0.0319$	$F_{IS} = 0.2198$	$N_M = 38.81$
Lagoons	$F_{ST} = 0.0156$	$F_{ST} = 0.0064$	$F_{IS} = 0.2576$

Table 10. Average F_{IS} and F_{ST} values and the number of migrants per generation (N_M) for Lake Erie and combined Presque Isle Bay specimens.

	Old Woman's Creek, Ohio	Long Point Bay, Ontario	Dunkirk Harbor, NY	Presque Isle Bay
Old Woman's Creek, Ohio	$F_{IS} = 0.155$	$N_M = 124.75$	$N_M = 12.31$	$N_M = 17.60$
Long Point Bay, Ontario	$F_{ST} = 0.0020$	$F_{IS} = 0.2606$	$N_M = 14.03$	$N_M = 13.34$
Dunkirk Harbor, NY	$F_{ST} = 0.0199$	$F_{ST} = 0.0175$	$F_{IS} = 0.2723$	$N_M = 73.28$
Presque Isle Bay	$F_{ST} = 0.0154$	$F_{ST} = 0.0184$	$F_{ST} = 0.0034$	$F_{IS} = 0.303$

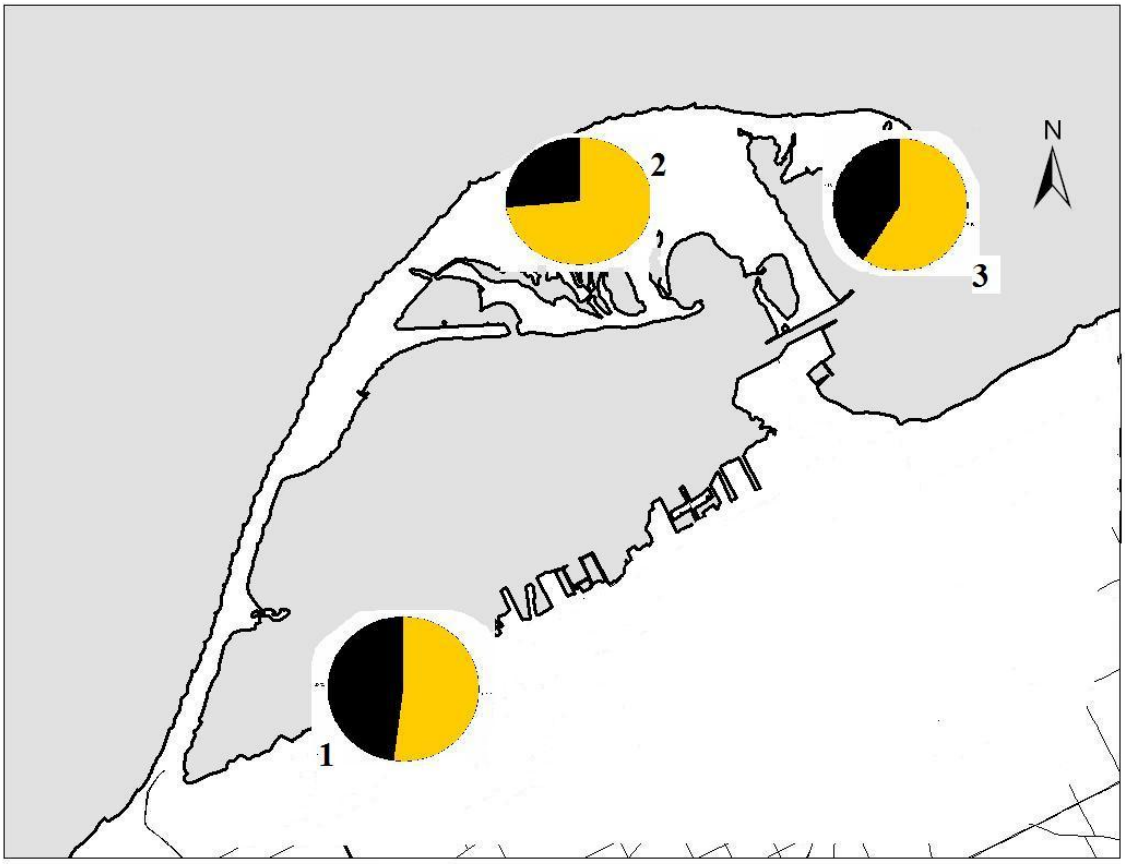

Figure 23. Percentage of bullheads possessing all *A. nebulosus* alleles (yellow) and percentage of bullheads with some *A. melas* alleles (black) for Presque Isle Bay; 1. Sara's Cove- 52% have all Brown bullhead alleles, 2. lagoons– 73% have all Brown Bullhead alleles, and 3. Thompson's Bay- 59% have all Brown Bullhead alleles.

Figure 24. Percentage of bullheads possessing all *A. nebulosus* alleles (orange) and percentage of bullheads with some *A. melas* alleles (grey) for Presque Isle Bay adjusted for the suggested presence of null alleles; 1. Sara's Cove- 57% have all Brown bullhead alleles, 2. lagoons– 90% have all Brown Bullhead alleles, and 3. Thompson's Bay- 78% have all Brown Bullhead alleles.

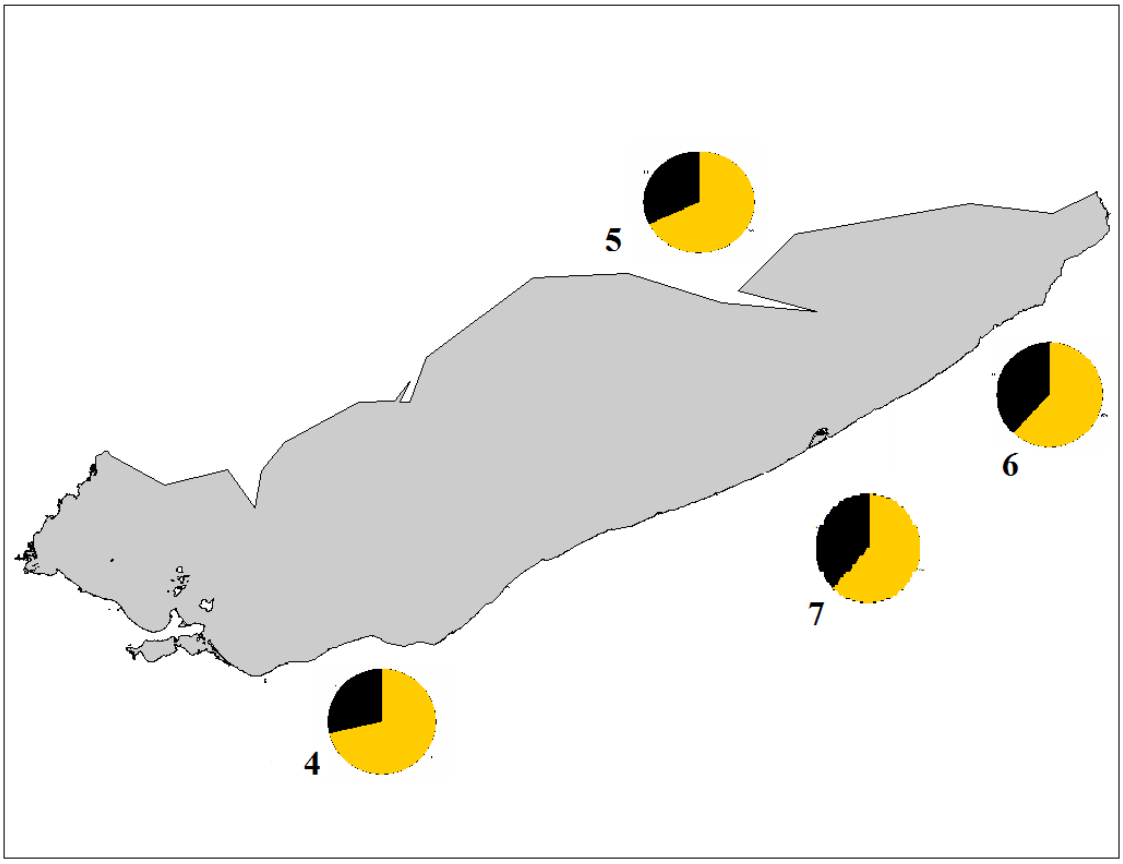

Figure 25: percentage of bullheads possessing all *A. nebulosus* alleles (yellow) and percentage of bullheads with some *A. melas* alleles (black) for the Lake Erie collections; 4. Old Woman Creek Ohio- 71% have all Brown Bullhead alleles, 5. Long Point Bay, Ontario - 68% have all Brown Bullhead alleles, 6. Dunkirk Harbor, New York- 62% has all Brown Bullhead alleles, and 7. Presque Isle Bay, Pennsylvania- 62% has all Brown Bullhead alleles.

Figure 26: percentage of bullheads possessing all *A. nebulosus* alleles (orange) and percentage of bullheads with some *A. melas* alleles (grey) for the Lake Erie collections after adjustment for the suggestion of null alleles; 4. Old Woman Creek Ohio- 75% have all Brown Bullhead alleles, 5. Long Point Bay, Ontario - 71% have all Brown Bullhead alleles, 6. Dunkirk Harbor, New York- 71% has all Brown Bullhead alleles, and 7. Presque Isle Bay, Pennsylvania- 75% has all Brown Bullhead alleles.

Chapter 5

Discussion

Hybridization among freshwater fishes is a common occurrence (Hubbs 1955), but is only known in a few Ictaluridae including *Noturus gyrinus* x *Noturus miurus* hybrid madtoms (Menzel and Raney 1973) and *Ameiurus nebulosus* x *Ameiurus melas* hybrid bullheads (Trautman 1981). The term hybridization is often difficult to define. Hybridization is usually employed in a broad sense to include crosses between genetically differentiated forms regardless of their current taxonomic status. Introgression refers to gene movement between species mediated by hybridization and backcrossing (Avise 2004). The occurrence and frequency of hybridization is related to environmental settings and reproductive ways of the parental species (Jenkins and Burkhead 1994). Hybridization under natural conditions is often associated with crowding of spawning fishes, and tends be facilitated when one species is rare and the other abundant (Stauffer et al. 1997). This is the likely situation for the endangered Black Bullhead and abundant Brown Bullhead in Presque Isle Bay and eastern portions of Lake Erie. Hybridization is also associated with habitat disturbances whereby two or more species may be forced into atypically close proximity during breeding thus increasing the chances of mismating. Turbidity may reduce the ability of a fish to visually discriminate other species form conspecific mates, and hybridization can occur with the introduction of non-native fishes (Jenkins and Burkhead 1994). Hybridization can also occur where there are environ-mental stresses. If the PCB loads in Presque Isle Bay initiated hybridization and hybrids

were less vulnerable for tumor formation, this could result in positive feedback system. Hybrid vigor may allow for the hybrids to reach maturity faster than the putative parents and breed. Those offspring again having traits superior to the putative species should then reach maturity faster and breed. And as this cycle continues, would be more hybrid individuals maturing faster without the presence of tumors.

5.1 Principal Component Analysis

Historically, identification of naturally occurring hybrids has depended on intermediacy of character states between the putative hybrid and the two parental forms (Stauffer et al. 1996). Principal component analysis was used to delimit naturally occurring hybrids and not the use of hybrid-description techniques that require *a priori* identification of the hybrid. Principal component analysis allows us to consider multivariate variability, since the components are composed of all of the initial characters and are in the directions of greatest variance within the data matrix. Hubbs (1955) reported that the vast majority of hybrids possess character indices intermediate of their parental forms. Characters of the hybrids may also lie outside the parental forms. The identification of F1 hybrids is likely using PCA, but the identification of F2 or backcrosses does not appear possible (Neff and Smith 1978), especially when backcrossing can produce an infinite combination of morphological traits.

The morphological graphs from this study do not form distinct intermediate clusters for hybrids relative to the putative species, but still identified a few intermediate hybrid specimens in the Lagoons and Thompson's Bay collections in Presque Isle Bay,

and also in the Old Woman Creek, Ohio and Dunkirk Harbor, New York samples. The morphological graphs also suggest heterosis in the Sara's Cove collection. Heterosis, also called hybrid vigor, is the increase in such characteristics as size, growth rate, fertility, and yield of a hybrid organism over those of its parents. It may also occur that a hybrid inherits such different traits from their parents that make them unfit for survival or quite possible, more susceptible to tumors and external abnormalities.

Brown Bullheads and Black Bullheads are phenotypically similar, and can be quite challenging to delimit in sympatric populations without having both representative species present. The importance of the gill raker counts in distinguishing between the two species of *Ameiurus* suggests an ecological separation of the two species in their mode of feeding or in some other particle-size-related aspect of their adaptation to their environment. As emphasized by the loadings of the principal components distinguishing the species, both of the gill raker counts are involved, as would be expected if there were a functional distinction involving the entire branchial basket. This example also suggests that principal components analysis is of use in identifying or confirming functional complexes of characters through the patterns of character loadings on the components.

5.2 Microsatellites

Molecular markers can be of great utility in diagnosing closely related species, even where morphological or other traditional markers fail or are ambiguous. (Avise 2004).

Microsatellites data indicates that gene flow has occurred in Presque Isle Bay and throughout Lake Erie in similar trends. All the intermediate specimens were identified as being backcrossed to the Brown Bullhead. Interspecific hybridization can be costly to the participants, typically yielding progeny with diminished fitness and resulting in hybrid zones that act as genetic sinks. Sometimes fitness of hybrid organisms surpasses those of their putative parents (Avise 2004). Some hybrid populations might also be the sources of adaptive evolution and lineage diversification by possessing novel recombinant genotypes (Avise 2004).

There seems to be small level of genetic differentiation between the sampled populations at each locus, but high levels within populations across Lake Erie. Inbreeding within a subpopulation is caused by the nonrandom mating of the members of that subpopulation, in that mating occurs more often than by chance alone, between closely related individuals. As closely related individuals will contain a large proportion of the same alleles due to common descent, their offspring will have a higher level of homozygosity, and conversely, a lower level of heterozygosity then expected. Positive F_{IS} values indicate heterozygote deficiency (inbreeding) compared with Hardy-Weinberg equilibrium expectations. A consequence known as Wahlunds' effect shows that as allele frequencies in two subpopulations deviate, the average observed heterozygosity in those populations will always be less than that expected from the pooled allele frequencies. Deviations from Hardy-Weinberg equilibrium may also be caused by the presence of null alleles. One method to detect such deviations is to compare the expected levels of heterozygosity to the observed levels of heterozygosity of alleles at a locus within a

population. Although null alleles lead to underestimated heterozygosity within samples, it is a minor source of error in estimating heterozygosity excess (Dakin and Avise 2004).

The high levels of F_{IS} (proportion of variation within a population) combined with the fact that backcrossed hybrids are present in all syntopic populations and significantly higher observed heterozygosities than expected from Hardy Weinberg Equilibrium found in all Lake Erie populations are all results of extensive hybridization between the two species.

Chapter 6

Conclusion

The question addressed in this study was to determine if hybridization has occurred between *Ameiurus nebulosus* and *Ameiurus melas* in Presque Isle Bay, Lake Erie. Results of the morphological and meristic analysis using principal component analysis indicate the majority of Brown Bullheads from Presque Isle Bay group with the reference Brown Bullhead population morphologically and not with the reference Black Bullheads. Collections from the Lagoons and Thompson's Bay each include an individual which maybe a hybrid, but what is likely being collected as a Brown Bullhead for the tumor studies in Presque Isle Bay, is morphologically a Brown Bullhead.

Genetically, over half of the Bullheads sampled were identified as having all *Ameiurus nebulosus* alleles, but multi-locus nuclear genotypes suggest the presence of extensive backcrossing between *Ameiurus nebulosus* and *Ameiurus melas* in Presque Isle Bay. The hybrid bullheads have also been reported from the western portion of Lake Erie prior to 1950 (Trautmann 1981) and were present in Presque Isle Bay in 2003 (Hunnicutt et al. 2005).

Presque Isle Bay has been under intensive environmental study due to high rates of liver and skin tumors in Brown Bullhead residing in this bay. It is not possible to state if *Ameiurus nebulosus* x *Ameiurus melas* hybrids are more susceptible to external abnormalities or more resistant to external abnormalities from this study. While hybrid specimens have higher external abnormalities (Figure 27 and 28), there is not a difference

between the pure Brown Bullhead and hybrid specimen collected in Presque Isle Bay regarding tumor and deformities rates (p-value = 0.663). The presence of tumors and deformities are related to age of the fish and contaminants in the sediments of the lakes (Pyron et al. 2001). High incidents of external abnormalities on Brown Bullheads and specimens backcrossed to Brown Bullheads indicate their sensitivity to contaminated sediment exposure. This sensitivity may be attributable to lack of scales and exposed skin, metabolic differences that result in formation of carcinogenic PAH metabolites, or extensive contact with contaminated sediments because of habitat requirements (Smith et al. 1994). Brown Bullheads are tolerant of very low dissolved oxygen concentrations and are able to feed on items correlated with these conditions. Brown Bullheads are also known to become sluggish and cease feeding in the late fall and bury themselves in soft, silt, mud and leafy material along the shore (Becker 1983). Long exposure to contaminated sediments may best explain the high incident of external abnormalities on Brown Bullheads from Presque Isle Bay, but further investigation into the role of hybridization and external abnormalities should be considered.

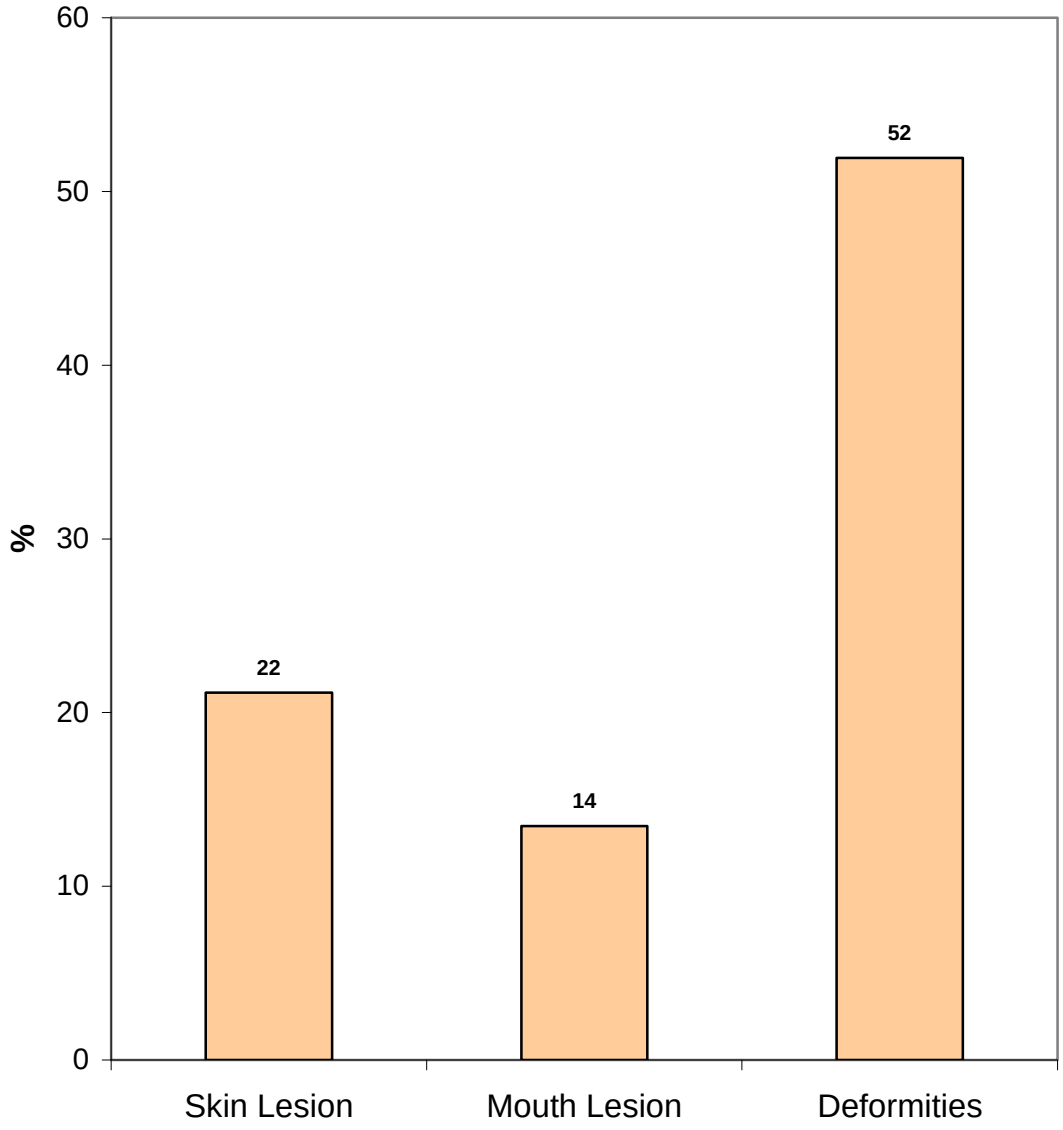

Figure 27. Tumor and deformity rates for individuals collected from Presque Isle Bay and identified as having all brown bullhead alleles, n = 52.

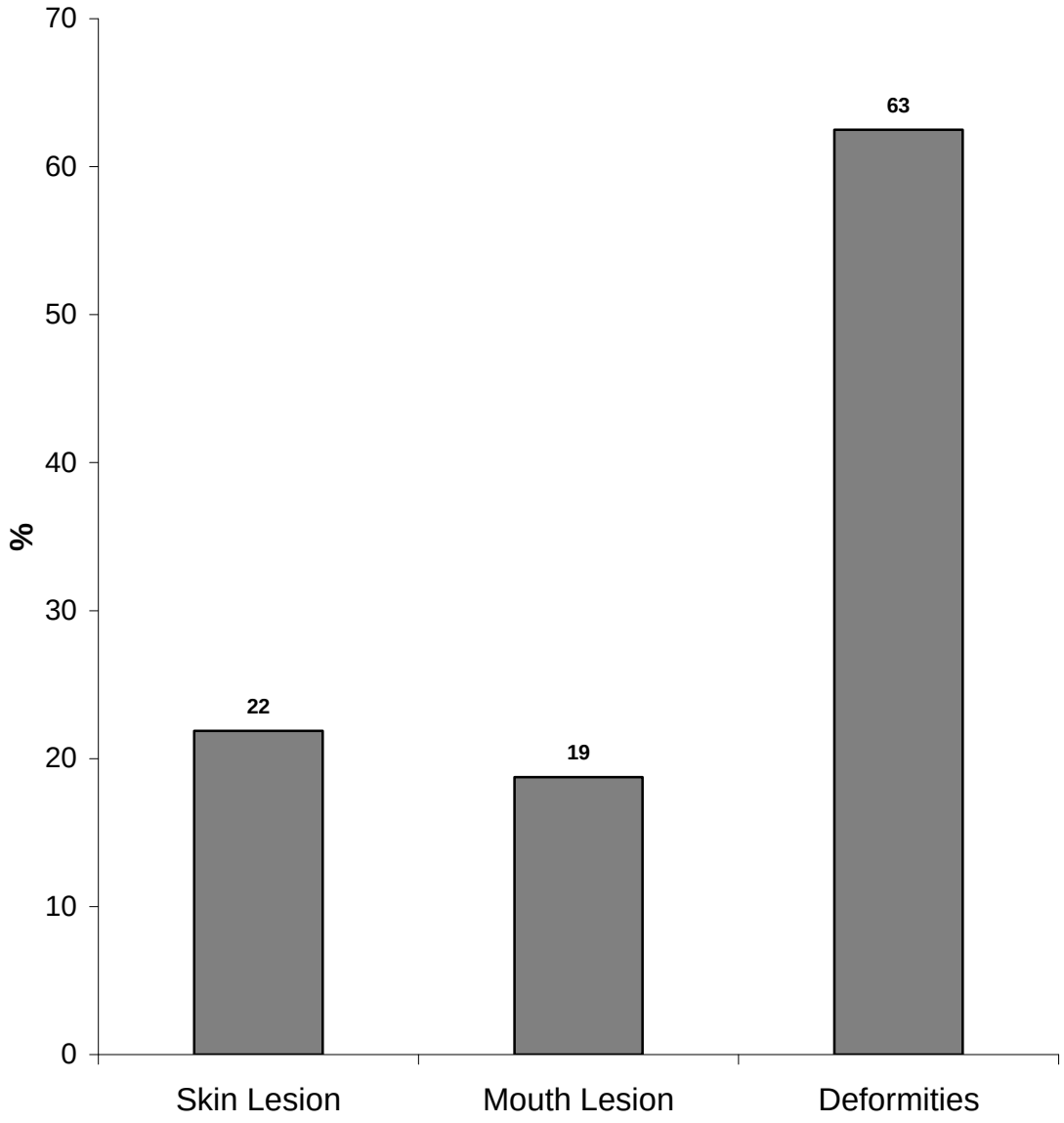

Figure 28. Tumor and deformity rates for individuals collected form Presque Isle Bay and identified as having some black bullhead alleles, n = 32.

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Appendix A

Descriptive Statistics

In tables A1- A31 the following abbreviations are used:

N = number of individuals

StDev = standard deviation

Table A1. Standard length (SL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	225.42	21.68	121.42	245.57
Petersburg, Pennsylvania	28	213.80	30.30	113.35	297.22
Sara's Cove, Presque Isle Bay	28	247.85	25.80	146.16	278.19
Lagoons, Presque Isle Bay	30	263.82	32.49	194.86	322.37
Thompson's Bay, Presque Isle Bay	28	269.06	24.28	205.11	313.67
Old Womans Creek, Ohio	28	242.56	22.91	197.79	283.47
Dunkirk Harbor, New York	22	222.96	38.54	164.83	303.11
Long Point Bay, Canada	28	212.72	23.85	182.98	275.89
Tamarack Lake, Pennsylvania	28	253.76	20.78	152.91	278.15

Table A2. Mean of corrected Head length (HL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.31	0.32	0.31	0.32
Petersburg, Pennsylvania	28	0.31	0.29	0.35	0.29
Sara's Cove, Presque Isle Bay	28	0.30	0.34	0.28	0.31
Lagoons, Presque Isle Bay	30	0.32	1.42	0.28	0.99
Thompson's Bay, Presque Isle Bay	28	0.29	0.31	0.29	0.29
Old Womans Creek, Ohio	28	0.29	0.34	0.29	0.29
Dunkirk Harbor, New York	22	0.29	0.30	0.28	0.29
Long Point Bay, Canada	28	0.29	0.30	0.29	0.30
Tamarack Lake, Pennsylvania	28	0.29	0.47	0.24	0.30

Table A3. Mean of corrected Head Width (HW) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.72	0.84	0.66	0.75
Petersburg, Pennsylvania	28	0.72	0.71	0.64	0.69
Sara's Cove, Presque Isle Bay	28	0.71	0.79	0.67	0.75
Lagoons, Presque Isle Bay	30	0.66	0.21	0.73	0.24
Thompson's Bay, Presque Isle Bay	28	0.72	1.00	0.68	0.79
Old Womans Creek, Ohio	28	0.71	0.89	0.69	0.74
Dunkirk Harbor, New York	22	0.68	0.89	0.60	0.78
Long Point Bay, Canada	28	0.69	0.87	0.67	0.74
Tamarack Lake, Pennsylvania	28	0.69	0.49	0.81	0.69

Table A4. Mean of corrected Post Orbital Head Length (POHL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.52	0.56	0.50	0.53
Petersburg, Pennsylvania	28	0.51	0.55	0.48	0.52
Sara's Cove, Presque Isle Bay	28	0.50	0.49	0.50	0.49
Lagoons, Presque Isle Bay	30	0.46	0.12	0.49	0.15
Thompson's Bay, Presque Isle Bay	28	0.51	0.56	0.49	0.52
Old Womans Creek, Ohio	28	0.53	1.10	0.50	0.89
Dunkirk Harbor, New York	22	0.51	0.54	0.49	0.52
Long Point Bay, Canada	28	0.51	0.52	0.49	0.51
Tamarack Lake, Pennsylvania	28	0.51	0.34	0.61	0.51

Table A5. Mean of corrected Horizontal Eye Depth (HED) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.12	0.19	0.13	0.14
Petersburg, Pennsylvania	28	0.12	0.08	0.15	0.11
Sara's Cove, Presque Isle Bay	28	0.12	0.13	0.15	0.12
Lagoons, Presque Isle Bay	30	0.11	0.03	0.12	0.03
Thompson's Bay, Presque Isle Bay	28	0.11	0.15	0.11	0.12
Old Womans Creek, Ohio	28	0.13	0.77	0.12	0.47
Dunkirk Harbor, New York	22	0.11	0.11	0.12	0.12
Long Point Bay, Canada	28	0.12	0.12	0.12	0.12
Tamarack Lake, Pennsylvania	28	0.11	0.11	0.14	0.13

Table A6. Mean of corrected Vertical Eye Depth (VED) of Ameiurus species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.11	0.15	0.15	0.13
Petersburg, Pennsylvania	28	0.12	0.08	0.14	0.11
Sara's Cove, Presque Isle Bay	28	0.11	0.12	0.14	0.11
Lagoons, Presque Isle Bay	30	0.10	0.02	0.12	0.03
Thompson's Bay, Presque Isle Bay	28	0.11	0.11	0.13	0.11
Old Womans Creek, Ohio	28	0.10	0.11	0.11	0.12
Dunkirk Harbor, New York	22	0.11	0.10	0.11	0.10
Long Point Bay, Canada	28	0.12	0.09	0.11	0.10
Tamarack Lake, Pennsylvania	28	0.11	0.10	0.12	0.11

Table A7. Mean of corrected Pre-Orbital Lenght (PRE) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.41	0.47	0.38	0.42
Petersburg, Pennsylvania	28	0.41	0.50	0.16	0.41
Sara's Cove, Presque Isle Bay	28	0.52	0.54	0.53	0.52
Lagoons, Presque Isle Bay	30	0.46	0.12	0.52	0.15
Thompson's Bay, Presque Isle Bay	28	0.52	0.58	0.53	0.53
Old Womans Creek, Ohio	28	0.49	0.86	0.12	0.51
Dunkirk Harbor, New York	22	0.42	0.50	0.38	0.48
Long Point Bay, Canada	28	0.52	0.51	0.50	0.51
Tamarack Lake, Pennsylvania	28	0.43	0.33	0.48	0.43

Table A8. Mean of corrected Cheek Depth (CD) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.23	0.33	0.19	0.24
Petersburg, Pennsylvania	28	0.22	0.28	0.17	0.22
Sara's Cove, Presque Isle Bay	28	0.23	0.33	0.19	0.26
Lagoons, Presque Isle Bay	30	0.22	0.07	0.21	0.08
Thompson's Bay, Presque Isle Bay	28	0.23	0.34	0.20	0.24
Old Womans Creek, Ohio	28	0.23	0.34	0.20	0.25
Dunkirk Harbor, New York	22	0.22	0.29	0.20	0.24
Long Point Bay, Canada	28	0.22	0.36	0.20	0.25
Tamarack Lake, Pennsylvania	28	0.20	0.20	0.23	0.22

Table A9. Mean of corrected Lower Jaw Lenght (LJL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.40	0.51	0.40	0.43
Petersburg, Pennsylvania	28	0.40	0.43	0.28	0.36
Sara's Cove, Presque Isle Bay	28	0.41	0.58	0.34	0.44
Lagoons, Presque Isle Bay	30	0.35	0.12	0.37	0.12
Thompson's Bay, Presque Isle Bay	28	0.38	0.57	0.38	0.43
Old Womans Creek, Ohio	28	0.37	0.60	0.34	0.43
Dunkirk Harbor, New York	22	0.37	0.59	0.31	0.44
Long Point Bay, Canada	28	0.36	0.49	0.34	0.39
Tamarack Lake, Pennsylvania	28	0.37	0.38	0.40	0.42

Table A10. Mean of corrected Head Depth (HD) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.49	0.55	0.46	0.49
Petersburg, Pennsylvania	28	0.49	0.59	0.46	0.55
Sara's Cove, Presque Isle Bay	28	0.48	0.62	0.40	0.51
Lagoons, Presque Isle Bay	30	0.43	0.13	0.43	0.14
B	28	0.48	0.56	0.46	0.50
Old Womans Creek, Ohio	28	0.44	0.51	0.41	0.48
Dunkirk Harbor, New York	22	0.45	0.61	0.42	0.54
Long Point Bay, Canada	28	0.45	0.59	0.43	0.49
Tamarack Lake, Pennsylvania	28	0.47	0.36	0.57	0.48

Table A11. Mean of corrected Body Depth (BD) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.21	0.24	0.19	0.23
Petersburg, Pennsylvania	28	0.21	0.19	0.26	0.21
Sara's Cove, Presque Isle Bay	28	0.25	0.34	0.23	0.27
Lagoons, Presque Isle Bay	30	0.24	0.29	0.21	0.24
Thompson's Bay, Presque Isle Bay	28	0.23	0.30	0.24	0.25
Old Womans Creek, Ohio	28	0.20	0.29	0.19	0.23
Dunkirk Harbor, New York	22	0.19	0.25	0.17	0.21
Long Point Bay, Canada	28	0.22	0.28	0.20	0.23
Tamarack Lake, Pennsylvania	28	0.21	0.25	0.21	0.22

Table A12. Mean of corrected distance from Snout to Dorsal Fin Insertion (SNDOR) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.42	0.44	0.42	0.43
Petersburg, Pennsylvania	28	0.42	0.40	0.46	0.40
Sara's Cove, Presque Isle Bay	28	0.40	0.44	0.38	0.41
Lagoons, Presque Isle Bay	30	0.40	0.44	0.39	0.39
Thompson's Bay, Presque Isle Bay	28	0.40	0.45	0.39	0.40
Old Womans Creek, Ohio	28	0.40	0.46	0.41	0.41
Dunkirk Harbor, New York	22	0.38	0.41	0.38	0.40
Long Point Bay, Canada	28	0.40	0.42	0.41	0.41
Tamarack Lake, Pennsylvania	28	0.39	0.43	0.37	0.40

Table A13. Mean of corrected distance from Snout to Pelvic Fin Insertion (SNPEL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.32	0.36	0.31	0.34
Petersburg, Pennsylvania	28	0.33	0.52	0.52	0.50
Sara's Cove, Presque Isle Bay	28	0.51	0.59	0.49	0.54
Lagoons, Presque Isle Bay	30	0.50	0.55	0.48	0.49
Thompson's Bay, Presque Isle Bay	28	0.49	0.54	0.49	0.50
Old Womans Creek, Ohio	28	0.48	0.53	0.48	0.50
Dunkirk Harbor, New York	22	0.48	0.53	0.45	0.51
Long Point Bay, Canada	28	0.49	0.51	0.47	0.48
Tamarack Lake, Pennsylvania	28	0.48	0.58	0.45	0.51

Table A14. Mean of corrected distance from Dorsal Fin Base Length (DFBL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.08	0.10	0.10	0.10
Petersburg, Pennsylvania	28	0.09	0.07	0.09	0.07
Sara's Cove, Presque Isle Bay	28	0.08	0.10	0.09	0.09
Lagoons, Presque Isle Bay	30	0.08	0.11	0.08	0.09
Thompson's Bay, Presque Isle Bay	28	0.09	0.07	0.10	0.09
Old Womans Creek, Ohio	28	0.08	0.09	0.08	0.08
Dunkirk Harbor, New York	22	0.08	0.07	0.08	0.07
Long Point Bay, Canada	28	0.08	0.10	0.07	0.08
Tamarack Lake, Pennsylvania	28	0.10	0.12	0.09	0.10

Table A15. Mean of corrected distance from Anterior Dorsal Fin Insertion to Anterior Anal Fin Insertion (ADAA) of *Ameiurus* species for each site

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.33	0.39	0.34	0.34
Petersburg, Pennsylvania	28	0.33	0.34	0.37	0.34
Sara's Cove, Presque Isle Bay	28	0.35	0.40	0.32	0.37
Lagoons, Presque Isle Bay	30	0.35	0.43	0.33	0.37
Thompson's Bay, Presque Isle Bay	28	0.35	0.32	0.36	0.36
Old Womans Creek, Ohio	28	0.34	0.38	0.34	0.37
Dunkirk Harbor, New York	22	0.34	0.40	0.33	0.36
Long Point Bay, Canada	28	0.34	0.39	0.32	0.36
Tamarack Lake, Pennsylvania	28	0.35	0.42	0.34	0.36

Table A16. Mean of corrected distance from Anterior Dorsal Fin Insertion to Posterior Anal Fin Insertion (ADPA) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.49	0.50	0.51	0.50
Petersburg, Pennsylvania	28	0.49	0.51	0.48	0.50
Sara's Cove, Presque Isle Bay	28	0.52	0.53	0.51	0.52
Lagoons, Presque Isle Bay	30	0.52	0.66	0.37	0.53
Thompson's Bay, Presque Isle Bay	28	0.53	0.51	0.54	0.53
Old Womans Creek, Ohio	28	0.50	0.61	0.39	0.49
Dunkirk Harbor, New York	22	0.52	0.56	0.51	0.53
Long Point Bay, Canada	28	0.51	0.53	0.50	0.52
Tamarack Lake, Pennsylvania	28	0.53	0.57	0.53	0.53

Table A17. Mean of corrected distance from Posterior Dorsal Fin Insertion to Anterior Anal Fin Insertion (PDAA) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.26	0.28	0.29	0.27
Petersburg, Pennsylvania	28	0.26	0.26	0.29	0.26
Sara's Cove, Presque Isle Bay	28	0.28	0.32	0.26	0.30
Lagoons, Presque Isle Bay	30	0.29	0.34	0.27	0.29
Thompson's Bay, Presque Isle Bay	28	0.28	0.30	0.28	0.28
Old Womans Creek, Ohio	28	0.27	0.30	0.28	0.28
Dunkirk Harbor, New York	22	0.28	0.35	0.25	0.29
Long Point Bay, Canada	28	0.28	0.34	0.27	0.29
Tamarack Lake, Pennsylvania	28	0.28	0.35	0.26	0.29

Table A18. Mean of corrected distance from Posterior Dorsal Fin Insertion to Posterior Anal Fin Insertion (PDPA) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.41	0.39	0.43	0.41
Petersburg, Pennsylvania	28	0.41	0.44	0.41	0.43
Sara's Cove, Presque Isle Bay	28	0.43	0.44	0.42	0.43
Lagoons, Presque Isle Bay	30	0.44	0.56	0.31	0.45
Thompson's Bay, Presque Isle Bay	28	0.44	0.45	0.44	0.45
Old Womans Creek, Ohio	28	0.42	0.54	0.31	0.42
Dunkirk Harbor, New York	22	0.44	0.50	0.42	0.47
Long Point Bay, Canada	28	0.43	0.44	0.43	0.44
Tamarack Lake, Pennsylvania	28	0.44	0.48	0.44	0.44

Table A19. Mean of corrected distance from Posterior Dorsal Fin Insertion to ventral point of least caudal peduncle (PDVC) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.53	0.52	0.56	0.55
Petersburg, Pennsylvania	28	0.54	0.52	0.58	0.54
Sara's Cove, Presque Isle Bay	28	0.55	0.83	0.25	0.56
Lagoons, Presque Isle Bay	30	0.56	0.57	0.56	0.56
Thompson's Bay, Presque Isle Bay	28	0.57	0.56	0.56	0.58
Old Womans Creek, Ohio	28	0.55	0.53	0.54	0.54
Dunkirk Harbor, New York	22	0.56	0.61	0.53	0.57
Long Point Bay, Canada	28	0.56	0.58	0.55	0.56
Tamarack Lake, Pennsylvania	28	0.56	0.56	0.57	0.55

Table A20. Mean of corrected distance from Posterior Anal Fin Insertion to Dorsal Point of Least Caudal Peduncle (PADC) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.19	0.23	0.20	0.21
Petersburg, Pennsylvania	28	0.19	0.17	0.24	0.19
Sara's Cove, Presque Isle Bay	28	0.20	0.17	0.28	0.22
Lagoons, Presque Isle Bay	30	0.19	0.22	0.18	0.20
Thompson's Bay, Presque Isle Bay	28	0.19	0.24	0.17	0.19
Old Womans Creek, Ohio	28	0.18	0.20	0.17	0.18
Dunkirk Harbor, New York	22	0.19	0.20	0.19	0.19
Long Point Bay, Canada	28	0.19	0.19	0.19	0.20
Tamarack Lake, Pennsylvania	28	0.18	0.18	0.20	0.19

Table A21. Mean of corrected distance from Anterior Dorsal Fin Insertion to Pelvic Fin (ADP2) of Ameiurus species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.23	0.29	0.25	0.26
Petersburg, Pennsylvania	28	0.23	0.20	0.29	0.22
Sara's Cove, Presque Isle Bay	28	0.27	0.32	0.30	0.30
Lagoons, Presque Isle Bay	29	0.26	0.29	0.24	0.27
Thompson's Bay, Presque Isle Bay	30	0.26	0.31	0.27	0.26
Old Womans Creek, Ohio	28	0.22	0.26	0.21	0.25
Dunkirk Harbor, New York	22	0.23	0.27	0.21	0.23
Long Point Bay, Canada	28	0.24	0.30	0.22	0.25
Tamarack Lake, Pennsylvania	28	0.23	0.28	0.24	0.26

Table A22. Mean of corrected distance from Posterior Dorsal Fin Insertion to Pelvic Fin (PDP2) . of Ameiurus species for each site

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.19	0.27	0.20	0.22
Petersburg, Pennsylvania	28	0.19	0.15	0.25	0.17
Sara's Cove, Presque Isle Bay	28	0.23	0.38	0.19	0.26
Lagoons, Presque Isle Bay	30	0.23	0.28	0.20	0.24
Thompson's Bay, Presque Isle Bay	28	0.23	0.30	0.21	0.23
Old Womans Creek, Ohio	28	0.18	0.24	0.17	0.21
Dunkirk Harbor, New York	22	0.19	0.24	0.17	0.20
Long Point Bay, Canada	28	0.21	0.28	0.19	0.22
Tamarack Lake, Pennsylvania	28	0.20	0.25	0.20	0.21

Table A23. Mean of corrected Caudal Peduncle Length (CPL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.15	0.20	0.16	0.17
Petersburg, Pennsylvania	28	0.15	0.14	0.17	0.15
Sara's Cove, Presque Isle Bay	28	0.15	0.16	0.16	0.15
Lagoons, Presque Isle Bay	30	0.14	0.19	0.14	0.15
Thompson's Bay, Presque Isle Bay	28	0.15	0.19	0.14	0.15
Old Womans Creek, Ohio	28	0.14	0.19	0.11	0.15
Dunkirk Harbor, New York	22	0.15	0.14	0.16	0.14
Long Point Bay, Canada	28	0.14	0.16	0.13	0.14
Tamarack Lake, Pennsylvania	28	0.15	0.17	0.16	0.15

Table A24. Mean of corrected Least Caudal Peduncle Length (LCPD) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.10	0.12	0.09	0.11
Petersburg, Pennsylvania	28	0.10	0.09	0.13	0.10
Sara's Cove, Presque Isle Bay	28	0.11	0.11	0.12	0.12
Lagoons, Presque Isle Bay	30	0.11	0.12	0.11	0.11
Thompson's Bay, Presque Isle Bay	28	0.11	0.13	0.10	0.11
Old Womans Creek, Ohio	28	0.11	0.11	0.11	0.11
Dunkirk Harbor, New York	22	0.10	0.12	0.10	0.11
Long Point Bay, Canada	28	0.11	0.13	0.10	0.12
Tamarack Lake, Pennsylvania	28	0.11	0.12	0.12	0.11

Table A25. Mean of corrected Anal Fin Base Length (AFBL) of *Ameiurus* species for each site.

Site	N	Mean	StDev	Minimum	Maximum
Clear Lake, Iowa	28	0.21	0.25	0.22	0.23
Petersburg, Pennsylvania	28	0.22	0.21	0.23	0.21
Sara's Cove, Presque Isle Bay	28	0.23	0.27	0.28	0.25
Lagoons, Presque Isle Bay	30	0.23	0.23	0.23	0.22
Thompson's Bay, Presque Isle Bay	28	0.23	0.38	0.12	0.24
Old Womans Creek, Ohio	28	0.21	0.23	0.21	0.22
Dunkirk Harbor, New York	22	0.22	0.28	0.10	0.22
Long Point Bay, Canada	28	0.22	0.21	0.22	0.22
Tamarack Lake, Pennsylvania	28	0.23	0.26	0.24	0.24

Table A26. Frequency distribution of the number of Dorsal fin rays (Dray) for *Ameiurus* species for each site.

Site	N	4	5	6	7	8	Mean	StDev
Clear Lake, Iowa	28		1	27	2		6.032258	0.314523
Petersburg, Pennsylvania	28	1	1	26			5.849896	0.423659
Sara's Cove, Presque Isle Bay	28		2	28			5.928571	0.262265
Lagoons, Presque Isle Bay	30		2	27			5.931034	0.257881
Thompson's Bay, Presque Isle Bay	28		1	26			5.962963	0.19245
Old Womans Creek, Ohio	28		1	25		1	6.037037	0.436902
Dunkirk Harbor, New York	22			19	3		6.136364	0.35125
Long Point Bay, Canada	28		3	25	1		5.931034	0.371391
Tamarack Lake, Pennsylvania	28			27	2		6.068966	0.257881

Table A27. Frequency distribution of the number of Anal fin rays (Aray) for *Ameiurus* species for each site.

Site	N	17	18	19	20	21	22	Mean	StDev
Clear Lake, Iowa	28	8	6	9	7			18.48	1.12
Petersburg, Pennsylvania	28			1	17	10		20.33	0.55
Sara's Cove, Presque Isle Bay	28	1		4	15	4	4	20.18	1.09
Lagoons, Presque Isle Bay	30	1	3	5	10	6	4	20.00	1.31
Thompson's Bay, Presque Isle Bay	28		1	5	13	8		20.11	0.89
Old Womans Creek, Ohio	28		6	7	10	3	1	19.48	1.34
Dunkirk Harbor, New York	22		2	3	7	7	2	20.23	1.11
Long Point Bay, Canada	28		1	7	9	9	3	20.21	1.05
Tamarack Lake, Pennsylvania	28		1	7	10	10	1	20.10	0.94

Table A28. Frequency distribution of the number of Pectoral fin rays (P1rays) for *Ameiurus* species for each site.

Site	N	6	7	8	9	Mean	StDev
Clear Lake, Iowa	28	1	2	27		7.87	0.43
Petersburg, Pennsylvania	28	1	5	22		7.74	0.53
Sara's Cove, Presque Isle Bay	28		2	28		7.93	0.26
Lagoons, Presque Isle Bay	30		4	24	1	7.90	0.41
Thompson's Bay, Presque Isle Bay	28		1	22	4	8.11	0.42
Old Womans Creek, Ohio	28		8	18	1	7.74	0.53
Dunkirk Harbor, New York	22	2	3	17		7.68	0.65
Long Point Bay, Canada	28		3	26		7.90	0.31
Tamarack Lake, Pennsylvania	28			29		8.00	0.00

Table A29. Frequency distribution of the number of Pelvic fin rays (P2rays) for *Ameiurus* species for each site.

Site	N	6	7	8	9	Mean	StDev
Clear Lake, Iowa	28		2	28		7.94	0.25
Petersburg, Pennsylvania	28			28		8.00	0.00
Sara's Cove, Presque Isle Bay	28		1	29		7.96	0.19
Lagoons, Presque Isle Bay	30		1	27	1	7.96	0.19
Thompson's Bay, Presque Isle Bay	28		1	24	2	8.04	0.34
Old Womans Creek, Ohio	28			26	1	8.04	0.19
Dunkirk Harbor, New York	22		1	19	2	8.05	0.38
Long Point Bay, Canada	28		1	27	1	8.00	0.27
Tamarack Lake, Pennsylvania	28		1	26	2	8.03	0.33

Table A30. Frequency distribution of the number of Epibranchial gill raker (EGR) for *Ameiurus* species for each site.

Site	N	3	4	5	6	7	Mean	StDev
Clear Lake, Iowa	28			25	2	3	5.23	0.56
Petersburg, Pennsylvania	28	2	26				3.93	0.27
Sara's Cove, Presque Isle Bay	28	13	17				3.54	0.51
Lagoons, Presque Isle Bay	30	5	19	5			4.00	0.60
Thompson's Bay, Presque Isle Bay	28	6	21				3.78	0.42
Old Womans Creek, Ohio	28	5	20	2			3.89	0.51
Dunkirk Harbor, New York	22	6	15	1			3.77	0.53
Long Point Bay, Canada	28	11	18				3.62	0.49
Tamarack Lake, Pennsylvania	28	5	24				3.83	0.38

Table A31. Frequency distribution of the number of Ceratobranchial gill raker (CGR) for *Ameiurus* species for each site.

Site	N	7	8	9	10	11	12	13+	Mean	StDev
Clear Lake, Iowa	28				5	12	8	6	11.61	1.26
Petersburg, Pennsylvania	28		10	18					8.63	0.49
Sara's Cove, Presque Isle Bay	28	8	18	3	1				7.89	0.69
Lagoons, Presque Isle Bay	30	1	12	16					8.48	0.69
Thompson's Bay, Presque Isle Bay	28	7	12	6	2				8.22	0.85
Old Womans Creek, Ohio	28		11	16					8.59	0.50
Dunkirk Harbor, New York	22		12	10					8.45	0.51
Long Point Bay, Canada	8	4	18	7					8.14	0.69
Tamarack Lake, Pennsylvania	28	2	14	13					8.38	0.62

Appendix B

Hybrid Index

A hybrid index was created for two individuals from Presque Isle Bay following Stauffer, Hocutt, and Denoncourt (1978);

$$H = [(X_H - u_1) / (u_2 - u_1)] \times 100$$

Where H = hybrid index, X_H = hybrid value, u_1 = value for *Ameiurus melas* and u_2 = value for *Ameiurus nebulosus*. An index value of 50 denotes exact intermediacy; over 50 indicates that the particular character is closer to *A. melas* and less than 50 indicates a closer resemblance with *A. nebulosus*.

Table B1. Comparison of the intergeneric hybrid *Ameiurus Nebulosus* x *Ameiurus melas* from Presque Isle Bay with pure species.

	Ameiurus melas				Hybrid				*Ameiurus nebulosus*				*Hybrid*
		n=30				n=2				n=27			*index*
Character	Min	Max	Mean		Min	Max	Mean		Min	Max	Mean		
SL (mm)	121.42	245.57	225.4197		281.08	283.9	282.49		113.35	297.22	158.4089		.....
HL	0.294946	0.324763	0.311124		0.306411	0.312402	0.309406		0.276444	0.370534	0.293939		*-106*
HW	0.202739	0.253354	0.225346		0.68318	0.689619	0.6864		0.179035	0.260962	0.198207		*
POHL	0.469379	0.557332	0.515097		0.522036	0.526957	0.524497		0.459524	0.531649	0.497811		*
HED	0.094401	0.147715	0.121778		0.123562	0.124727	0.124145		0.114374	0.176813	0.14819		*-81.9*
VED	0.088865	0.152767	0.114607		0.105455	0.106449	0.105952		0.106	0.155448	0.138276		**
PRE	0.384697	0.452797	0.413275		0.515089	0.519945	0.517517		0.14607	0.444696	0.397787		*
CD	0.165665	0.272309	0.225556		0.221387	0.223474	0.222431		0.152316	0.243441	0.190495		*-119*
LJL	0.347347	0.479312	0.398883		0.391869	0.395563	0.393716		0.261429	0.380746	0.330793		*-121*
HD	0.135718	0.167346	0.152571		0.150088	0.151594	0.150841		0.133735	0.183591	0.150105		*-102*
BD	0.187145	0.723193	0.224904		0.25044	0.252953	0.251697		0.208431	0.323599	0.232838		*
SNDOR	0.397606	0.450968	0.424508		0.426418	0.430696	0.428557		0.371751	0.455492	0.393786		*
SNPEL	0.302524	0.345205	0.323892		0.473089	0.477835	0.475462		0.456696	0.524956	0.486259		*-51.5*
DFBL	0.072444	0.103101	0.084875		0.08429	0.085136	0.084713		0.068272	0.103426	0.092571		**
ADAA	0.225621	0.362638	0.329439		0.316414	0.319589	0.318001		0.315834	0.402294	0.335949		**
ADPA	0.397332	0.517614	0.48892		0.522332	0.527572	0.524952		0.484076	0.549292	0.512253		*
PDAA	0.21923	0.296497	0.262288		0.247587	0.250071	0.248829		0.249028	0.361006	0.270331		**
PDPA	0.386428	0.437307	0.407916		0.439451	0.443859	0.441655		0.397735	0.46669	0.428841		*
PDVC	0.509024	0.563864	0.533495		0.54727	0.552761	0.550015		0.529031	0.589322	0.557947		*-94*
PADC	0.16247	0.214905	0.187275		0.165586	0.167248	0.166417		0.173217	0.255404	0.200617		**
ADP2	0.185048	0.274863	0.231756		0.253188	0.255728	0.254458		0.222933	0.339921	0.257648		*-87.7*
PDP2	0.142752	0.240294	0.192276		0.272561	0.275295	0.273928		0.172768	0.323776	0.219179		*
CPL	0.133258	0.174171	0.154175		0.152025	0.153551	0.152788		0.13763	0.172469	0.156848		**
LCPD	0.08401	0.119178	0.099408		0.116273	0.11744	0.116857		0.09898	0.170534	0.111999		*
AFBL	0.190879	0.240377	0.214306		0.243572	0.246015	0.244794		0.214824	0.265549	0.238155		*
DRay	5	7	6.032258		6	6	6		4	6	5.888889		*-106*
Aray	17	20	18.48387		18	18	18		19	21	20.33333		**
P2Rays	7	8	7.935484		8	8	8		8	8	8		*-92.7*
P1RAYS	6	8	7.870968		8	8	8		6	8	7.740741		*
TGR	5	7	5.225806		5	5	5		3	4	3.925926		*-156*
BGR	10	15	11.6129		8	9	8.5		8	9	8.62963		**

*Hybrid value greater than the mean for either parent.

** Hybrid value lower than the mean for either parent.

Appendix C

Table C1. Fluorescently labeled microsatellite primer sets for *Ameiurus nebulosus.*

Primer	Sequence	Microsatellite	length
Aneb16F	5' ATA TGA TAC TGA AAA CAG GTT GCC 3'	(GATA)24	~287
Aneb16R	5' GCT CCA AAT GTG TGC AAT TAG TAG 3'		~300
Aneb37F	5' CTT CCG AAC ATG CTG GGG TAT G 3'	(CTAT)12(CTGT)10	~267
Aneb37R	5' GAC TGC GGT TGC TGA TAT GGC 3'		
Aneb39F	5' AGC TTA GCT GCT GTC CTG CTA TCA CAC 3'	(GTAT)16	~240
Aneb39R	5' GCT GTC GCT TAC GGC CAT ATT 3'		
Aneb42F	5' AGC AAA CAC TTC TAT CCC AAA C 3'	(GTTT)10	~217
Aneb42R	5' CTA AAG ACC CAC CTC CTA CG 3'		
Aneb51F	5' GCT TAT AGA GAC CCA CAG TTA T 3'	(GATA)8(GAT)(GATA)15	~238
Aneb51R	5' TTT GAG CTA CTA GGA TCC C 3'		
Aneb61R	5' GTG TGC CTG AAC AAG CTC 3'	(CAGA)8	
Aneb61F	5' TGG GTT GAA AAT GAT GTA ATT C 3'		~255
Aneb63F	5' CTA ACT AAC TAG CCA ACA AAC C 3'	(CTAT)7	~167
Aneb63R	5' CGC ATG TTT TAT TTT CTC AA 3'		
Aneb64F	5' GCT GCA GCT GCC ACT ACT GCT GTG ACC 3'	(GTAT)8	~161
Aneb64R	5' TCC AAT CTT CAC CAA ATC TCG C 3'		
Aneb86F	5' CCA GCA GAG GAA CTG ATT AG 3'	(CTAT)13	~264
Aneb86R	5' ATT TCC TAC TGA CAG ACG GAT A 3'		

Appendix D

Allele Frequencies at microsatellite Locus Aneb16, Aneb37, Aneb61, Aneb63, and Aneb64 for the Presque Isle Bay collections; Lake Erie collections; and Tamarack Lake and Wisconsin collections.

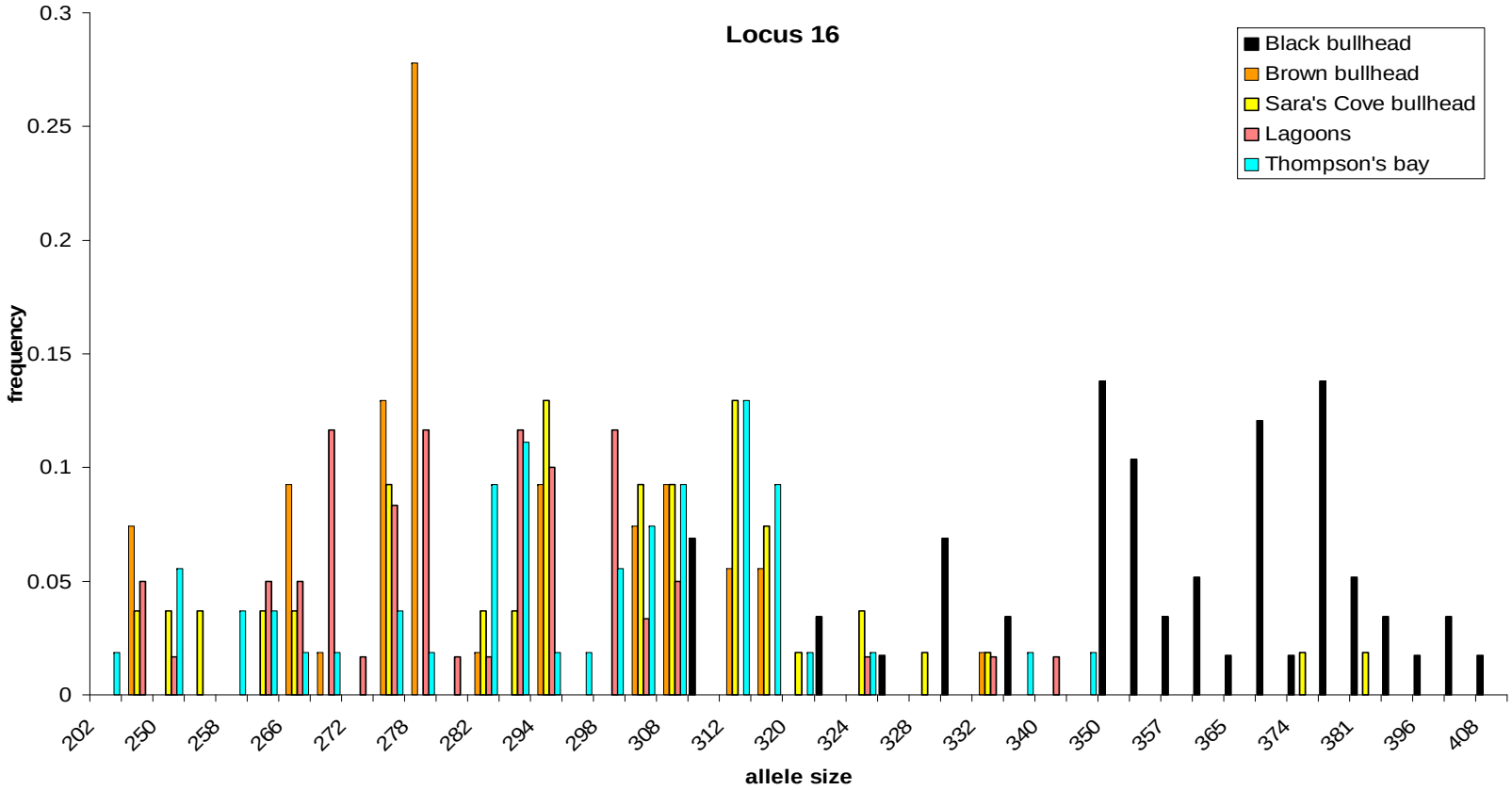

Figure D1. Allele frequencies at microsatellite locus 16 from samples of Presque Isle Bay populations.

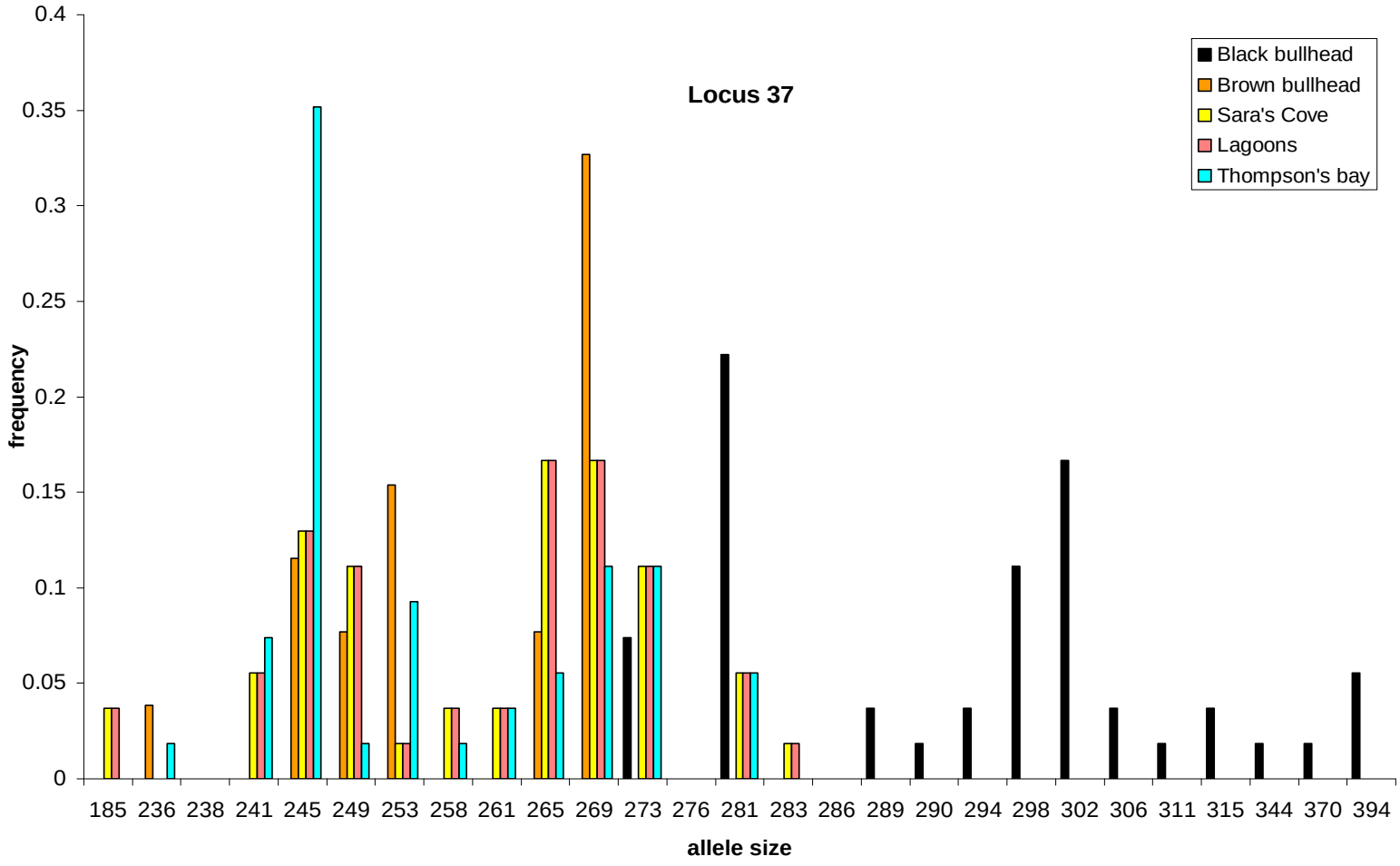

Figure D2. Allele frequencies at microsatellite locus 37 from samples of Presque Isle Bay populations.

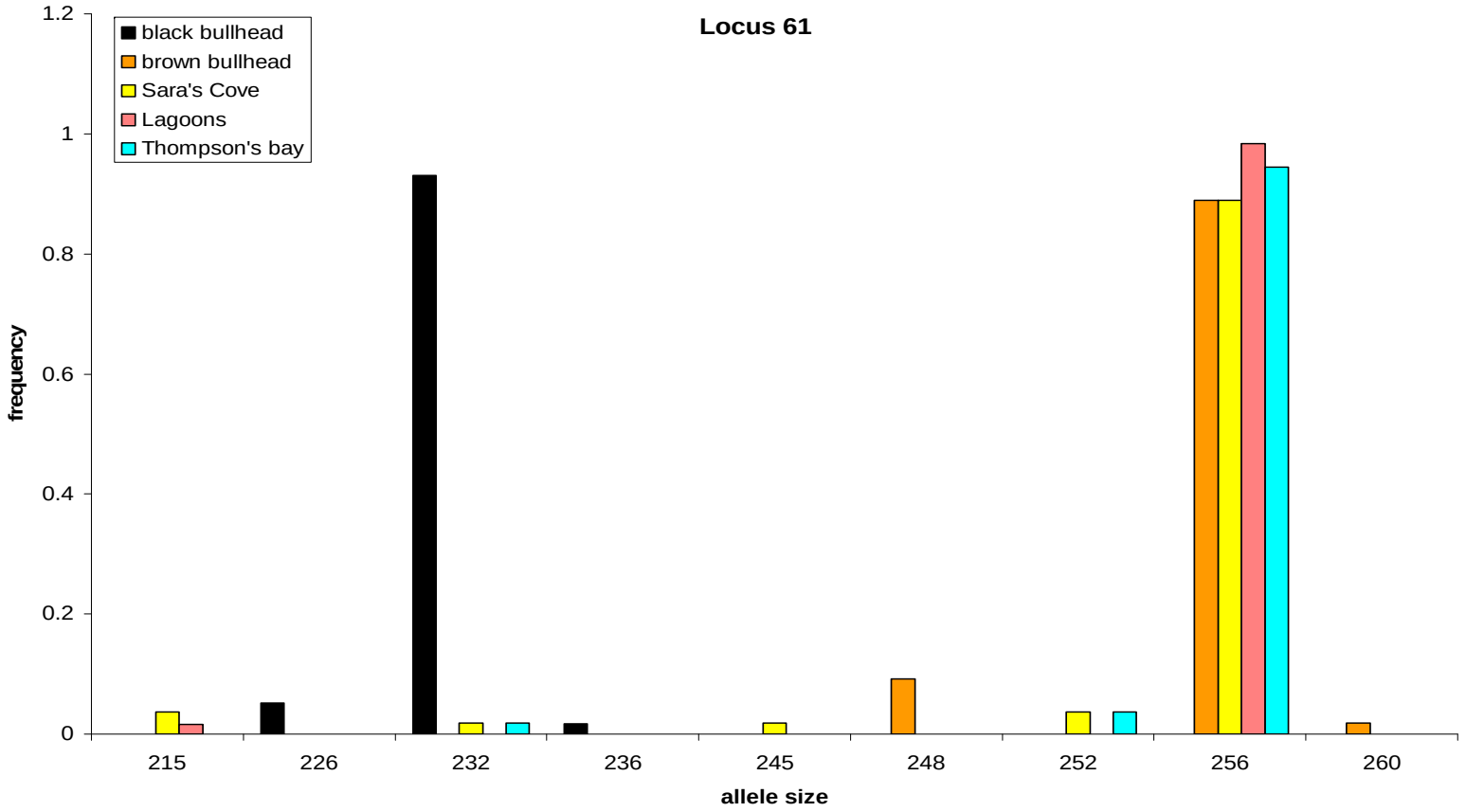

Figure D3. Allele frequencies at microsatellite locus 61 from samples of Presque Isle Bay populations.

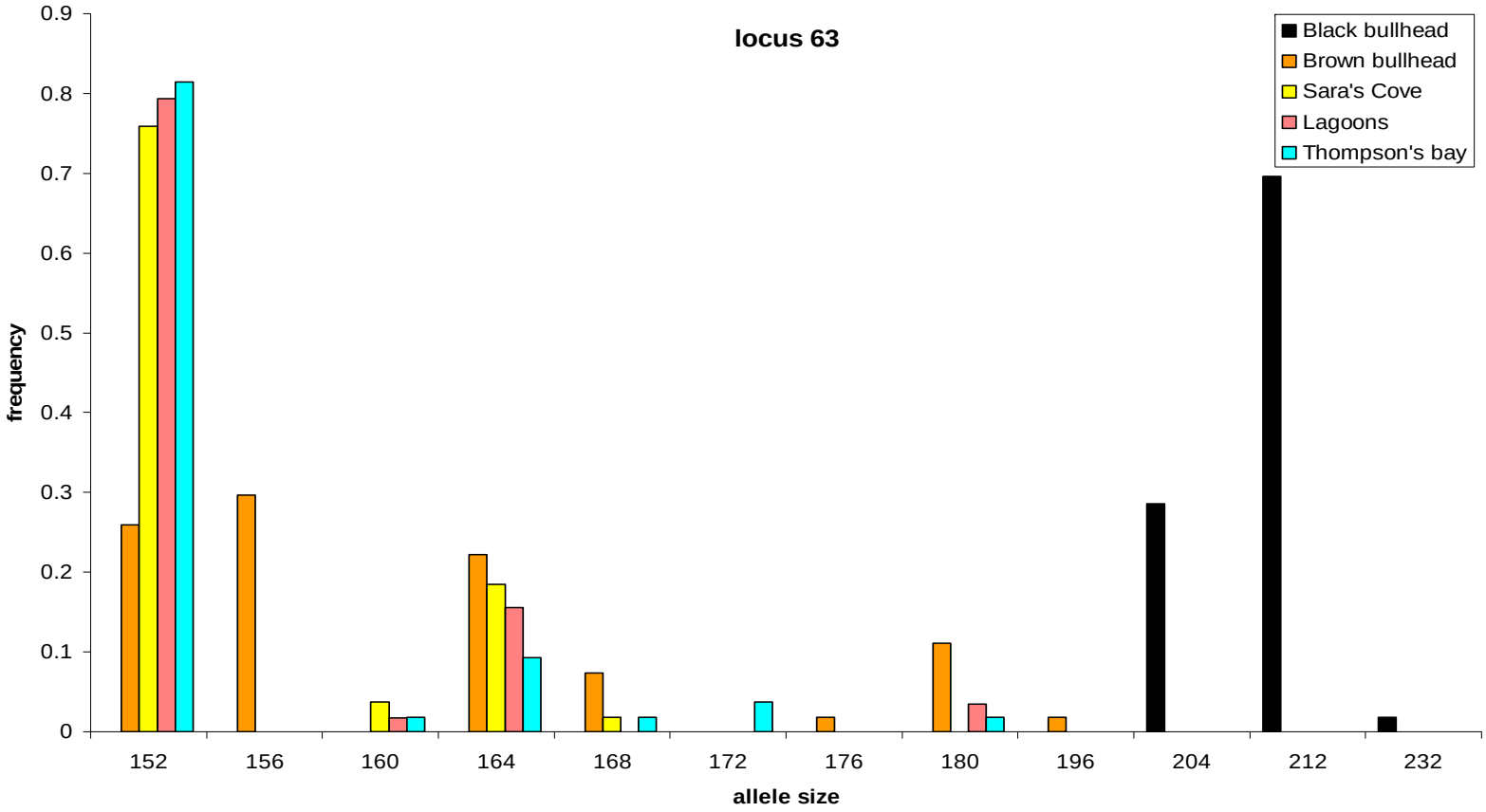

Figure D4. Allele frequencies at microsatellite locus 63 from samples of Presque Isle Bay populations.

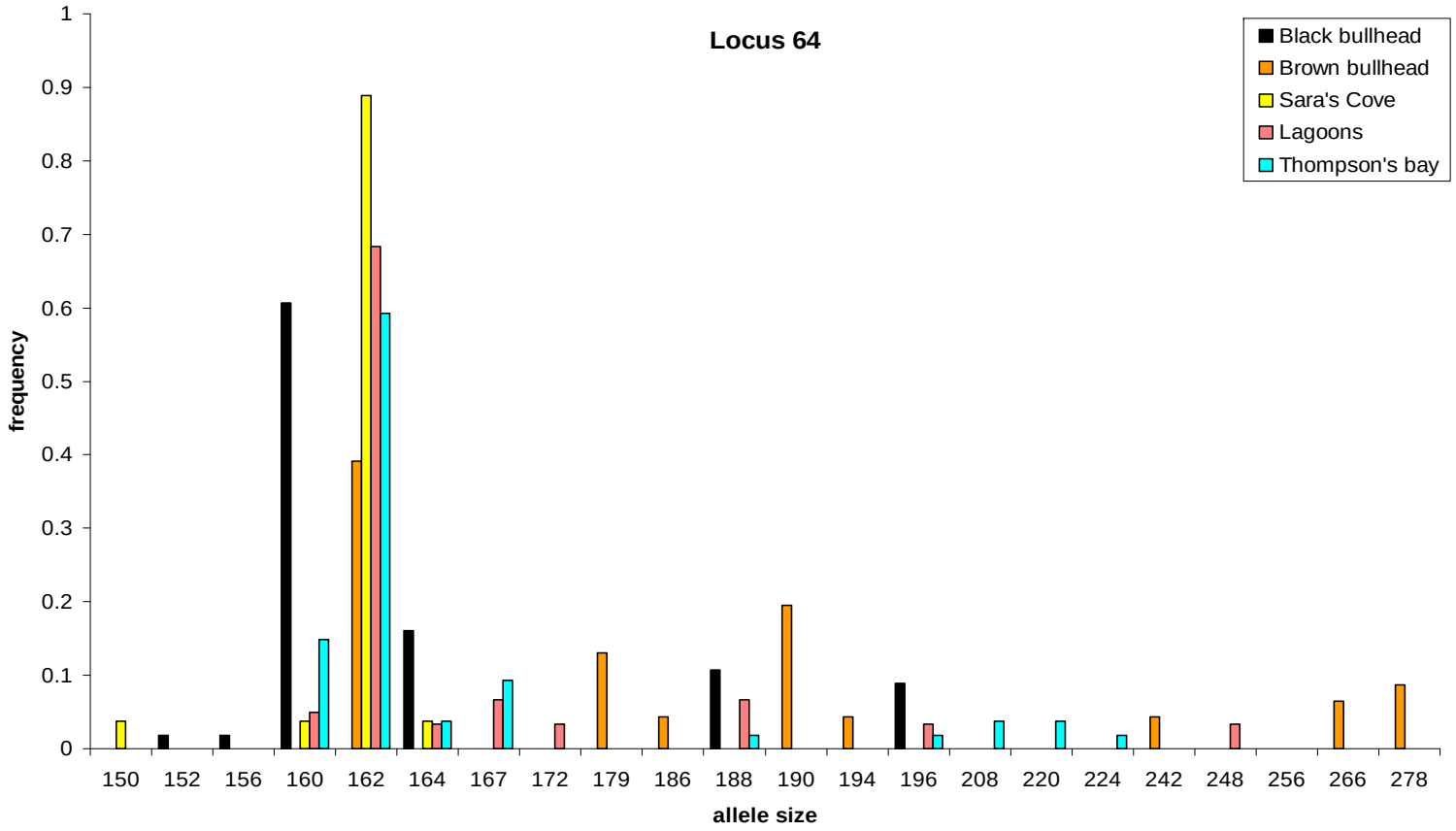

Figure D5. Allele frequencies at microsatellite locus 64 from samples of Presque Isle Bay populations.

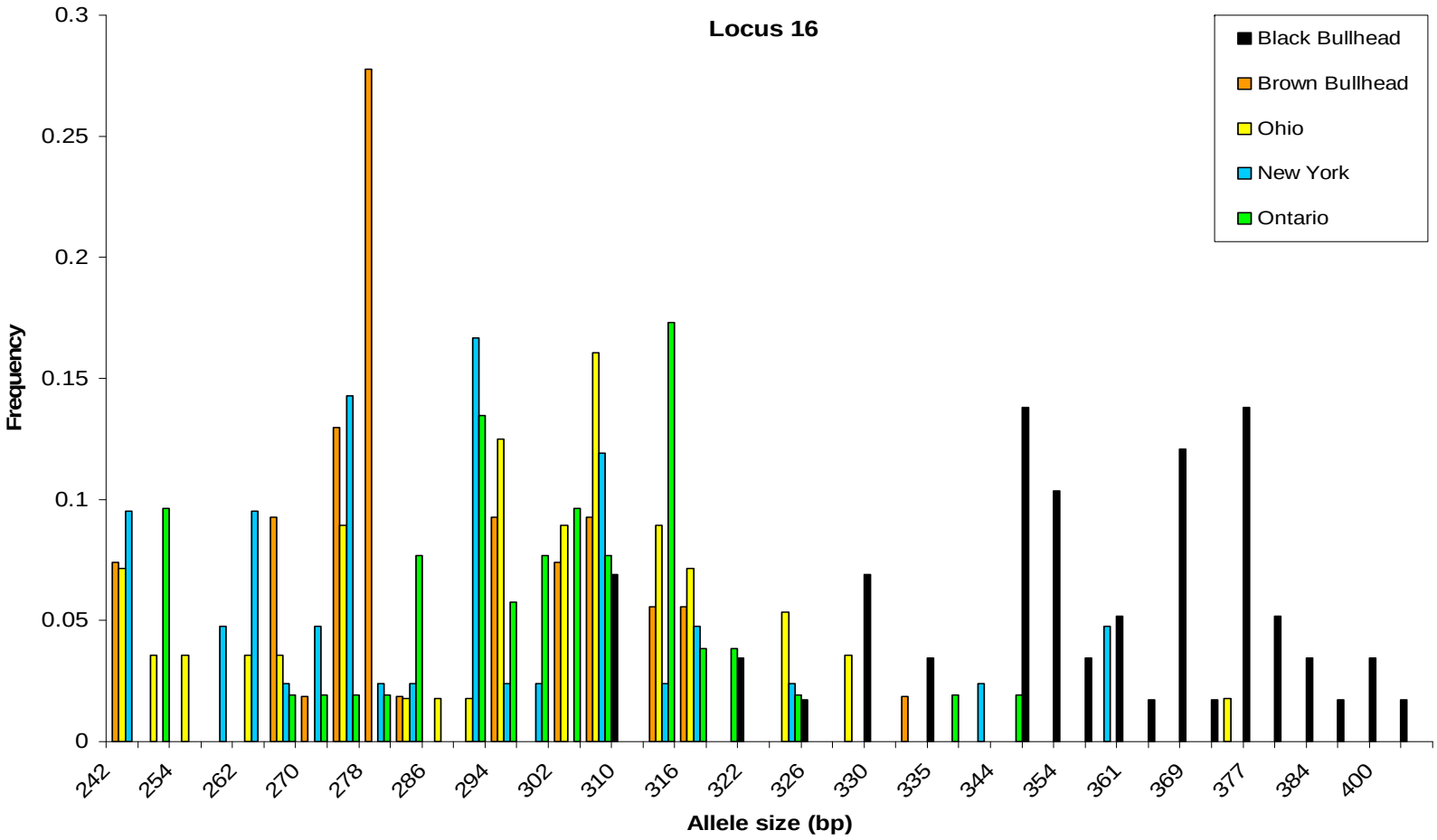

Figure D6. Allele frequencies at microsatellite locus 16 from samples of Lake Erie populations.

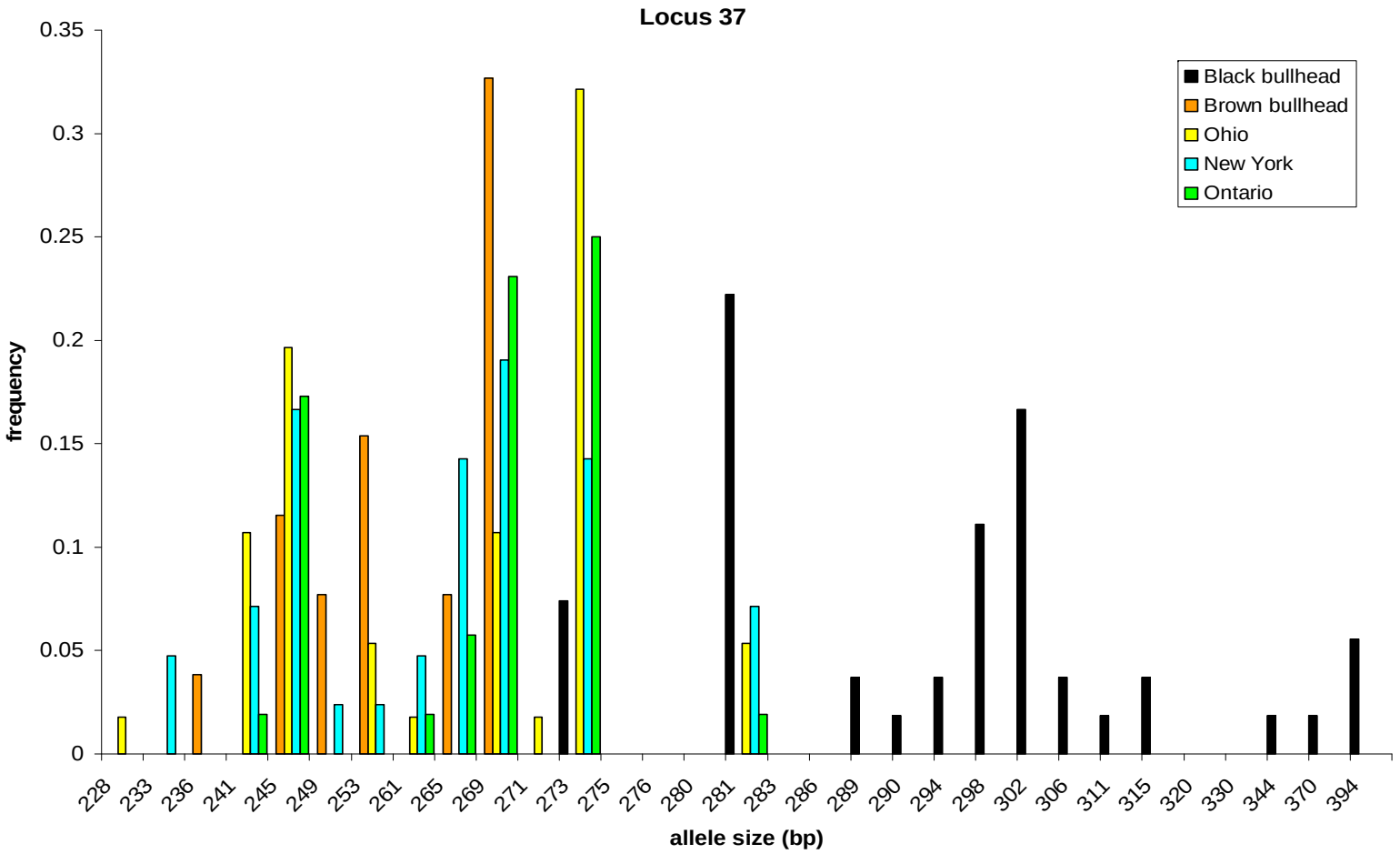

Figure D7. Allele frequencies at microsatellite locus 37 from samples of Lake Erie populations.

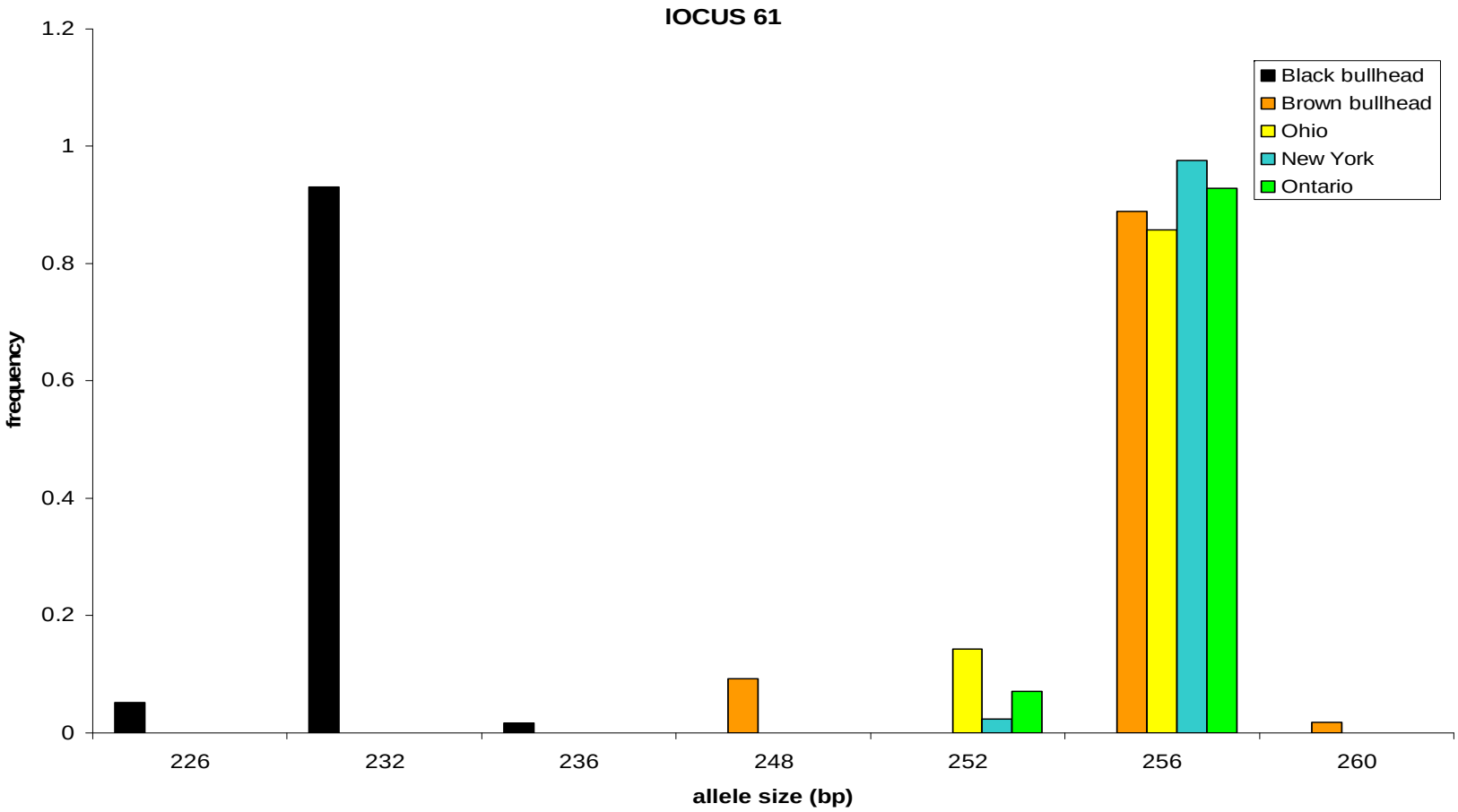

Figure D8. Allele frequencies at microsatellite locus 61 from samples of Lake Erie populations.

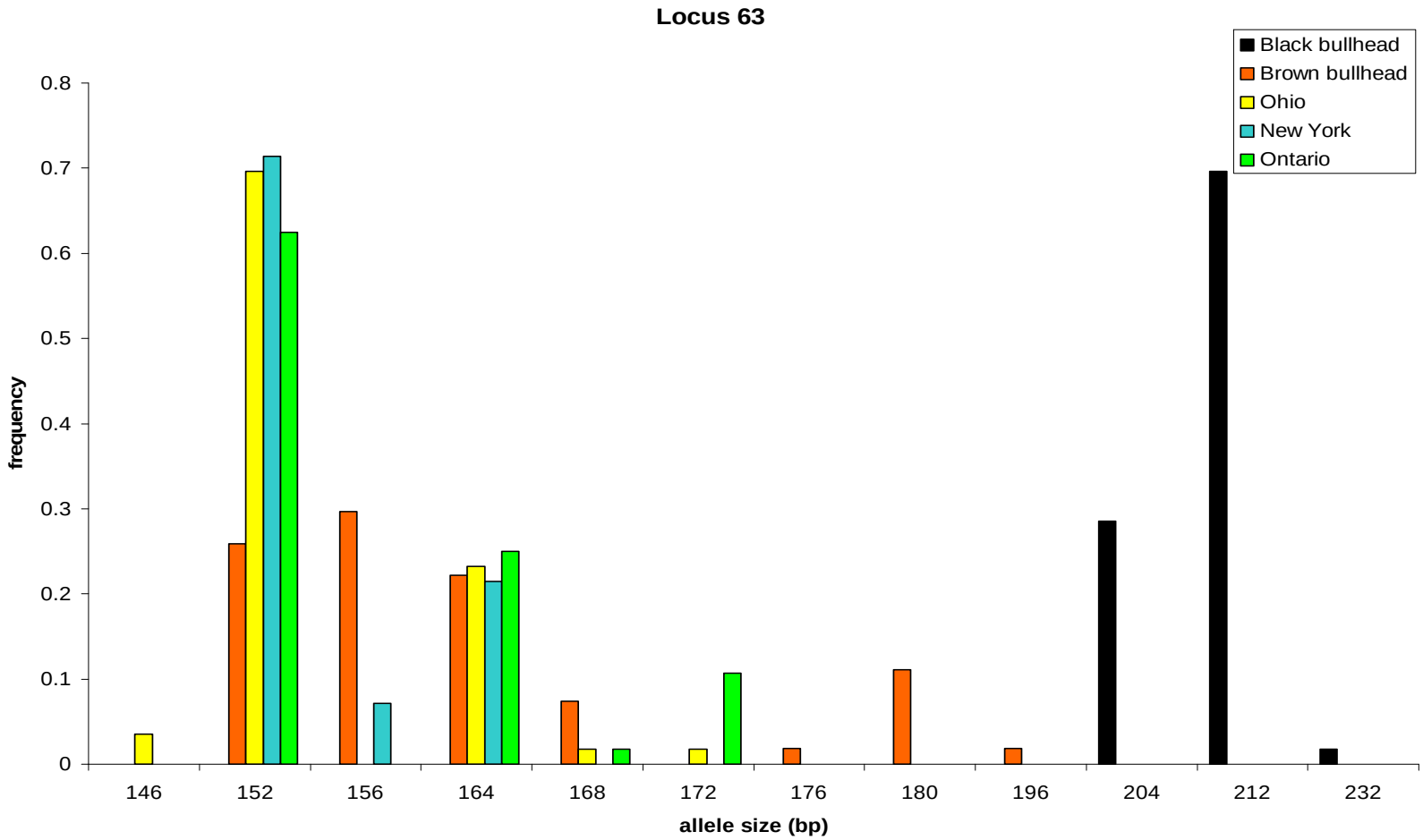

Figure D9. Allele frequencies at microsatellite locus 63 from samples of Lake Erie populations.

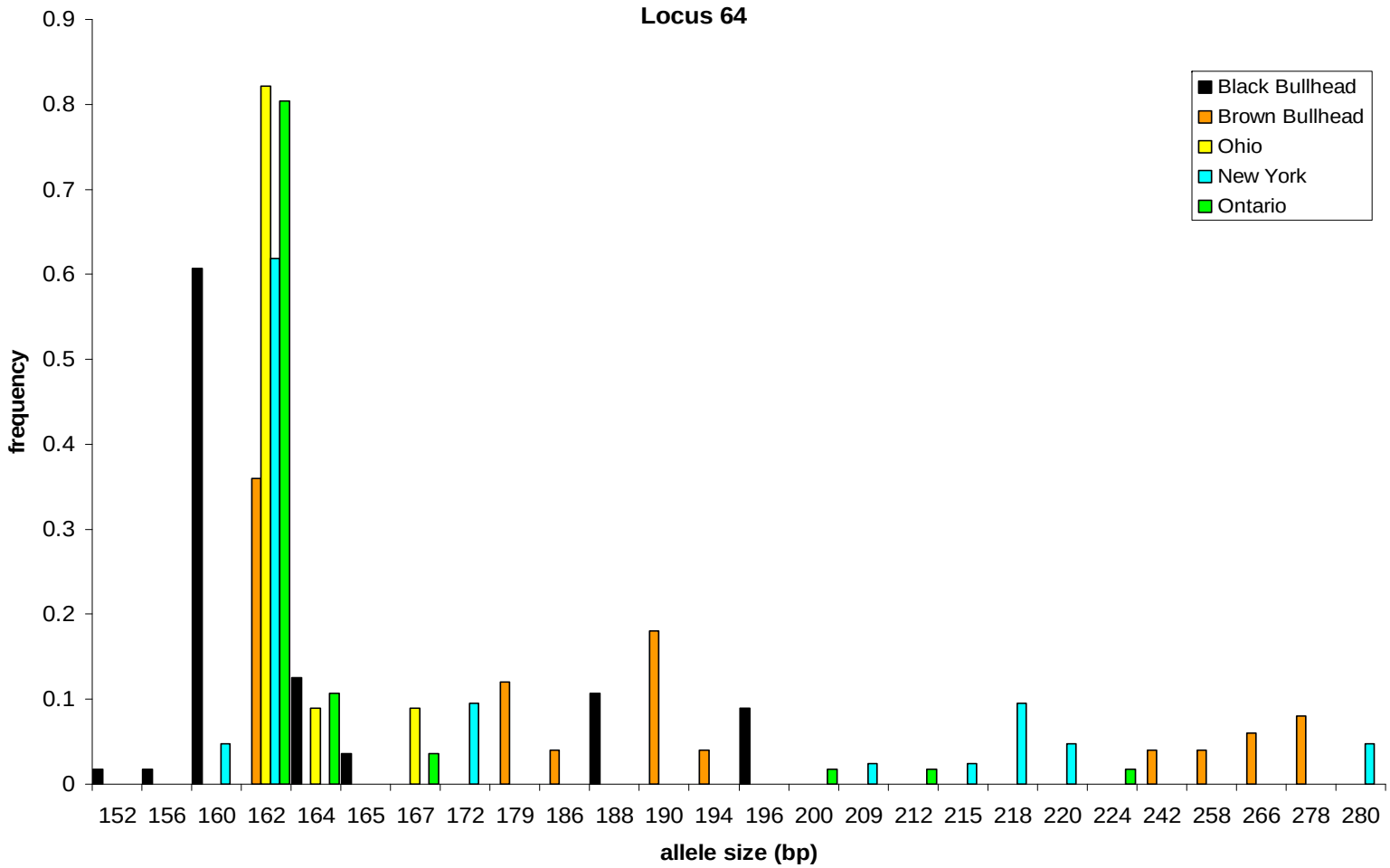

Figure D10. Allele frequencies at microsatellite locus 64 from samples of Lake Erie populations.

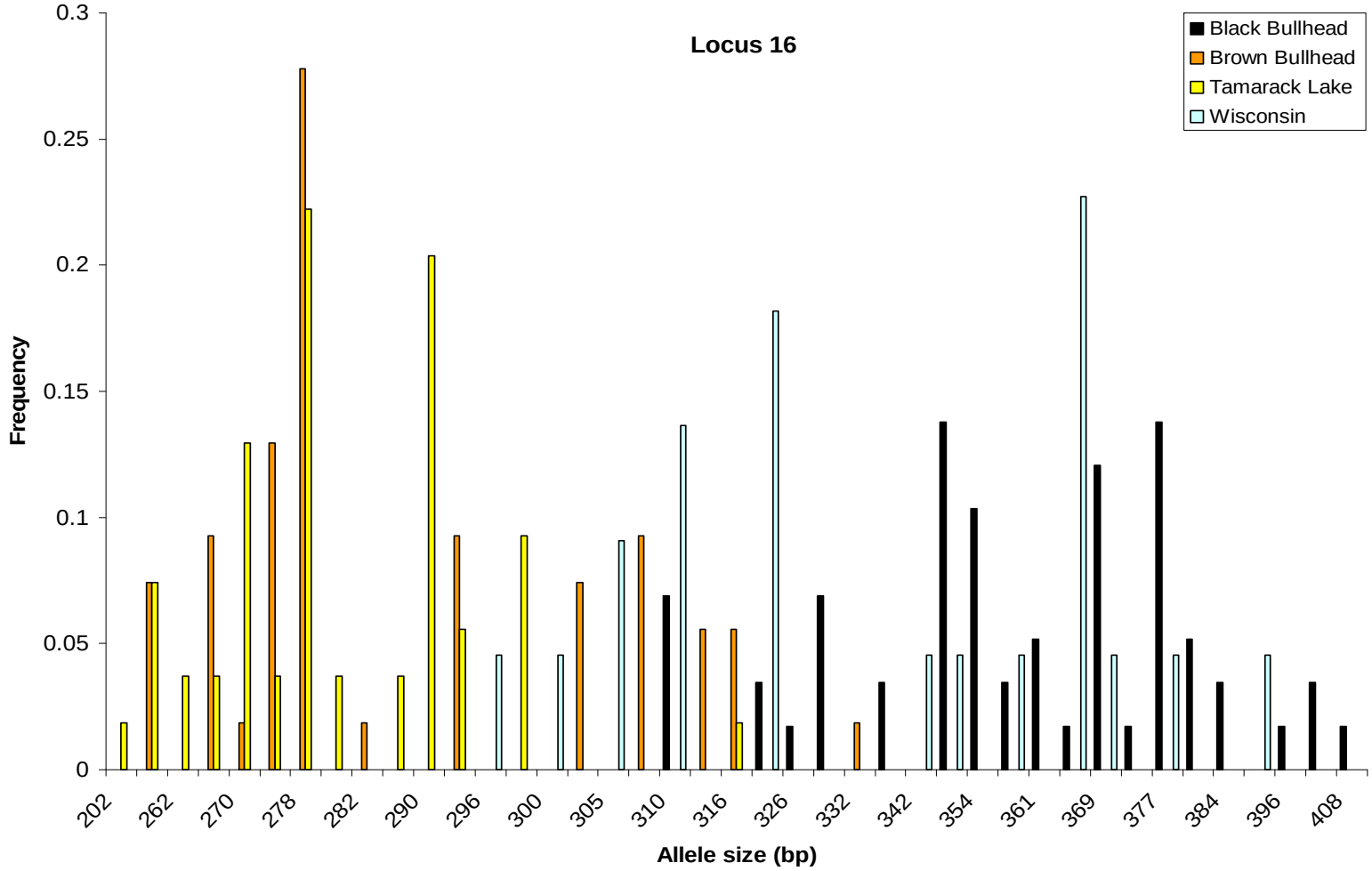

Figure D11. Allele frequencies at microsatellite locus 16 from samples of Tamarack Lake and Wisconsin populations.

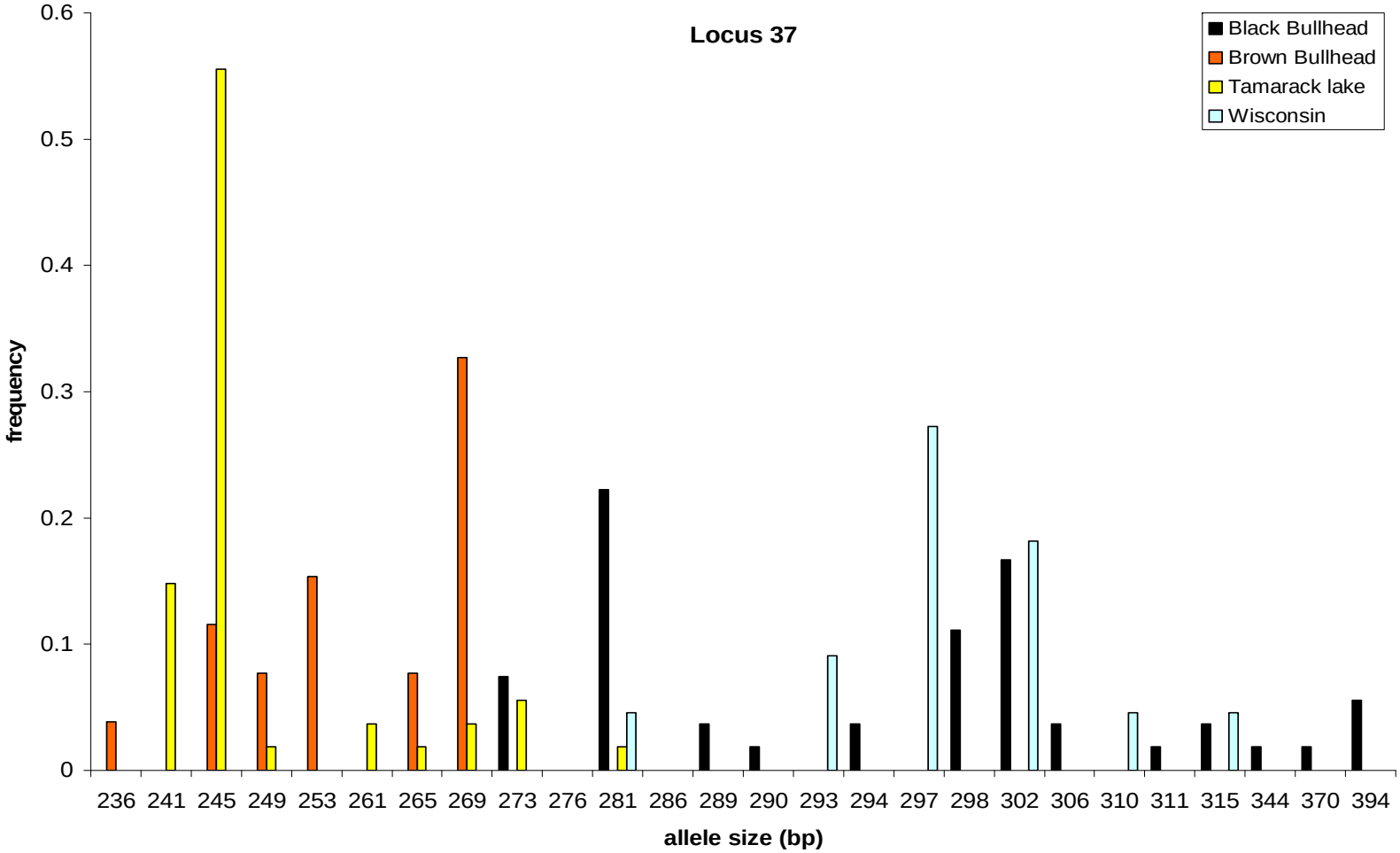

Figure D12. Allele frequencies at microsatellite locus 37 from samples of Tamarack Lake and Wisconsin populations.

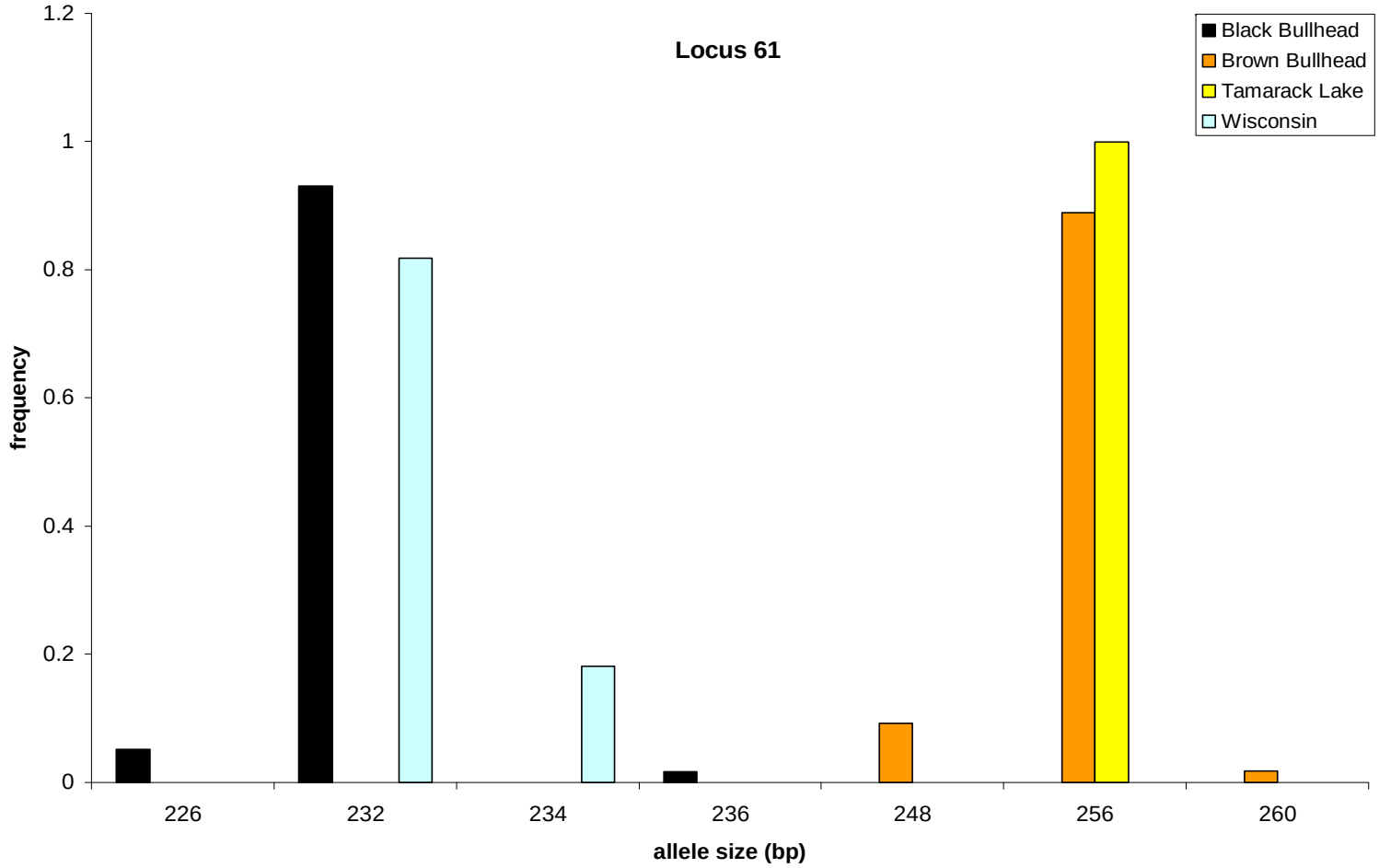

Figure D13. Allele frequencies at microsatellite locus 61 from samples of Tamarack Lake and Wisconsin populations.

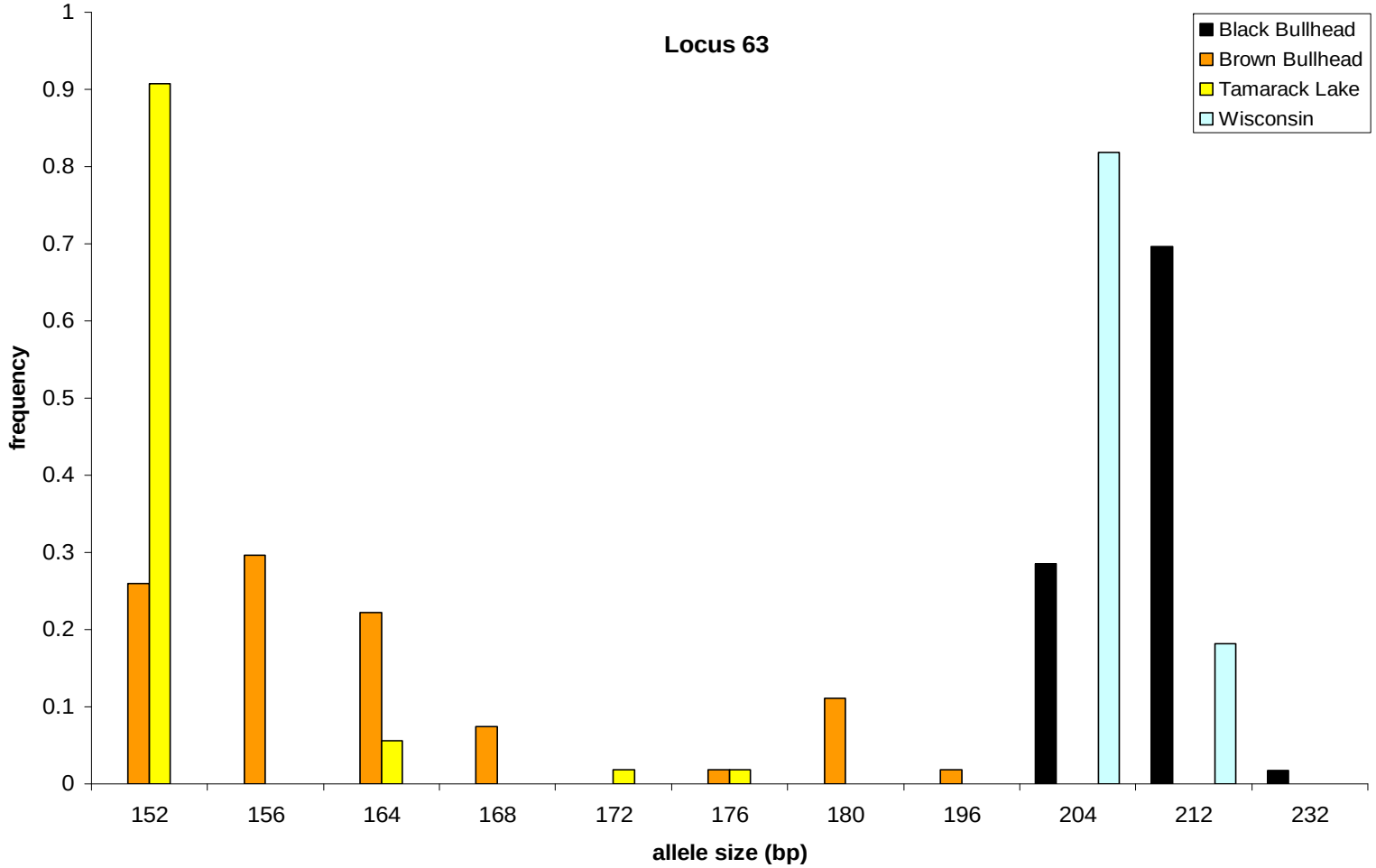

Figure D14. Allele frequencies at microsatellite locus 63 from samples of Tamarack Lake and Wisconsin populations.

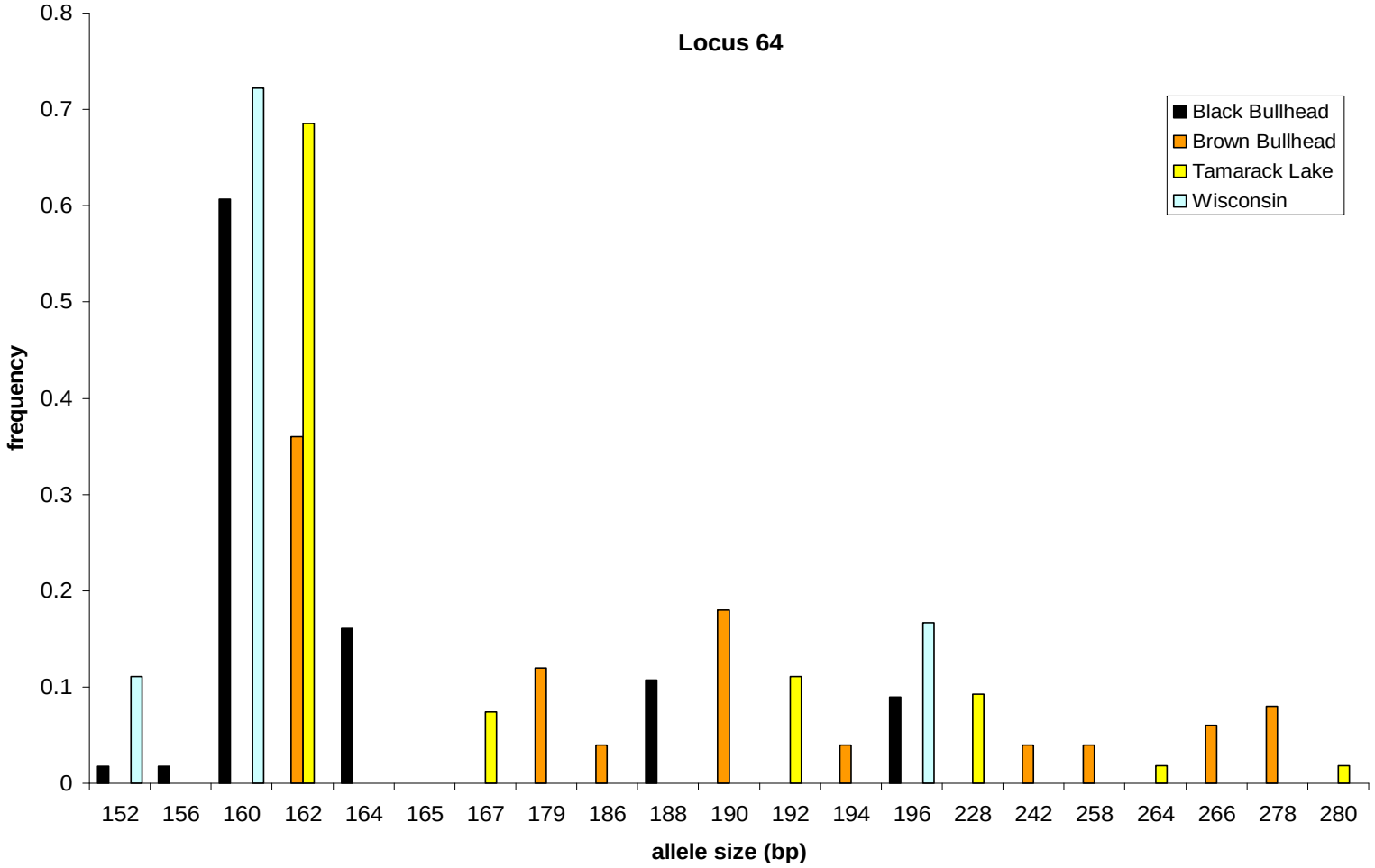

Figure D15. Allele frequencies at microsatellite locus 64 from samples of Tamarack Lake and Wisconsin populations