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Mitochondrial Haplotype Analysis of *Pomoxis nigromaculatus* Inhabiting Three Georgian Lakes

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Abstract

Pomoxis nigromaculatus, more commonly referred to as black crappie is indigenous to fresh water streams and lakes in the eastern United States and supports an important recreational fishery. We examined the genetic population structure of black crappie inhabiting three Georgian Lakes, Lake Sidney Lanier, Lake Seminole and Hartwell Lake. DNA sequencing of 229 fish samples, utilizing the DNA barcode marker cytochrome oxidase subunit I (COI) revealed 27 polymorphic sites which defined nine haplotypes. Only haplotype 2 was shared between all sample sites with six other haplotypes being unique for individual lakes, for an overall haplotype diversity of 0.734. Tajima's D and Fu's tests were implemented to assess departures from neutral expectations. Fst pairwise comparisons were statistically significant among all populations of black crappie evaluated in this study.

Keywords

Pomoxis nigromaculatus, Black Crappie, Cytochrome Oxidase, COI, Haplotype, Mitochondria

1. Introduction

Pomoxis nigromaculatus, more commonly referred to as black crappie, calico bass, grass bass, moonfish or speckled bass is a freshwater species in the sunfish family, Centrarchidae, and is native to the eastern United States [1]. It is indigenous to freshwater lakes and streams from the Great Lakes south to the Gulf of Mexico, as far north to North Dakota and eastern Montana, and east to the Appalachians [2] [3]. Black crappie has a silver-gray to white laterally compressed body with uneven black speckles along their sides. Black crappie has 7 - 8 dorsal

spines along with dorsal and anal fins that are similar in size and shape [4]. Both male and female black crappie reaches sexual maturity by the age of two and may live an average of seven years.

Although black crappie does not support a commercial fishery, the species is an important recreational fishery. According to the 2011 US Census Report, black crappie is the fourth most popular sporting fish sought out by [5]. Due to its popularity, the range of black crappie has been expanded through stocking and can now be found throughout the United States [6]. With the geographical expansion of black crappie by humans, it is important to genetically assess naturally established populations to assist in the management of species.

Visual identification of this species can be problematic because of similar morphological characteristics shared with white crappie, *Pomoxis annularis*. In previous studies, molecular markers such as isozymes and microsatellites have been utilized to help distinguish black crappie from white crappie and have been applicable to detect hybridization between the two species of crappie [7] [8] [9]. Molecular genetics techniques have also supported fisheries management by revealing reproductively isolated populations which require the formation of management units that assist in the generation of conservation strategies [10]. Another beneficial and reliable molecular tool for species identification and population delineation is DNA barcoding.

In 2003, Herbert *et al*. introduced DNA barcoding as a more rapid and reliable method for species identification. DNA barcoding uses short (~650 bp) nucleotide sequences to serve as unique species identifiers. These unique identifiers arise due to the possibility of four choices at each nucleotide position, which could lead to the creation of billions of unique identifiers when analyzing sequences that are hundreds of bp in length [11]. DNA's ability to substitute at the third base nucleotide adds to its characteristics for producing unique identifiers. The mutational rate of mitochondrial DNA (mtDNA) which is higher than that of nuclear DNA, may contribute to increased genetic variation between species. Also, mtDNA does not contain introns, is inherited maternally and tends not to exhibit the effects of recombination if only one lineage is present [12] [13].

Cytochrome oxidase subunit 1 (CO1) gene is the most commonly applied universal barcode, which codes for a mitochondrial protein involved in the Electron Transport Chain [14]. The CO1 mtDNA sequence is often used in forensic analysis, wildlife management, and species identification [15]. Furthermore, research indicates that CO1 is relatively conserved and therefore, an ideal gene for comparison of phylogenetic relationships [16]. The CO1 gene reveals a sequence longer than 650 bp long [15] [17]. Longer DNA lengths will provide more efficient identification labels [13]. Researchers have demonstrated that genetic barcoding has been beneficial in population genetic studies on invertebrates [18] [19] [20] and vertebrates [21] [22], such as fish [23] [24] [25]. Barcoding has the ability to detect both interspecies and intraspecies variation [26], [27] whether genetic samples are obtained from larval, juvenile or adult life stages [26] [28]

[29]. The aim of the present study is to identify CO1 haplotypes among black crappie residing in three Georgian lakes (Lake Sidney Lanier, Lake Seminole, and Hartwell Lake) and examine the population structure among species inhabiting the aforementioned water systems utilizing universal barcode COI.

Lake Lanier and Lake Seminole are reservoirs formed from the waters of the Chattahoochee River, which is part of the Chattahoochee River Basin [30]. The Chattahoochee River is the longest river in Georgia, and has been fragmented by 13 mainstream impoundments [30]. With the construction of the Buford dam in 1956, Lake Sidney Lanier was created for flood control, hydroelectric power generation, and recreational usage (Georgia Department of Natural Resources). Lake Lanier is a 15,378 ha. reservoir that provides water to the Atlanta, Georgia metropolitan area and is located approximately 560 km northeast of Lake Seminole (Georgia Department of Natural Resources). Lake Seminole is a 14,973 ha. reservoir was constructed in 1957 [31]. The lake borders the southwestern part of Georgia and the Floridian panhandle (Georgia Department of Natural Resources). It forms at the junction of the Flint and Chattahoochee Rivers and is dammed by the Jim Woodruff Lock and Dam before flowing into the Apalachicola River (Georgia Department of Natural Resources). Hartwell Lake is a 22,649 ha. reservoir at the border of Georgia and South Carolina. It was created from the construction of the Hartwell Dam in 1955 [32]. The Hartwell dam is one of the dams located along the Savannah River and is approximately 11 km downstream the Seneca and Tugaloo River [32]. There is no detectable hydrological connectivity between the two lakes formed by the Chattahoochee River and Hartwell Lake located on the Savannah River, but all three water systems contain natural occurring populations of black crappie.

2. Materials and Methods

Promoxis nigromaculatus, balck crappie specimens were collected by fisheries biologists of the Georgia Department of Natural Resources and researchers at Georgia Gwinnett College. Collection permits were issued by the Georgia Department of Natural Resources and Wildlife Resources Division. Fin clips were removed by nonlethal means from fish inhabiting three lakes in Georgia: Hartwell Lake (n = 29), Lake Sidney Lanier (n = 116), Lake Seminole (=81) and preserved in 90% ethanol for genetic analysis (**Figure 1**).

To obtain mitochondrial DNA sequence data, mitochondrial DNA was isolated utilizing Chelex-100 resin based on the protocol of Walsh *et al.* [33]. The COI gene was amplified with the following primer sequences CO1_VF2_t1-5' TGT AAA ACG ACG GCC AGT CAA CCA ACC ACA AAG ACA TTG GCA C 3' and CO1_FISHR2_T1-5' CAG GAA ACA GCT ATG ACA CTT CAG GGT GAC CGA AGA ATC AGA A '3 [34]. PCR reactions were carried out in a 25μl volume consisting of 12.5 μl of GoTaq® Green Master Mix (Promega™); 1 μl of each forward and reverse primer; 1 μl of DNA template; and 9.5 μl of nuclease-free water. Thermal cycling profile consisted of an initial denaturation step

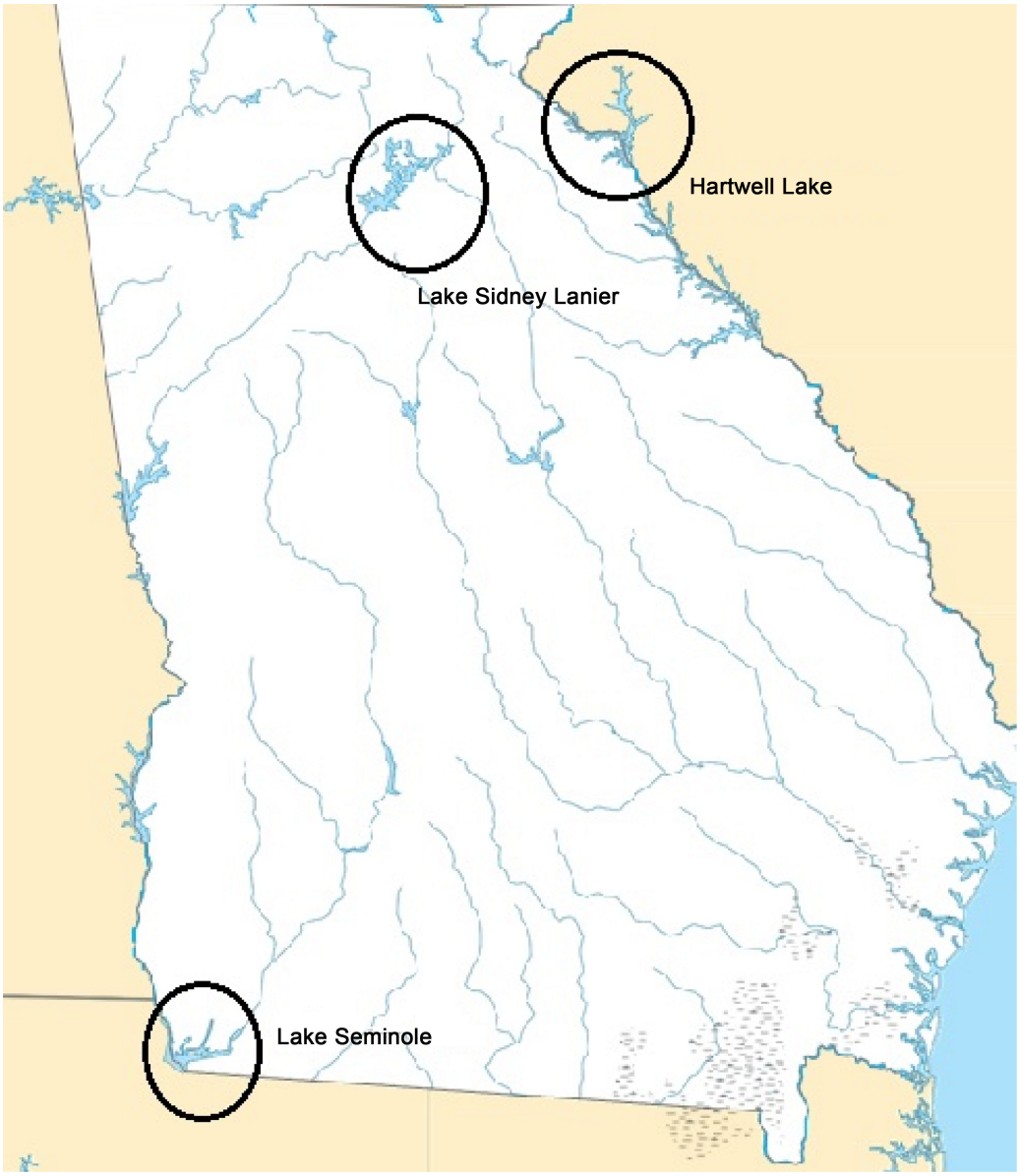

Figure 1. Map of sampling locations for Black crappie: Hartwell Lake (HL) (n = 29), Lake Sidney Lanier (LL) (n = 116), Lake Seminole (LS) (n = 81).

of 94˚C for 2 minutes followed by 35 cycles of 94˚C for 30 seconds, 52˚C for 45 seconds, and 72˚C for 1 minute, with a final extension at 72˚C for 10 minutes. All PCR products were run on 1% agarose gel with SYBR green and Tris-Borate-EDTA (TBE) buffer and visualized with UV translumninator. Positively amplified PCR product were then purified utilizing ExoSAP-IT (Affymetrics), 2 µl of ExoSAP-IT per 5 µl of PCR product. The mixture was incubated at 37˚C for 15 minutes, and incubated at 80˚C for 15 minutes to inactivate reagent. The cleansed PCR product was sent to University of Georgia Genomics Facility in Athens, Georgia for sequencing on a Applied Biosystems 3730xl 96-capillary DNA Analyzer. Chromatograms were verified and trimmed in Finch TV version 1.4.0. Sequences were then exported to Clustal X2.1 for alignment [35]. The

aligned sequences were uploaded to DnaSP 5.10 to determine haplotype diversity (*h*) and nucleotide diversity (π) [36]. Partitioning of variance within and among populations was calculated utilizing Arlequin 3.5.1.2 by an analysis of molecular variance (AMOVA) [37]. A minimum spanning tree was created in Hapstar to reveal the most likely connections among haplotypes and lakes [38].

3. Results

Nine haplotypes (Hap 1 - Hap 9) were recovered from sequencing partial CO1 gene for 226 individuals. Hap 2 was identified in all three locations and was the most abundant haplotype representing thirty-eight percent of the population. Of the nine haplotypes defined, only Hap 8 was unique to one individual calculated to be 0.4% of the total sample size. Lake Lanier had five haplotypes represented in 116 individuals (haplotypes 1, 2, 3, 4 and 5). LS revealed six haplotypes for 81 individuals (haplotypes 1, 2, 3, 7, 8 and 9) and HL's 29 samples contained two haplotypes, Hap 2 and Hap 6. Haplotype 6 was unique to HL, while Hap 5 and Hap 4 were only identified within LL and Hap 7, 8, and 9 were only observed in LS (Table 1).

Table 1. Sample size, number of haplotypes and haplotype frequencies for each sample site.

Location	Haplotypes	n	Haplotype Fequency
LL	Hap 1	24	0.21
	Hap 2	77	0.66
n = 116	Hap 3	4	0.03
	Hap 4	4	0.03
	Hap 5	7	0.06
LS	Hap 1	47	0.58
	Hap 2	8	0.10
n = 81	Hap 3	17	0.21
	Hap 7	5	0.06
	Hap 8	1	0.01
	Hap 9	3	0.04
HL	Hap 2	1	0.03
n = 29	Hap 6	28	0.97
All Locations	**Haplotypes**	**n**	**Haplotype Frequency**
	Hap 1	71	0.31
	Hap 2	86	0.38
	Hap 3	21	0.09
n = 226	Hap 4	4	0.02
	Hap 5	7	0.03
	Hap 6	28	0.12
	Hap 7	5	0.02
	Hap 8	1	0.00
	Hap 9	3	0.01

Haplotype diversity, *h* was low for HL (*h* = 0.069) and moderate for LL (*h* = 0.515) and LS (*h* = 0.612) (Table 2). Nucleotide diversity, π ranged from 0.0001 for HL to 0.0052 for LS. While haplotype and nucleotide diversity were similar among samples from LL and LS. HL samples had the lowest nucleotide diversity value 0.0001 which is representative of two alleles with one polymorphic site (Table 2).

Results from AMOVA indicated an overall genetic variation within populations (65.76%) which was much larger than the variation among populations (34.24%). Pairwise F_{ST} values among populations were all significant. The most significant differentiation in pairwise comparisons was determined to be between LL and HL (F_{ST} = 0.606), both located within two different river basins (Table 3). The lowest pairwise F_{ST} value was observed between LL and LS (F_{ST} = 0.216) which reside within the same river basin. Tajima's D values were negative for all population except LL. Tajima's D values were negative for LS and HL indicating an excess of low frequency polymorphisms, but positive for LL signifying low levels of both low and high frequency polymorphisms. Fu's F statistic were positive for LL and LS, but negative for HL. Neither neutrality statistic were significant.

The minimum-spanning tree depicts mutational relationship between mtDNA haplotypes among all lakes for CO1 (Figure 2). The shaded areas within the circles are proportional to the frequency of each haplotype. The prevalent haplotype was Hap 2, which was separated by one to two mutations for the majority of haplotypes and 21 nucleotide substitutions between Hap 7. Haplotype network can be divided into three groups that over lap at Hap 2, which corresponds with the location of the three sample sites.

Table 2. Sample size, number of haplotypes, number of polymorphic sites, haplotype diversity and nucleotide diversity of different lakes.

Location	n	n_h	s	*h*	π	*D*	Fs
LL	116	5	3	0.515	0.001	0.833	0.058
LS	81	6	25	0.612	0.005	-0.970	4.698
HL	29	2	1	0.069	0.0001	-1.149	-1.183

Sample size = n; number of haplotypes = n_h; number of polymorphic sites = s; haplotype diversity = h; nucleotide diversity = π; Tajimas D = D and Fu's Fs = Fs.

Table 3. AMOVA F_{ST} results (lower diagonal) and their significance level (upper diagonal).

Location	LL	LS	HL
LL	-	*	*
LS	0.21597	-	*
HL	0.60576	0.43807	-

Denotes significant difference < 0.050.

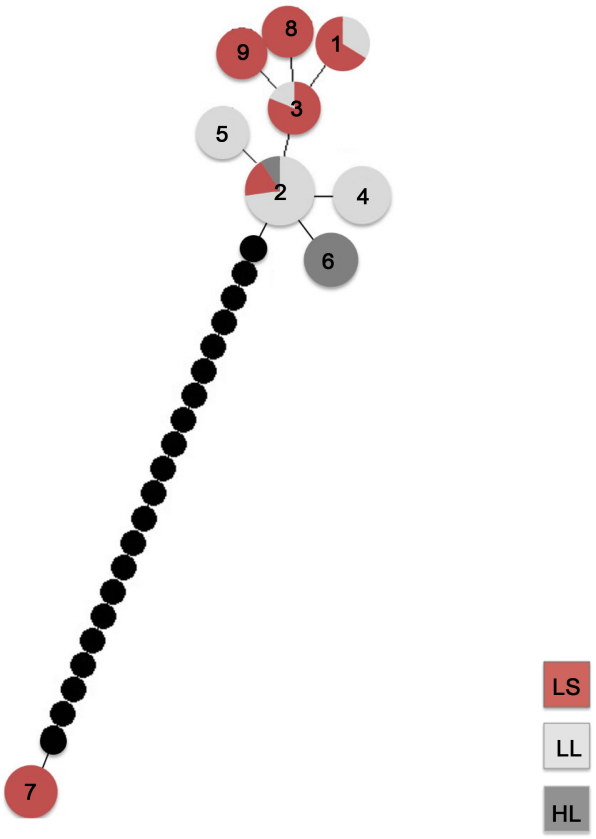

Figure 2. Minimum spanning tree of *P. nigromaculatus* haplotypes. Small black circles are mutational steps, branches indicate single mutations and numbers represent individual haplotypes. Large circle denotes most common haplotype, Hap 2.

4. Discussion

In this study we examined genetic variability utilizing mitochondrial COI sequences to detect genetic structuring in three Georgian Lakes. Mitochondrial analysis through pairwise comparison Fst values indicated significant genetic variation among three Georgian Lake populations of black crappie. We believe there are several factors that may attribute to the statistical significance revealed through AMOVA, such as migratory behavior, distance, hydrological barriers and historical river drainage connectivity.

Migratory behavior of black crappie may be an obstacle to gene flow among interconnected lakes. This species exhibits diel and seasonal variation in depth and distance from shore. Black crappie oscillation between offshore and littoral habitat maybe triggered by food availability, predation or the need to locate optimal spawning habitat [39]. Preliminary data has suggested that black crappie could possess home ranges, but have the potential to migrate considerable distances with no preference for upstream or downstream movement [40] and [41]. Guy *et al.* [42] has reported a median home range of 15.8 ha. for a related species, white crappie in a South Dakota lake. Black crappie have been observed to

have cove fidelity during spawning seasons at a rate of 80%.

It should be noted that migratory studies discussed were performed on smaller lakes ranging from 11 ha. to 1151 ha. compared to the lakes reported in this study, that ranged in size from 14,973 ha. to 22,662 ha. Georgian lakes sampled in this study contained greater depths, thermoclines and lack of suitable habitat proximal to dams which may affect migratory behavior downstream. Migratory behavior has the potential to affect gene flow between Lake Lanier and Lake Seminole which could be relevant for management of the species in the categorization of black crappie species on the Chattahoochee River into isolated sub population. Hartwell Lake's black crappie migratory behavior would not support significantly different Fst values due to the lack of hydrological connectivity to Lake Lanier and Lake Seminole. We can assume that Fst values are a product of reproductive isolation, drift and mutation.

The length and hydrological barriers of the Chattahoochee River support the observed significant Fst values. The river is estimated to be 698 km. and contains 13 dams and three lock-and-dam facilities. Lake Lanier and Lake Seminole are approximately 552 km. apart and separated by 14 dams including three locks. Man-made barriers were implemented in the 1800's when timber dams were constructed to grind corn mealand later in the twentieth century hydro electrical dams were erected. With numerous impediments and hundreds of kilometers to traverse, genetic fragmentation has been maintained and reinforced through genetic drift and mutation. These populations have only been separated physically for an estimated 189 years, but maintain shared alleles (Hap 1, Hap 2, Hap 3). Gene flow in riverine systems are more inclined to move downstream instead of upstream [43]. However, even this connectivity may be filtered by barriers and distance. In essence fish population structure can be altered by hydroelectric and low-head dams and has been observed in several species [43] [44] [45].

Common haplotypes observed between river systems may be explained through historical connectivity between southern Atlantic Slope Rivers and three Gulf Slope Rivers during the Oligocene Epoch [46]. The intraspecific genetic variation detected in black crappie sample sites on the Chattahoochee River (Lake Lanier and Lake Seminole) and Hartwell Lake located on the Savannah River maybe attributed to repeated periods of isolation and recolonization among riverine systems. Maurakis and Lipscomb [47], researched 19 river drainages on the Atlantic Slope detecting the presence of 124 shared native species of cyprinid fishes indicating connectivity among the rivers of the Atlantic slope river drainage and the Gulf slope. Specifically four species of Nortropis were shared between the southern Atlantic slope and the Apalachicola drainage. It has been hypothesized that the Savannah River, a member of the southern Atlantic Slope and the Chattahoochee River belonging to the present day Gulf Drainage once both drained into the southern Atlantic drainage. The constant fluctuations in sea levels during the Oligocene, Miocene, Pliocene, and Pleistocene epoch could have allowed for genetic isolation and intermittent gene flow, which may have led to a decrease in genetic variation [48].

5. Conclusion

In summary, the research presented in this study represents a baseline understanding of black crappie genetic structure through COI barcoding and possible conditions for genetic fragmentation within water systems. The populations of black crappie in this study have low to moderate haplotype diversity and low nucleotide diversity, which may affect their ability to respond to a rapidly changing environment, especially when coupled with increased fishing pressure. This species is not a highly migratory and increased gene flow through the removal of man-made barriers and efficient fish passage may allow for the accumulation genetic variation. While mtDNA haplotype analysis revealed significant differentiation among the three lakes, future research should include an increase in sample sites on the Chattahoochee River Basin to allow for an analysis of isolation by distance and the detection of shared and unique haplotypes more proximal to Lake Lanier and Lake Seminole. Additionally, researchers should increase the sample size for Hartwell Lake to better represent a 22,649 ha. lake, which may resolve Tijima's D value and Fu's Fs statistic. Continued sampling and genetic analysis could provide wildlife management with a powerful molecular tool to ensure sustainable usage of our freshwater resources.

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