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Assessment and Deployment of Genetic Tools for North American River

Otters (*Lontra canadensis*)

By

Kelsey Lawton

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of
Science in Environmental Science

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Table of Contents

Acknowledgments 4

Abstract..... 5

Introduction..... 6

Background 7

Introduction to North American River Otters 7

Haplotyping with Cytochrome b 7

Microsatellites and Parentage 8

The Exclusion Method Paternity Test 9

AZA NARO Species Survival Plan 9

Goal and Objectives..... 10

Methods..... 11

DNA Purification, Amplification and Sequencing 11

Paternity Test Methods 12

Phylogenetic and Network Analyses 13

Development of a NY Otter Locality Map..... 15

Results and Discussion..... 15

Conclusions and Future Work..... 27

References..... 29

Appendix..... 33

Table of Tables

Table 1: *Haplotype IDs assigned to each Otter in the Project*.....18

Table of Figures

Figure 1: Demonstration of the Bootstrap Consensus containing the 5 Major Haplogroups.....17

Figure 2: Demonstration of a Parsimony Network containing 32 Unique Haplotypes.19

Figure 3: Demonstration of Monomorphic Loci on an Agarose Gel.....20

Figure 4: Demonstration of Polymorphic Loci on an Agarose Gel.....21

Figure 5: Demonstration of an Anomalous Three Allele Individual at Locus RIO19.....22

Figure 6: Demonstration of Offspring with Nonparental Allele.....23

Figure 7: Demonstration of Offspring with Nonparental Allele.....24

Figure 8: Demonstration of Otter Locality and Haplogroup Distribution within NY State Watershed
Boundaries (whole state view HUC 8).....26

Table of Appendix Figures

Figure A1: Demonstration of a Paternity Test Gel on Agarose Gel (RIO11&12).....33

Figure A2: Demonstration of a Paternity Test Gel on Agarose Gel (RIO17&18).....33

Figure A3: Demonstration of a Paternity Test Gel on Agarose Gel (RIO11).....33

Figure A4: Demonstration of a Paternity Test Gel on Agarose Gel (RIO12).....34

Figure A5: Demonstration of a Paternity Test Gel on Agarose Gel that failed to Amplify
(RIO13).....35

Figure A6: Demonstration of a Paternity Test Gel on Agarose Gel (RIO15).35

Figure A7: Demonstration of a Paternity Test Gel on Agarose Gel (RIO16).....36

Figure A8: Demonstration of a Paternity Test Gel on Agarose Gel (RIO17).....36

Figure A9: Demonstration of a Paternity Test Gel on Agarose Gel (RIO19).....37

Figure A10: Demonstration of a Paternity Test Gel on Agarose Gel (RIO20).....37

Figure A11: Demonstration of Otter Locality and Haplogroup Distribution within NY State Watershed

Boundaries (Central and Western state view HUC 8).....37

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Abstract

North American River Otters (*Lontra canadensis*) once thrived in large numbers in most wetlands of North America. However, due to human pressures such as trapping, habitat loss and pollution, these carnivorous mammals have decreased in numbers significantly in the last 100+ years, becoming extirpated in many traditionally inhabited areas. The use of genetic data from molecular techniques (PCR, DNA sequencing) can guide the identification and management of populations used for relocations to better maintain genetic diversity in both wild and captive otter populations and help inform the Association of Zoos and Aquariums (AZA) breeding programs. Management organizations are concerned with the possibility of multiple paternity in the management of small captive populations. Therefore, molecular tests that show positive paternity/parentage are useful for managing small populations. This study deployed a suite of ten previously developed microsatellite loci as tests of parentage in a few families and to show a proof of concept that the set of loci would be suitable for *Lontra canadensis* populations. The study confirmed parentage for one family unit and cast out sire parentage for another. This study used (1122 of 1140 bp) mitochondrial DNA sequences from the cytochrome *b* locus to reconstruct relationships of 31 haplotypes from many US localities from both AZA and wild populations totaling over 100 individuals (~40% of the captive AZA population). Haplotype relationships reveal (1) 5 haplogroups, (2) shallow divergences (0-0.5%) among lineages and (3) a moderate divergence (0.7-1%) between haplogroup V from the Atlantic US coast and the remaining US haplogroups. The data reveal the AZA population maintains a mixture of 24 haplotypes and 5 haplogroups, with most animals within one large haplogroup (II) and fewer in the remaining haplogroups identified. The NYS samples represent 7 unique haplotypes plus 2 shared haplotypes (with AZA) within 3 haplogroups.

Introduction

Genetic diversity within and among populations is viewed as an indication of a population's health; therefore, the greater the level of genetic diversity, the greater the potential fitness level of a population (Markert et al, 2010). Many otherwise monogamous mammal species may participate in extra-pair copulation (Cohas, 2009) and exhibit multiple paternity (Dean, 2010) particularly when under high stress conditions (i.e., high density). While in large populations these behaviors may maintain genetic diversity within a population, they could pose a threat to small captive populations because captive populations can have lower genetic diversity relative their wild counterparts (i.e., number of haplotypes present (Jiang, 2005)). In addition, the uncertainty of these otters' reproductive behaviors has raised concern in both private and public wildlife organizations regarding the possibility of multiple paternity in the management of small populations even though there are few if any published records of this occurring. Alternative reproductive pairing behavior can make managing genetic diversity and inbreeding in captive populations more challenging (Boakes, E. et al. 2007)

Understanding parentage patterns in captive North American River Otters (NARO) will be facilitated by the application of paternity testing that will enable us to infer extra-pair copulation through parent-offspring genotyping. This information is also critical to ensuring genetic diversity within captive populations. We are developing a NARO phylogeographic hypothesis with an mtDNA locus and paternity testing with microsatellite loci. Both results will aid zoos and aquariums by informing them about NARO reproduction in both wild and captive populations. In addition to increasing the knowledge about NARO genetic diversity and parentage behavior, this paternity test will also help decrease the chance of consanguineous breeding in captive populations.

Background

Introduction to North American River Otters

North American River Otters (NARO) are carnivorous mammals found in most of North America (Newak, 1983) that typically weigh anywhere from 3-14 kg, and have thick dense bodies with webbed digits, small ears, and nostrils that can be closed when underwater (Newak, 1983). Their diet mostly consists of fish, frogs, crabs, and other aquatic invertebrates (Newak, 1983). Wild populations of NARO faced threats from the fur trade in the early 1900s, persecution from fishermen, industrialization which led to significant habitat loss, and pollution from the misuse of pesticides (Newak, 1983). This led to regional extinction (extirpation) and much later reintroduction efforts from the Catskills and Adirondack Mountains into parts of central and western New York State (Spinola, R. 2008).

Like many northern latitude carnivores NARO display delayed implantation (Newak, 1983) of embryos in which the embryonic blastocyst will stay dormant rather than implant to the uterus immediately after fertilization (Newak, 1983). Evidence shows that this delay correlates to an increasing photoperiod after the vernal equinox (Fenelon, J. C., 2014). Delayed implantation is important to understand because of the female otter's reproductive anatomy. Mustelids have a bicornate uterus (a "U" shaped uterus) that can potentially allow the simultaneous gestation of more than one litter and the possibility of multiple paternity (Baitchman, E. J., 2000). In addition to their physical attributes, it is also important to understand their genetics.

Haplotyping with Cytochrome b

Phytogeography and haplotyping are essential tools for understanding populations of different species. This had been done with mitochondrial DNA (mtDNA) in the past with species of otters such as the Marine Otter and the Eurasian Otter (Trinca, C. S, 2012 and Hwang, J. Y.,

2018). Mitochondrial DNA is useful and interesting in many ways including the fact that it does not recombine as nuclear DNA does and therefore relies on clonal inheritance typically from the maternal DNA. In addition, mtDNA lacks repair mechanisms for replication errors, unlike nuclear DNA, and therefore mutates and evolves more quickly than nuclear DNA (roughly 10x faster). These mutations can also be attributed to its lack of introns which results in a higher chance of mutation events (insertion/deletion) of coding regions (Awise 1994). Lastly, because these cells typically contain only one mtDNA haplotype (homoplasmic), there are ample copies of the mitochondrial genome in each cell, reducing difficulties when extracting DNA from either blood or tissue samples. Cytochrome *b* was chosen due to its moderately high sequence variation between and within species (Kocher 1989). However, something with even faster rates of mutation were needed for a successful paternity test.

Microsatellites and Parentage

Faster sequence substitution rates occur in microsatellites loci, even faster than mtDNA. Also known as simple sequence repeats, microsatellites are short motifs (usually 1-6 bp) repeated numerous times at a locus (Grover, 2012). These sequences can be found in various locations of the eukaryotic genome and one microsatellite can have several length polymorphisms corresponding to the number of repeats present per locus (Guo et al. 2009). These polymorphisms make microsatellites a useful tool for developing a paternity test because although these are sequence repeats, they can vary slightly from each other (Vieira, M. 2016). Each unique sequence will have a corresponding band on a gel that can then be visually compared to other otters to determine parentage.

Microsatellite loci are relevant to parental studies due to their high mutation rates that are between 10^3 and 10^6 per cell generation (Vieira, M. L. C., 2016). When used for parentage

studies, multiple unlinked microsatellite loci are typically tested within populations. Similarity and dissimilarity among potential parents and offspring can be used as a test of paternity. This is done via the exclusion method where alleles between maternal, paternal, and offspring DNA are compared. Incompatible patterns are excluded from the possible parentage pool. (Jones, A. G., 2003). Offspring that possess alleles not present in the known maternal or paternal genotypes. This would improve upon parent/offspring identification when compared to the AZA Breeding program standards as of right now.

The Exclusion Method Paternity Test

The development of a paternity test allows for more straight forward analysis of parentage in NARO. Paternity could be identified with near certainty, which would lead to genetically tested Studbook records. This could then result in the stabilization of or even increase genetic diversity within captive populations, leading to healthier individuals. Finally, we would also gain insight into NARO reproduction and parentage behavior. These improvements in informed captive breeding were observed as a result of a 2015 study of a captive population of endangered African Penguins, after which the researchers found that the inaccuracies in a studbook can have consequences on the genetic future of the animals involved. They also found that a paternity test was a useful tool for the management of captive populations (Labuschagne et al., 2015). A statistical paternity test requires an entire population of potential sires. We are considering an application of this type of testing to the entire AZA population of male NARO.

AZA NARO Species Survival Plan

Although NARO are not currently endangered, it is crucial to maintain genetic diversity within captive populations because the greater the genetic diversity, the better equipped that species is to adapt to environmental changes and stressors (Markert, 2010). Furthermore,

breeding animals in a low diversity population can be detrimental to the animals' health and wellbeing (Animal Care & Management n.d.). Member institutions of the Association of Zoos and Aquariums (AZA) currently house 267 North American river otters in breeding programs (D. Hamilton, 2020). NARO breeding pairs are chosen during an annual population management session with the studbook keep and a population biologist. The studbook contains the lineage of every otter in the program (Animal Care & Management n.d.). There are low parentage uncertainties in facilities where there is a single breeding pair; however, many facilities house females with several males to increase the chance of breeding by giving the female mate choice (D. Hamilton, pers. comm.). In the case of a female housed with more than one male, each male is given an equal probability of being the sire (D. Hamilton, pers. comm.). Incorrect identification of the sire can lead to underestimated and overestimated parentage probability and inadvertent consanguineous breeding or a prevention of outbreeding. Both are detrimental to the genetic diversity of a small captive population (Markert, J., 2010).

Goal and Objectives

This project used genetic data from molecular techniques (PCR, DNA sequencing) to enhance a guide for identification and management of populations used for relocations to better maintain genetic diversity in both wild and captive otter populations and help inform the Association of Zoos and Aquariums (AZA) breeding programs. The objectives of this project were: (1) determine the current mitochondrial DNA haplotypes and haplogroups of captive and wild populations of North American river otters, (2) produce a set of relationships among NARO haplotypes using phylogenetic and network analyses, (3) determine suitable microsatellite loci for paternity testing, (4) use an exclusion method of paternity testing to investigate parentage of

two NARO family units, and (5) produce a locality map for NYS otter samples and match them to haplogroup and watershed.

Methods

DNA Purification, Amplification and Sequencing

The technique used to clean the DNA was from the manufacturer protocol of the E.Z.N.A.® Blood DNA Mini Kit (Omega Bio-tek, Inc. 400 Pinnacle Way, Norcross, GA 30071). Per the manufacturer's instructions, 250 μL of blood was incubated with 25 μL Proteinase and 250 μL BL Buffer at 65°C overnight. Next, the sample was passed through a cleaning column and then cell debris was washed away with manufacturers supplied buffers (HBC Buffer and DNA Wash Buffer). Next, the cleaned DNA was eluted off the column with 100 μL of manufacturers supplied Elution Buffer. The full 100 μL of purified DNA of each sample was divided into 80 μL stored at -80°C (permanent genomic stock DNA) and 20 μL stored at -20°C (working stock DNA). Splitting the samples into two locations protects against possible contamination of the entire sample, and cryostorage of 80 μL better preserves the samples.

A DNA sequence alignment was developed using mitochondrial DNA from generated PCR products sent to GeneWiz (South Plainfield, NJ) for sequencing. Each PCR contained 12.5 μL of GoTaq, 1 μL of primer L14724, 1 μL of primer H15915, 10 μL of autoclaved nano pure water, and 0.5 μL of the DNA sample, for a total volume of 25 μL . If the DNA did not amplify well, then 1 μL of MgCl₂ solution was added to each sample, and the water decreased by 1 μL to maintain the 25 μL total volume. In addition to the samples, a blank was made without DNA to ensure that there is no contamination in the samples. The sample is then run through a cycle of PCR. The PCR protocol used was 94°C for 2min for initial denaturing, 94°C for 1min, 54°C

annealing for 1min, 72°C extension for 1min for 35 cycles, then a final 72°C extension for 2min. Once finished, the samples will be stored in a freezer at -20°C.

Next, gel electrophoresis was run to ensure no contamination occurred, and that the PCR worked properly. First, a 1% Agarose gel was made with 0.5grams of Agarose and 1% TAE Buffer to bring the total volume to 50ml. This mixture was heated until all the Agarose was dissolved. 5 µL of Gel-Red (Biotium, Inc., Fremont, CA) is then added to mixture. This allows the bands of DNA to be seen under UV light. It was then poured into the mold with a comb to accommodate the number of wells needed. When set, the comb was removed, forming wells and the gel rotated so that the wells are on the negative side of the Gel Electrophoresis box. The box was filled with more 1% TAE buffer to cover the gel. 1 µL of ladder was added to the first well, then the blank in the second, and the remaining samples loaded into the remaining wells. The gel box was plugged into the power supply and allowed to run for ~ 20 minutes at 200volts or until the bands moved down $\frac{3}{4}$ of the gel. When done, the were imaged under the UV light of an Azure Biosystems c600 and an image was captured and placed in a notebook. Once it was confirmed that the samples amplified properly and there was no contamination, the samples were sent to GeneWiz. GeneWiz sent the DNA sequences of both the forward and reverse primers for future downloading.

Paternity Test Methods

Amplification of 10 microsatellite loci (RIO11-RIO20) from previous research with microsatellites used in multiple otter species (Beheler, A. S et al., 2005) was performed on two family units from AZA facilities. To test the viability of the primers, PCR amplifications were performed on otter samples from our 5 main haplogroups before being attempted with DNA from family units. Each reaction contained 12.5ul of GoTaq, 1ul of the forward RIO primer and

1ul of the reverse RIO primer, 9.5ul of dH₂O and 1ul of DNA for a total reaction volume of 25ul. All the PCR products were then run on a 50ml 1% agarose gel at 100V to check if it successfully amplified and to see any possible allele differences or evidence of heterozygosity. Once all the primers were checked, the primers that resulted in possible heterozygotes or allele differences were run on a 2% gel in a small gel box at 80V to prevent any curving of the bands. This allowed for better separation of the bands and determination of the best microsatellites to use for the test of parentage. Finally, family units were determined. Each family unit -maternal, siblings, and possible paternal, was run on a 200ml 2% agarose gel with a single primer at 65V and imaged regularly to check the progression of the gel. Finally, the gel was imaged and labeled. With each primer, the test of paternity became more and more clear.

Phylogenetic and Network Analyses

DNAS_Tar Lasergene SeqMan Pro (SeqMan Pro, v17.2) was used to make contiguous sequences (contigs) by combining individual trace files. MegAlign Pro (MegAlign Pro, v17.2) combined contigs into a multiple sequence alignment. These alignments were used to construct phylogenetic trees using PAUP* (Swofford, D., 2003) and haplotype networks using TCS (Clement M, et al., 2000).

Two types of analyses were used to recover our phylogenetic trees, a maximum parsimony analysis and a maximum likelihood analysis. Maximum parsimony searches all possible trees and finds those that possess the least number of character trait changes along branches within a tree. Another important feature of the parsimony criterion is its use of a subset of data called informative nucleotide sites. Informative sites are those which have two or more alternative states shared by two or more taxa. Informative sites are characterized by their ability to discriminate among alternative tree topologies on the basis of the number of character state

changes required by each (Li and Graur 1991). Hence, under the parsimony criterion, tree and branch length pertain only to informative sites.

The maximum likelihood tree reconstruction method uses individual nucleotide change likelihoods along branches to produce the tree with the highest probability (Felsenstein, 1988), produced a model of DNA sequence evolution and an algorithm to evaluate the likelihood of a particular topology, conditional on the model and a set of DNA data. Any topology can have its likelihood assessed under the model for a DNA data set, and the topology with the highest likelihood is taken as the best estimate of the phylogeny.

The maximum likelihood estimation procedure involves the conversion of a substitution rate matrix for each site, to a probability matrix by integration of linear equations which describe the original rates. This gives a probability matrix for each substitution type at each site. The algorithm then searches for the topology which maximizes the site-substitution probabilities (Swofford and Olsen 1990).

Finally, bootstrap resampling process was used for both parsimony and likelihood tree searches. Bootstrapping calculates a value that indicates the number of times out of 100 that a branch remained the same after reconstruction. This involves reconstructing best trees from pseudo-replicate matrices of the original data set and summarizing these results in a single best tree with frequencies on branches that represent the percentage a clade was reconstructed during the bootstrap analysis (out of 100%). Clade frequencies in the pseudo-replicate trees may be interpreted as relative levels of support (Felsenstein, 1988). Interpretation of bootstrap values must be qualified by noting their sensitivity to the size and structure of the dataset (Sanderson 1989), but to date this approach is by far the most practical for assessing the phylogenetic resolving power of large sequence alignments.

Due to a combing effect on the tree analysis a minimum spanning network was constructed via the TCS. Minimum spanning networks are drawn by connecting DNA sequences that first differ by a single mutation, then two mutations and so on until all samples are included in the network (Bertorelle, G.2009). This network was drawn to include polymorphic sites and their location, as well as median vectors.

Development of a NY Otter Locality Map

In order to develop a NY otter locality map, locality information for 20 otters obtained from the New York State Department of Environmental Conservation (NYS DEC) was input into ArcGIS online which then located the samples on a NYS base map. There are 19 localities identified on the base map, one locality was shared by 2 of the 20 otter samples. Each location was marked with icons and colors that corresponded to the color assigned to each of the five different haplogroups. Finally, a HUC 8 NY watershed boundary layer was added into the map via the add layer function. The label for each of these watersheds was turned on and the layer made transparent so that other labels such as county names could be seen. The watershed boundaries are critically important for otter distribution because otters rely heavily on freshwater tributaries for food, shelter, and navigation within their home range.

Results and Discussion

A multiple sequence alignment was used to produce both a phylogenetic tree of haplotypes and a haplotype network. The phylogenetic analysis recovered five major haplogroups (I-V) and two ungrouped haplotypes (*Figure 1*). Statistical support for the haplogroups was recovered using a bootstrap of both phylogenetic analyses (maximum likelihood and parsimony). The bootstrap values from the maximum likelihood analysis are shown in parentheses and the parsimony values are not in parentheses. The ungrouped otters

representing two unique haplotypes would likely represent new haplogroups with more complete population sampling.

Taxon identification labels in *Figure 1 and Table 1* contain up to three numbers followed by two letters. These correspond to the otter's lab extraction number and its US state of origin. Table 1 was constructed containing each haplotype labeled from A-AF with each corresponding otter sample ID. Haplotypes are organized into colored groups corresponding with the tree. There is a large amount of diversity within the group of otters we sampled. In most cases, geographical distance correlates with genetic distance; however, this is not the case with all otter populations. For example, we expected otters from states on the west coast (AK, WA, OR) to be the most divergent from the remainder of the otters because of their large geographical distance from them, however, they are more similar genetically to other subgroups of otters within one of the larger haplogroups. In addition, there are relatively small areas within the overall North American range of NARO that contain and disproportionately large amount of haplotype diversity. For example, states like FL and NY are represented in several haplogroups and NY alone contains nine of the 31 unique haplotypes. Haplogroup number five (V; Fig. 1) possesses the most divergent DNA sequence and is approximately 1 % divergent from all the other NARO groups. Since mtDNA evolves at approximately 1% per million years these specific haplotypes are approximately 750K to 1 million years old (Mueller, 2006).

Bootstrap consensus tree

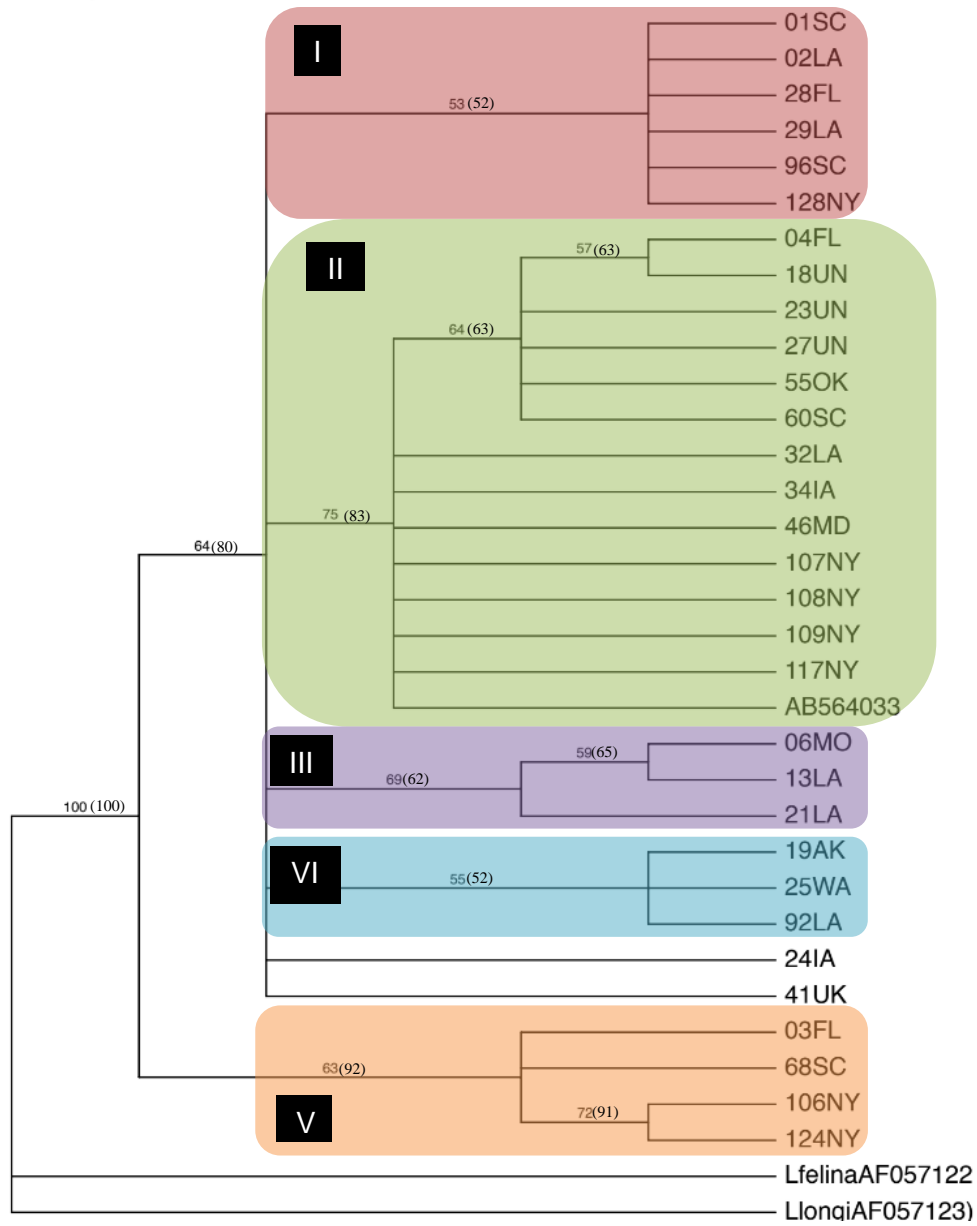


Figure 1: Demonstration of the Bootstrap Consensus containing the five major haplogroups.

Phylogenetic tree showing 31 recovered haplotypes grouped into five haplogroups (labeled I-V) and two ungrouped haplotypes. The bootstrap values for the maximum likelihood analysis are in parentheses and the parsimony bootstrap values are not.

Table 1: Haplotype IDs assigned to each otter in the project.

The code under the column “Otter” which consists of 2-3 numbers and 2 letters corresponds to that otter’s number assigned to it in this study and the otter’s state.

Haplotype ID	Otter	Haplotype ID	Otter	Haplotype ID	Otter	Haplotype ID	Otter
A	01SC		104NY		57LA		65MO
	07LA		105NY		58LA		69MO
	08NC		112NY	J	27UNK	V	13LA
	09NC		114NY		39LA		30TX
	10LA		115NY		40KY		31UNK
	12MO		122NY		44KY		61UNK
	14LA		123NY		72FL	W	21LA
	15LA		126NY	K	55OK		22LA
	16LA		127NY	L	60SC		38MS
	26UNK	B	02LA	M	32LA		54UNK
	36LA		11LA	N	34IA	X	19AK
	37SC		33LA		101NY		20AK
	42LA		70LA		102NY	Y	25WA
	47LA		71LA		111NY		74OR
	48LA		82UNK		116NY		84LA
	49LA		83UNK		119NY		90WA
	50LA	C	28FL		120NY		AF057121
	51OR	D	29LA		125NY	Z	92LA
	62NC		76LA	O	46MD	AA	24IA
	63NC	E	96SC	P	107NY	AB	41UNK
	66NC	F	128NY	Q	108NY		45SC
	67NY	G	04FL		110NY	AC	03FL
	75UNK		59FL		113NY		05GA
	85SC		97FL	R	109NY		17FL
	86UNK		98FL	S	117NY	AD	68SC
	87MD		99FL		118NY	AE	106NY
	88MD	H	18UNK	T	AB564033	AF	124NY
	91UNK	I	23UNK	U	06MO		
	100LA		56LA		35MO		

A parsimony network was created using TCS (Clement M. et al., 2000) and rooted with closely related marine and neotropical otters as outgroups with sequences from GENBANK (*Fig. 1*).

The parsimony network depicts close relationships among four of the five haplogroups (I-IV) with no more than four single nucleotide polymorphisms (SNP = DNA sequence difference)

between the closest member haplotypes. In contrast, the branch leading to haplogroup V shows

twice the number of changes with at least 9 to its next nearest haplotype. Haplogroup V shows a much larger genetic distance (1.5%) from its closest relative haplogroup, compared to smaller genetic distances (<0.5%) among the four other haplogroups. Given genetic distance is directly correlated to the time to descendants shared their most recent common ancestor, the greater genetic distance of haplogroup V from the remaining NARO haplogroups indicates that it shares a much deeper split from the ancestor of the other four haplogroups than they do with one another.

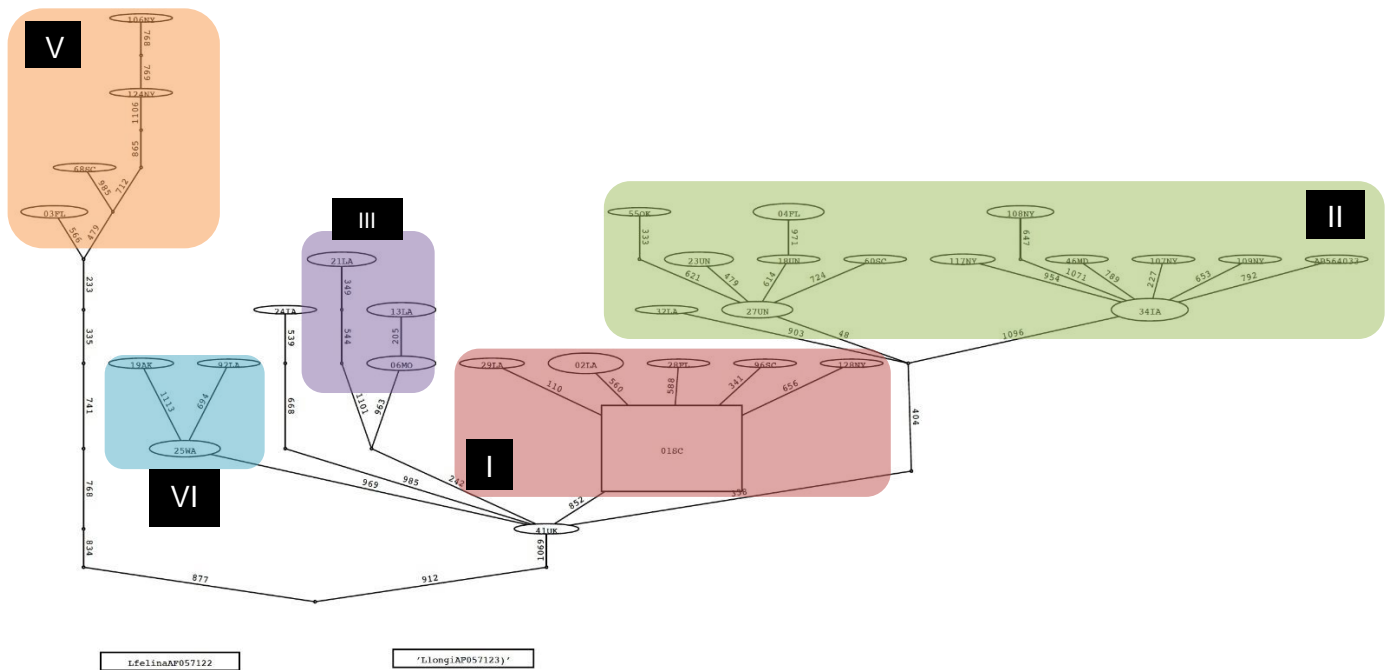


Figure 2: Demonstration of a Parsimony Network containing 32 unique haplotypes. Each haplogroup is highlighted in the same color as the phylogenetic tree. Network node sizes correspond to the number of otters in each haplogroup. Each number along the network branches represent a SNP, or polymorphic nucleotide site.

The paternity testing consists of PCR and gel electrophoresis of alleles from 10 different genes (loci) that resolved maternal, paternal and offspring genotypes. The alleles amplified during the PCR appear as bands on agarose gel images. Diploid genotypes (two alleles per locus) appear as a single bright band (homozygote = identical alleles) or two bands that appear close together but separate on the gel (heterozygote = non-identical alleles) for any individual otter in each lane on the gel. For example, *Figure 3* shows a single band per individual representing identical alleles in each otter depicting a monomorphic locus (all homozygous genotypes) from the otters in family unit one that were amplified using the primers RIO15 and RIO16.

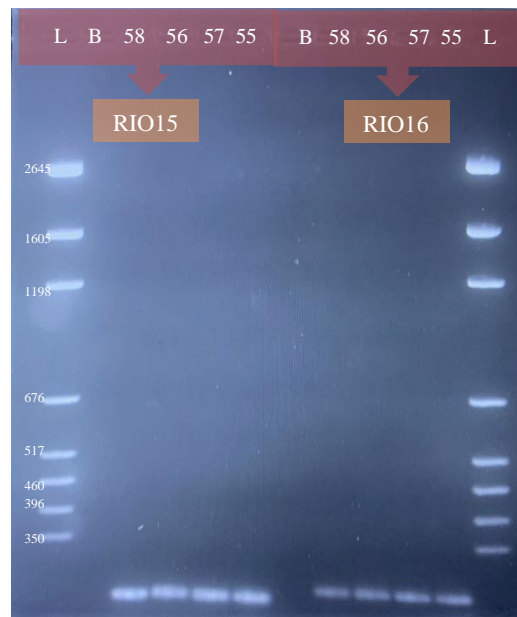


Figure 3: Demonstration of monomorphic loci on an agarose gel.

Agarose gel showing homozygous genotypes in Family Unit 1 amplified with primers RIO15 and RIO16 run on a 2% 200ml gel at 65V. For all loci with Family unit 1 (Fig. 3, 4 , 5, A1 and A2) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5) the offspring samples (2 in this family) , (6) the paternal sample, and (7) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

The same family unit 1 amplified with loci RIO 13 and 14 (*Figure 4*) shows multiple bands in several individuals (heterozygous genotype) that are examples of polymorphic loci

(they show polymorphism). The gel in *Figure 4* contains both heterozygous and homozygous genotypes. Locus RIO13 exhibits three different heterozygous genotypes in the maternal, paternal and offspring lanes each with two different size alleles that appear as separate but closely spaced bands in each lane. Locus RIO14 exhibits three homozygous genotypes among the maternal and offspring lanes and a heterozygous genotype in the paternal lane. The offspring in family unit 1 always possess alleles that are present in both parents. Confirming the potential parentage of the maternal and paternal otters for these offspring with loci RIO13, RIO14, RIO15 and RIO16

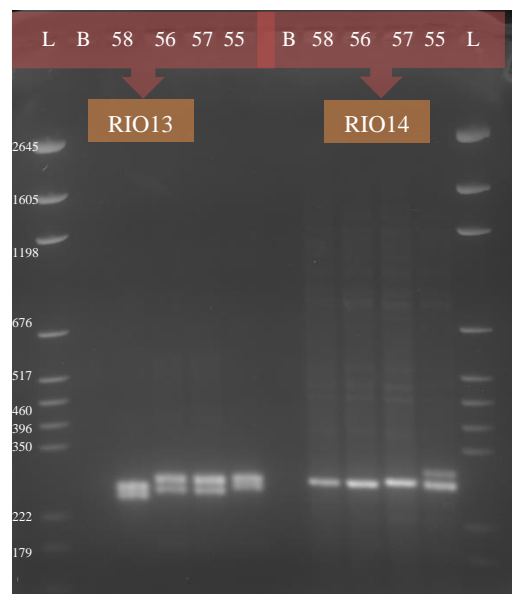


Figure 4: Demonstration of polymorphic loci on an agarose gel.

PCR of Family unit 1 with microsatellite loci RIO13 and RIO14 showing heterozygous genotypes in all family members at locus RIO13 and both heterozygous and homozygous genotypes in family members at locus RIO14. For all loci with Family unit 1 (Fig. 3, 4, 5, A1 and A2) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5) the offspring samples (2 in this family) , (6) the paternal sample, and (7) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

Family unit 1 with locus RIO20 (*Figure 5*) results also show both heterozygous (maternal sample) and homozygous (paternal and offspring samples) genotypes. Unexpected results were found with Family unit 1 and locus RIO19. The maternal, paternal and offspring #1 samples from Family unit 1 exhibit typical diploid heterozygous genotype (2 bands = 2 alleles), while offspring #2 exhibits what appear to be 3 bands corresponding to 3 different size alleles (an apparent triploid locus).

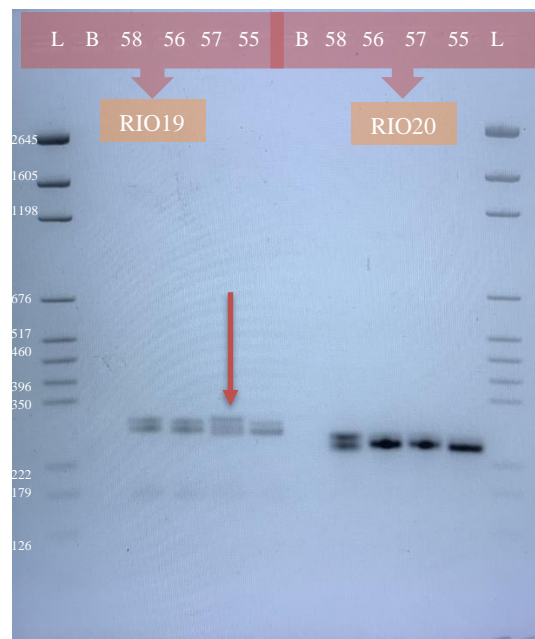


Figure 5: Demonstration of an anomalous three allele individual at locus RIO19.

PCR of Family unit 1 with microsatellite loci RIO19 and RIO20 showing heterozygous genotypes in all family members at locus RIO19 and both heterozygous and homozygous genotypes in family members at locus RIO20. Arrows shows apparent triploid genotype for locus RIO19 in offspring #2 (otter 57). For all loci with Family unit 1 (Fig. 3, 4, 5, A1 and A2) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5) the offspring samples (2 in this family), (6) the paternal sample, and (7) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

Triple copies of alleles are possible within an otherwise diploid genome as a result of a duplication event followed by a transposition (relocation of the duplicated allele) and mutation.

Also known as “jumping genes”, this process is the result of an allele copy moving to another

position in the genome with subsequent mutations that can result in size variation (McClintock B.1929). Although this does not happen often, the location of these microsatellites that we are using as primers are located within gene introns (noncoding regions) and therefore are under little to no selective pressure which allows mutations such as this to occur (Whittaker, J. C, et al, 2003). This experiment was run again and confirmed same result.

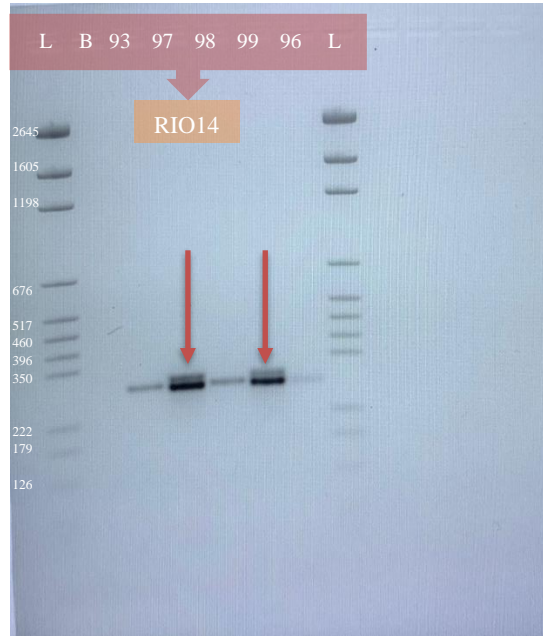


Figure 6: Demonstration of offspring with nonparental allele.

PCR of Family unit 2 with microsatellite locus RIO14 shows (arrows) nonparental alleles in offspring #1 and #3 (otters 97 and 99). For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

The genotypes shown in *Figure 6* depict offspring alleles do not present in either parent. The nonparental allele is shown as a slightly higher band just above the very dark lower band in offspring #1 and #3 (otters 97 and 99). This higher band is not present in either the maternal or the paternal sample lane (otters 93 and 96). Offspring #1 and #3 (otters 97 and 99) show a heterozygous genotype, while offspring #2 (otter 98) possess the single parental allele and

appears to be a homozygote just as the parents possess. A heterozygous genotype is highly improbable for two homozygous parents with the same allele at a single locus.

The genotypes shown in *Figure 7* depict an offspring allele that is not present in either parent.

The nonparental allele is shown as a slightly higher band just above a slightly fuzzy lower band in offspring #2 (otters 98). This higher band is not present in either of the other offspring #1 and #3 (otters 97 and 99), nor the maternal or the paternal sample lanes (otters 93 and 96). Offspring #1 and #3 (otters 97 and 99) show a homozygous genotype, while offspring #2 (otter 98) appears to be a heterozygote with and an allele not found in any of the other family members. This possession in offspring #2 (otter 98) of a unique allele not found in either putative dam and sire casts significant doubt on the parentage in this family unit.



Figure 7: Demonstration of offspring with nonparental allele.

PCR of Family unit 2 with microsatellite locus RIO18 shows (arrow) nonparental alleles in offspring #2 (otter 98). For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

Both *Figures 6 and 7* show discrepancies between offspring and parental genotypes in Family unit 2. In *Figure 6*, the two offspring 97 and 99 possess a higher allele (higher in the gel image) than is present in either parent. If the identity of the homozygous mother is correct, the possession of a nonparental allele in two of the offspring means one of two events must have occurred, (1) an identical mutation occurred separately in both offspring #1 and #3, or (2) there has been a misidentification of the sire. A misidentification seems much more likely because the same exact mutation occurring twice in offspring that are not twins is unlikely. This is further supported by the results using RIO14 that shows offspring 98 (*Figure 7*) again possessing a higher allele than either parent possess. One may also notice in *Figures 5, 6, and 7* that some bands are much brighter than others, even in the case of heterozygotes. This is due to the competitive nature of PCR. If a sample contains two different alleles, one of them may show a higher affinity for the priming sites and is likely to be amplified at a greater rate, therefore resulting in a brighter band.

The NY Otter Locality map (*Figure 8*) was generated from samples obtained from the NYS Department of Environmental Conservation. This study found nine haplotypes represented in three haplogroups among the NYS samples. The NYS DEC in the late-1990s reintroduced 279 (River Otter - NYS DEC. (n.d.) otters from the Catskills and Adirondacks to the Finger Lakes and Western NY. All three haplogroups from in and near the Catskills are represented in the otter samples from the Finger Lakes and Western NY regions. It is interesting that all three haplogroups are represented both in areas of the NYS that likely have possessed continuous otter populations (Catskills and Central NY) and those areas of the state the likely saw extirpation of their original otter populations (Finger Lakes and Western NY). One particularly interesting feature is the presence of otters from our most divergent haplogroup in two different drainages in Oneonta and Sodus NY separated by at least two other drainage systems. I hypothesize three possible

explanations for the co-occurrence of otters from the same haplogroups at multiple localities and separate drainages in Central and Western NY: (1) the continuous historical existence of NYS extant otter populations in and between both areas, (2) human induced reintroduction per the 1996 DEC reintroduction project, and (3) recent dispersal from one location to the other over generations subsequent to previous extirpation in one of the areas. Any single one of these hypotheses, or a combination could account for the co-occurrence of otters in separated NYS drainages. The limited NYS otter population sampling in this study (20 samples total, and none from the Adirondacks) is insufficient to discriminate among these three possible explanations because distributions gaps could be due to inadequate sampling. It is possible that a more continuous and complete otter population sampling regimen might eventually be able to discriminate a co-occurrence mechanism among these three hypotheses.

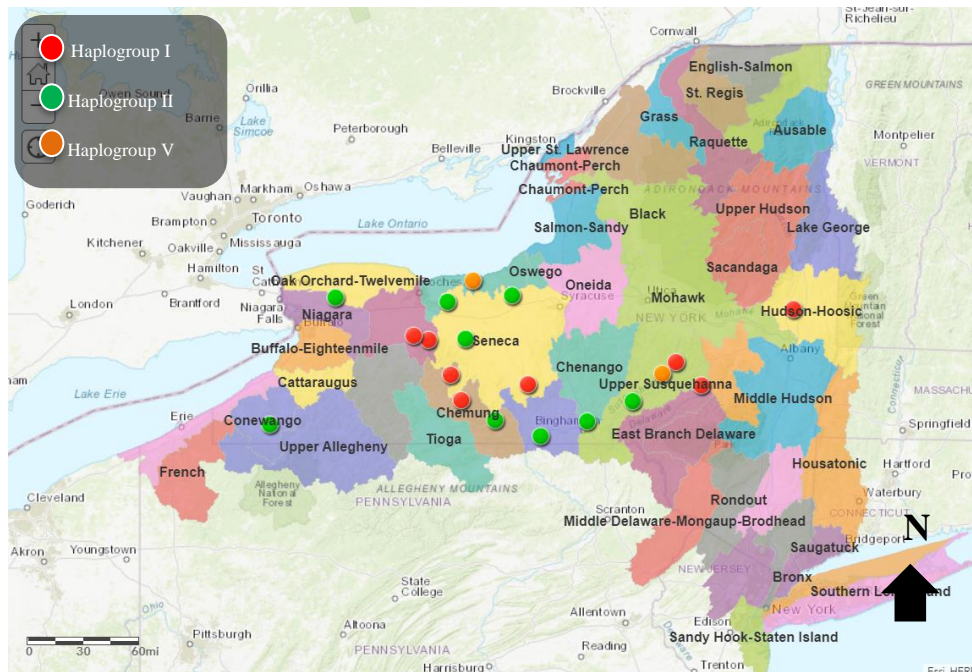


Figure 8: Demonstration of Otter Locality and Haplogroup Distribution within NY State Watershed Boundaries (whole state view HUC 8).

A statewide base map showing NYS otter localities and haplogroup membership within watershed boundaries. Localities depict otter samples provided by the NYS DEC (19) and Cornell University (1).

Conclusions and Future Work

High regional haplotype diversity found in NYS and Florida could be beneficial for future conservation efforts. It has also the case that the otters from Louisiana (LA) represent 22.7% (29/128 otters) of the AZA population, and 32.3% of the AZA haplotype diversity. Because many of the LA otters possess identical haplotypes the use of LA otters in relocation/repopulation efforts must be coupled with close genetic tracking of released LA otters to avoid overrepresentation of any single haplotype. (see *table 1*). In addition, genetic diversity from the nuclear genome in the form of microsatellite loci diversity should be included in relocation/repopulation efforts so that they do not exclusively focus on mitochondrial diversity.

Given the divergence levels discovered in this study, I hypothesize that most all the haplotype diversity revealed in this study is primarily the result of population divergence due to Pleistocene Epoch glacial cycles alternatively isolating and re-introducing otter populations and distribution routes.

The successful application of microsatellite genotyping in this study confirms that a paternity testing project could be developed using the entire AZA population as a candidate for a statistical paternity test since these require an entire population of potential sires to include or exclude any particular male otter from being a potential sire. This project may serve as a case study to be used with numerous other species in captivity that are not kept in single mating pair enclosures. Although many of the AZA breeding species could benefit greatly from a paternity test, gorillas and birds are two of the most discussed groups in need of one. Furthermore, gel electrophoresis of microsatellite loci is inexpensive and once mastered could be adopted by facilities breeding captive animals to confirm or reject parentage assignments and maintain healthy populations.

The mapping of the NYS otters in this project has discovered that genetic diversity remains in NYS among several drainages. Insufficient sampling prevented discrimination among several possible explanations for the co-occurrence of related otters in non-contiguous drainages. It is possible that these knowledge gaps are due to insufficient sampling rather than lack of extant populations; however, extensive field sampling is necessary to gather sufficient data to answer questions about distribution gaps, population origins and relatedness of otter populations.

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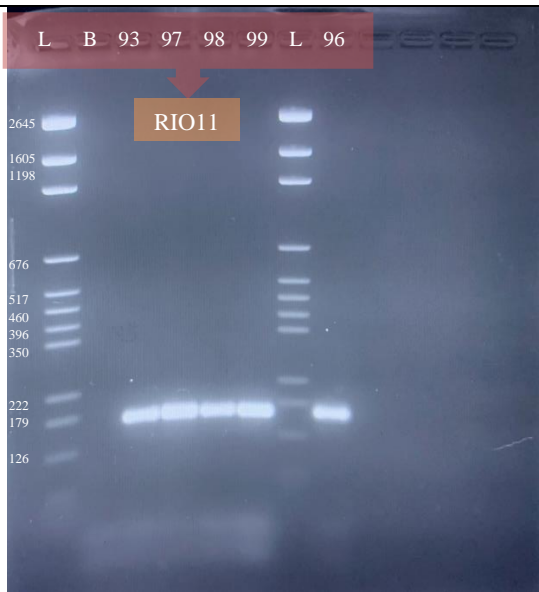
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Appendix

	<p>Figure A1: Demonstration of a Paternity Test Gel on Agarose Gel (RIO11&12). PCR of Family unit 1 with microsatellite loci RIO11 and RIO12 showing homozygous genotypes in family members at both loci. For all loci with Family unit 1 (Fig. 3, 4, 5, A1 and A2) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5) the offspring samples (2 in this family) , (6) the paternal sample, and (7) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.</p>
	<p>Figure A2: Demonstration of a Paternity Test Gel on Agarose Gel (RIO17&18). PCR of Family unit 1 with microsatellite loci RIO17 and RIO18 showing both heterozygous and homozygous genotypes in family members at both loci. For all loci with Family unit 1 (Fig. 3, 4, 5, A1 and A2) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5) the offspring samples (2 in this family) , (6) the paternal sample, and (7) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.</p>
	<p>Figure A3: Demonstration of a Paternity Test Gel on Agarose Gel (RIO11). PCR of Family unit 2 with microsatellite locus RIO11 showing homozygous genotypes in family all members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows:</p>



(1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.



Figure A4: Demonstration of a Paternity Test Gel on Agarose Gel (RIO12). PCR of Family unit 2 with microsatellite locus RIO12 showing homozygous genotypes in all family members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.

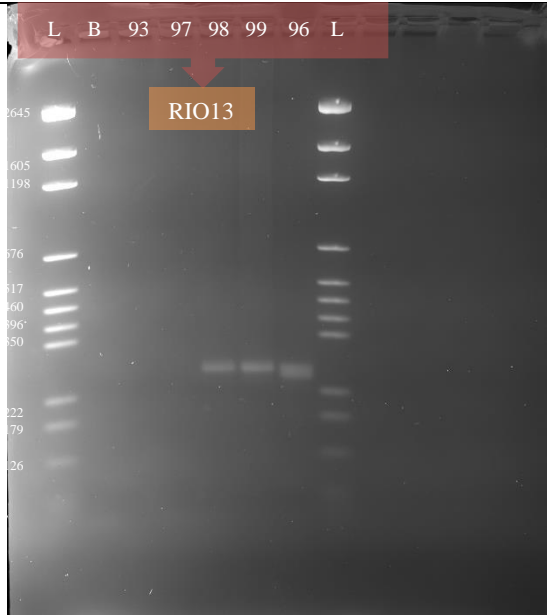


Figure A5: Demonstration of a Paternity Test Gel on Agarose Gel that failed to Amplify (RIO13). PCR of Family unit 2 with microsatellite locus RIO13 showing a heterozygous paternal (otter 96) and homozygous genotypes in offspring #2 and #3 (otters 98 and 99). For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

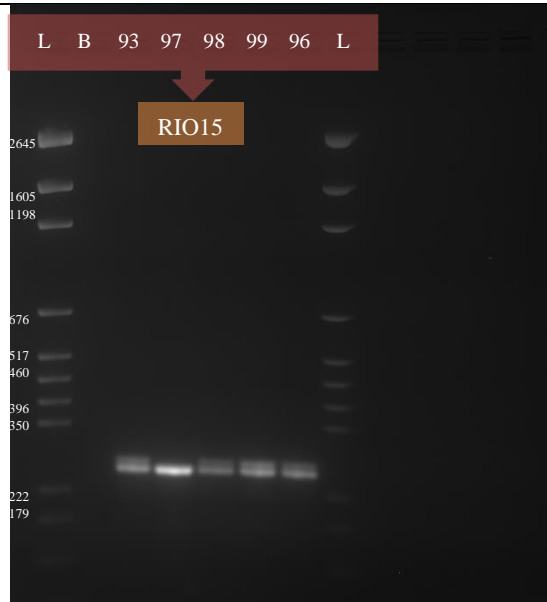


Figure A6: Demonstration of a Paternity Test Gel on Agarose Gel (RIO15). PCR of Family unit 2 with microsatellite locus RIO15 showing heterozygous genotypes the maternal, paternal and offspring #2 and #3 (otters 98 and 99) samples and a homozygous genotype in offspring #1 (otter 97). For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

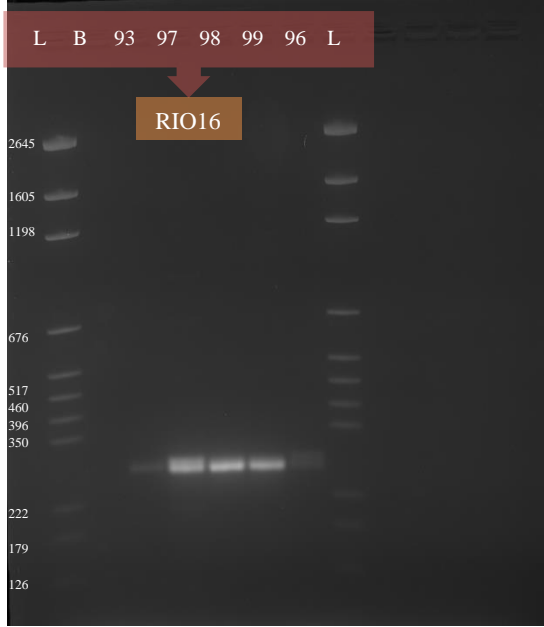


Figure A7: Demonstration of a Paternity Test Gel on Agarose Gel (RIO16). PCR of Family unit 2 with microsatellite locus RIO16 showing both heterozygous and homozygous genotypes in family members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.



Figure A8: Demonstration of a Paternity Test Gel on Agarose Gel (RIO17). PCR of Family unit 2 with microsatellite locus RIO17 homozygous genotypes in all family members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by otter sample numbers.

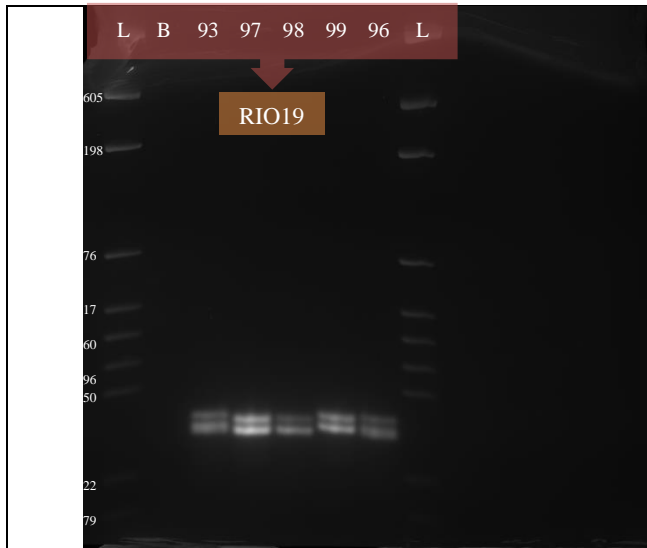


Figure A9: Demonstration of a Paternity Test Gel on Agarose Gel (RIO19). PCR of Family unit 2 with microsatellite locus RIO19 showing heterozygous genotypes in all family members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.



Figure A10: Demonstration of a Paternity Test Gel on Agarose Gel (RIO20). PCR of Family unit 2 with microsatellite locus RIO20 showing both heterozygous and homozygous genotypes in family members. For all loci with Family unit 2 (Fig. 6, 7, and A3-A10) the lanes are loaded from left to right as follows: (1) a DNA ladder to help determine allele size, (2) the negative control for that PCR experiment, (3) the maternal sample, (4&5&6) the offspring samples (3 in this family), (7) the paternal sample, and (8) DNA ladder. Lane labels: L=DNA Ladder, B=Negative control followed by other sample numbers.

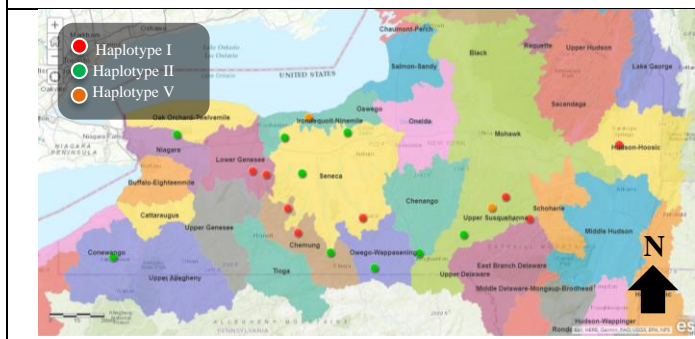


Figure A11: Demonstration of Otter Locality and Haplogroup Distribution within NY State Watershed Boundaries (Central and Western state view, HUC 8). A Central and Western NY base map showing NYS otter localities and haplogroup membership within watershed boundaries.

