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Determining Relatedness among Captive North American River Otters (*Lontra canadensis*) to Create a SPP (Species Survival Plan) Complaint Breeding Recommendation

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Abstract

The North American river otter (*Lontra canadensis*) is a semi-aquatic mustelid found throughout North America. *L. canadensis* is currently listed as a species of least concern by the International Union for the Conservation of Nature, after successful reintroduction programs to restore the historic range. To prevent future extinctions, the Association of Zoos and Aquariums (AZA) created Species Survival Plans (SSPs) to preserve organisms' genetic diversity and demographic stability in captive populations. In order to comply with SSP regulations within their breeding program, four wild caught individuals (Lily, Sam, Shadow, and Frances), housed at the Potawatomi Zoo (South Bend, Indiana, USA), were assessed for relatedness. DNA was extracted from blood samples, eight microsatellite loci per sample were amplified, fragment analysis was conducted in Geneious v10.1.3, and Lynch genetic distance was calculated in RStudio v2.11.1. Results reveal that the Frances and Sam dyad shows the greatest genetic distance, while Lily and Sam have the least genetic distance of possible male-female dyads. Based on these findings, Frances should be given the opportunity to breed with Sam and discouraged from breeding with Shadow. In order to capture the genetic diversity of wild caught individuals in captive populations, Lily and Shadow should be bred with other river otters brought into the zoo or transferred to another zoo to breed.

1. Introduction

North American river otters (*Lontra canadensis*) are semi-aquatic carnivores in the family Mustelidae[1]. Their primary food source is fish, but their diet can include insects, birds, reptiles, mammals, amphibians, and crustaceans[1]. River otters were once found throughout North America in freshwater riparian areas and coastal habitats, with one of the largest ranges for mammals on the continent[1,2]. However, due to habitat loss, pollution, urbanization, and unregulated trapping, local extinctions occurred[2,3]. In the 1970s, reintroduction programs were utilized to restore the species to its historical range, and it has been one of the most successful carnivore reintroductions due to the size of the range restored[2,4]. As a result, the North American river otter was listed as a species of least concern (LC) by the International Union for the Conservation of Nature (IUCN) in 2015[3].

Although the North American river otter is currently listed as LC, breeding programs are still utilized to maintain a reserve population[5]. The Association of Zoos and Aquariums (AZA) created Species Survival Plans (SSPs) in 1981 in order to maintain captive populations of animals while also maintaining genetic diversity and demographic stability[6]. SSPs include Breeding and Transfer Plans for each taxon as well[7]. Ascertaining relatedness among individuals is therefore an important step before animals are bred to keep in compliance with their SSPs.

Microsatellite analysis is a molecular genetic technique that can be utilized for determining individual relatedness. A microsatellite is an intergenic, short, tandem repeat of a specific DNA motif within a genome[8]. These repeats are one to ten nucleotides in length and are abundant in eukaryotic genomes[8]. Analysis of polymorphisms in microsatellite

loci show that they are variable within populations, making them useful in distinguishing among individuals[9]. To maintain genetic diversity as required by SSPs, mating should occur between individuals of high genetic distance. Analysis of microsatellite length differences can be employed to determine genetic distance among potential mates.

In this study, eight microsatellite loci were examined to determine the relatedness of four wild-caught North American river otters from Potawatomi Zoo in South Bend, Indiana: 11 year old intact female Lily, 11 year old intact female Frances, 10 year old intact male Sam, and 10 year old intact male Shadow. These animals were captured eight to nine years ago from a population in Louisiana. Louisiana otters have been sources for captive breeding and reintroduction throughout North America[2]. Data generated from this project will be used to make recommendations to the Potawatomi Zoo's river otter SSP.

2. Materials and Methods

2.1 DNA Extraction

Blood samples from 4 river otters (two females: Lily and Frances; two males: Sam and Shadow) were collected by head veterinarian Dr. Ronan Eustace at the Potawatomi Zoo in South Bend, Indiana, frozen, and sent on dry ice to the University of North Carolina Asheville. Whole genomic DNA extractions were performed on the blood samples from each otter using a Qiagen™ DNeasy Blood and Tissue Kit with manufacturer-provided protocol. A NanoDrop™ spectrophotometer was used to determine DNA concentration and purity for each sample, followed by 1% agarose gel electrophoresis to further confirm the presence of high molecular weight DNA.

2.2 Polymerase Chain Reactions and Analysis

Polymerase chain reactions (PCRs) for loci Rio01, Rio06, Rio15, Rio16, and Rio18 were conducted in 16.2 µL reactions consisting of: 3 µL DNA extract, 1.5 µL New England BioLabs 10x standard *Taq* reaction buffer, 0.45 µL *Taq* DNA polymerase, 1.5 µL dNTP mixture, 1.2 µL 25 mM $MgCl_2$, 7.05 µL PCR water, 0.15 µL M13-labeled forward primer, 0.15 µL 6-FAM fluorophore, and 0.3 µL reverse primer[10,11]. A BIO RAD T100 Thermal Cycler was used with the following PCR parameters: 95°C for 2 min, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, repeated for 28 cycles, followed by 72°C extension for 10 min[10,11]. Presence of PCR products was confirmed with 1% agarose gel electrophoresis using a Promega 100 bp ladder. The PCR products were mixed with a GeneScan 500 size standard with LIZ dye, then sent to Yale University DNA Analysis Facility to determine fragment length. Fragments analysis was conducted in Geneious v10.1.3 to determine allele lengths at each locus for all 4 river otters[12]. PCRs for Rio11, Rio13, and Rio19 were conducted in 16.2 µL reactions consisting of: 3 µL DNA extract, 1.5 µL New England BioLabs 10x standard *Taq* reaction buffer, 0.45 µL *Taq* DNA polymerase, 1.5 µL dNTP mixture, 1.2 µL 25 mM $MgCl_2$, 7.05 µL PCR water, 0.3 µL fluorescently-labeled forward primer, and 0.3 µL reverse primer. PCR conditions were as above, but annealing temperatures were lowered to 55°C; for Frances at locus Rio19, an annealing temperature of 58°C was used[10,11]. Presence of PCR products was confirmed with 1% agarose gel electrophoresis using a Promega 100 bp ladder, and PCR products were prepared for analysis as stated above.

2.3 Genetic Distance Calculations

Lynch pairwise genetic distance was calculated for all otters at all loci using the polysat package v1.7 in R v2.11.1[13,14,15]. In Lynch genetic distance calculations, 0 indicates no difference in the alleles examined, and 1 indicates differences in all alleles examined. Representative genetic distances used are described in Table 1.

Table 1. Average Lynch genetic distance based on relationship.

Consanguinity	Average Lynch Genetic Distance
Parent-offspring	0.50
Siblings	0.50
Grandparent-grandchild	0.75
Cousins	0.875

3. Results

NanoDrop™ spectrophotometer measurements revealed adequate DNA concentrations and purities for all 4 river otters (Table 2).

Table 2. Results from a NanoDrop™ spectrophotometer.

NanoDrop™ Results	Lily	Sam	Frances	Shadow
260/230	5.16	0.88	0.89	1.32
260/280	2.67	1.50	1.58	1.96
DNA Concentration (ng/µL)	5.40	5.60	5.30	4.00

Results from agarose gel electrophoresis confirmed the presence of high molecular weight DNA in all 4 river otter samples (Figure 1).

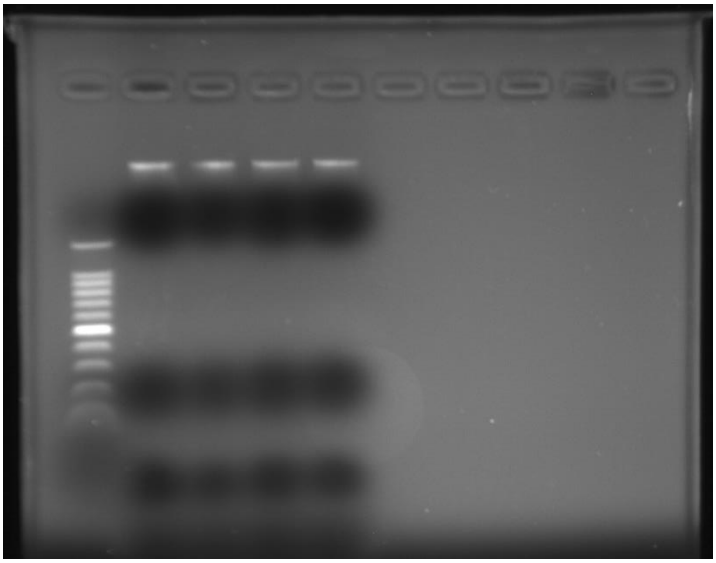

Figure 1. 1% Gel Electrophoresis Results for DNA Extraction

Figure 1 Gel electrophoresis confirmed successful DNA extraction from all 4 river otters: Lily (lane 2), Sam (lane 3), Frances (lane 4), and Shadow (lane 5); lane 1 is Promega 100 bp ladder.

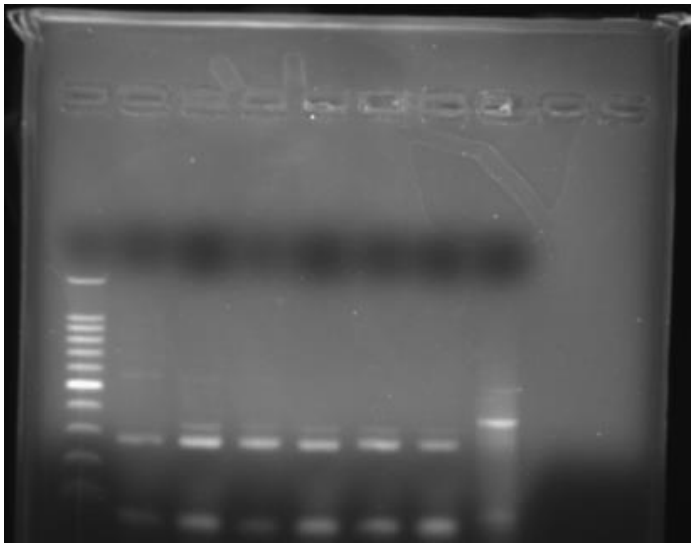

Figure 2. 1% Gel Electrophoresis Results for PCR Products with Florescent Primer

Figure 2 Representative photo from 1% gel electrophoresis confirming successful PCR with fluorescent primer: lane 1 is Promega 100 bp ladder, lane 2-7 show Rio01 for 3 individuals run at 55°C and 58°C annealing temperatures, lane 8 shows Rio16.

The microsatellites amplified were 150-300 bp in length; PCR amplification of each microsatellite was confirmed by examining that bp range on 1% agarose gel (Figure 2).

A representative file from Geneious demonstrated successful analysis for allele length, due to high intensity and similar shape of peaks (Figure 3). This specific representation shows two peaks in the microsatellite range, revealing that this individual is heterozygous at this locus.

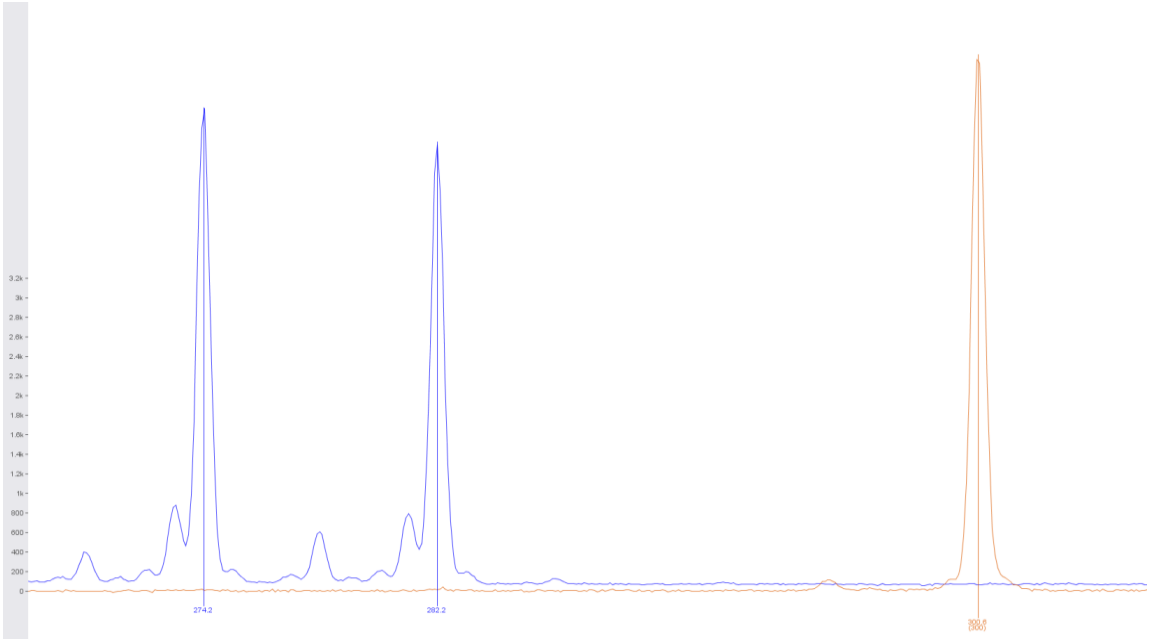

Figure 3. Representative fragment analysis results in Geneious.

Figure 3 Screenshot of Geneious results for Sam at locus Rio18. Orange peak is 300 bp on GeneScan 500 ladder, and blue peaks represent 274 bp and 282 bp microsatellite DNA fragments.

Lynch genetic distance calculations for male-female dyads revealed Sam and Frances have the greatest genetic distance, while Lily and Sam have the least genetic distance (Table 3).

Table 3. Lynch genetic distance between each dyad of four individuals, as calculated using RStudio.

	Frances	**Lily**	**Sam**	**Shadow**
Frances	-	0.6875	0.7292	0.6042
Lily	0.6875	-	0.5000	0.6875
Sam	0.7292	0.5000	-	0.5000
Shadow	0.6042	0.6875	0.5000	-

4. Discussion

These results provide support for the notion that staff at the Potawatomi Zoo should breed Sam and Frances to maintain SSP guideline of genetic diversity. Currently, no statements exist on how much genetic distance is required for breeding in captive breeding programs, but of the possible dyads at the Potawatomi Zoo Frances and Sam are the least related potential breeding pair. More importantly, these findings also show that, of the possible male-female dyads, Shadow and Frances should receive interventions to inhibit breeding due to low genetic distance between the two. In fact, a genetic distance of 0.5 is the average for full siblings and parent-offspring pairs. To prevent breeding between Shadow and Frances, it is recommended that they are separated into different enclosures. In the future, immunocontraceptives or hormonal contraceptives may be available for both male and females that could allow individuals that should not breed to be in the same enclosure[16,17,18].

The trapper who caught these four individuals claimed they were collected from different areas of a single river, but *L. canadensis* has the ability to travel long distances[19]. Therefore, two individuals caught from two different areas may be closely related. This could account for the low genetic distance between Shadow and Frances. Due the river otters being wild-caught, the genetic diversity they could bring into captive populations is advantageous. To take advantage of the genetic diversity present in these individuals, the zoo could bring in more individuals to breed with Lily and Shadow to increase genetic diversity in their zoo exhibit. They could also transfer potential offspring to other zoos. Alternatively, Lily or Sam could be moved to other AZA zoos to breed.

These river otters have been in captivity for about eight years at Potawatomi Zoo. During most of this time, no breeding behavior was seen. This could be attributed to disruption of reproduction in captive environments due to small spaces, altered sexual behavior, or health and husbandry issues[20]. Furthermore, it is known that successful captive breeding of river otters has decreased[21]. Due to this, the SSP for *L. canadensis* has emphasized research in reproduction[17]. North American river otters are seasonal breeders, with breeding occurring in the spring followed by embryonic diapause for seven to nine months and gestation for an average of 71 days[21]. In order to help captive breeding, these patterns must be understood before assisted reproductive techniques (ART) can be accurately implemented for North American river otter. ART includes embryo transfer, artificial insemination (AI), gamete cryopreservation, and in vitro fertilization (IVF)[20]. Preliminary research shows that fecal hormone monitoring and collection of high quality sperm through electroejaculation could be helpful techniques in performing AI and sperm cryopreservation, but AI has not been performed in North American river otters[21,22]. Unfortunately, ART will not be available to help these individuals breed, but future generations could be helped with these techniques, therefore SSP should continue to prioritize this research.

Although reintroduction was successful and *L. canadensis* is listed as LC, a current threat to wild populations is water pollution. In areas where water quality is poor, populations are small or missing entirely[3]. If population decline is seen again in this species, similar actions to the SSP for Maned wolves (*Chrysocyon brachyurus*) can be employed. In the case of the Maned wolf, SSP began wild conservation efforts, including health and ecological studies, genetic diversity assessment, and public education[23]. If water pollution escalates and is harming wild populations, SSP for *L. canadensis* can begin similar education programs for communities whose activities limit North American river otters through water pollution. Moreover, environmental contamination in the water can cause disease in river otters, especially through biomagnification[24]. River otters are also affected by different parasites and microbes[24]. If

environmental containments result in increased disease, SSP can begin health studies, similar to the Maned wolf SSP, to ensure that wild river otter populations are stable[23].

5. Conclusion

The use of microsatellite amplification and fragment analysis allowed the best possible breeding pair among the Potawatomi Zoo's North American river otters to be determined. These findings will help to maintain a SSP compliant captive population that has the opportunity to increase the genetic diversity of not only the Potawatomi Zoo's captive population, but other AZA zoos populations as well. These methods and data can be used for future studies to determine breeding pairs that are SSP compliant.

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7. References

1. Michael J. Johnson, "North America River Otter," *Bayland Ecosystems Species and Community Profiles*, January 2000, https://www.waterboards.ca.gov/sanfranciscobay/water_issues/hot_topics/Berressa/references/2000Species_and_Community_Profiles.pdf.

2. Emily K. Latch and others, "Deciphering Ecological Barriers to North American River Otter (*Lontra canadensis*) Gene Flow in the Louisiana Landscape," *Journal of Heredity* 99, no. 3 (2008): 265.

3. Thomas L. Serfass and others, "*Lontra canadensis*," *The IUCN Red List of Threatened Species* (2015).

4. Rebecca A. Mowry and others, "Genetics and the Successful Reintroduction of the Missouri River Otter," *Animal Conservation* 18, 2 (2015): 196.

5. Austin L. Hughes, "MHC Polymorphism and the Design of Captive Breeding Programs," *Conservation Biology* 5, no. 2 (1999): 249.

6. Michael Hutchins and Robert J. Wise, "Beyond Genetic and Demographic management: The Future of the Species Survival Plan and Related AAZPA Conservation Efforts," *Zoo Biology* 10, 4 (1991): 285.

7. Association of Zoos and Aquariums, "Species Survival Plan Programs," AZA.org, https://www.aza.org/species-survival-plan-programs.

8. Yogeshwar D. Kelkar and others, "What is a Microsatellite: A Computational and Experimental Definition Based Upon Repeat Mutational Behavior at A/T and GT/AC Repeats," *Genome Biology and Evolution* 2 (July 2010): 620.

9. William S Klung and others, *Essentials of Genetics*, 8th ed. (New York: Pearson, 2013), 211.

10. Amanda S. Beheler and others, "Development of Polymorphic Microsatellite Loci for North American River Otter (*Lontra Canadensis*) and Amplification in Related Mustelids," *Molecular Ecology* 4, 1 (2004): 56.

11. Amanda S. Beheler and others, "Ten New North American River Otter (*Lontra Canadensis*) and Their Utility on Related Mustelids," *Molecular Ecology* 5, 3 (2005): 602.

12. M. Kearse and others, "Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data,"*Bioinformatics*, 28, 12 (2012): 1647.

13. Michael Lynch and Kermit Ritland, "Estimation of Pairwise Relatedness with Molecular Markers," *Genetics* 152, 4 (1999): 1753.

14. R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

15. L Clark and M Jasieniuk, "polysat: an R package for polyploid microsatellite analysis." *Molecular Ecology Resources* 11, 3 (2011): 562.

16. Lowell A. Miller and others, “Comparative Efficacy of Two Immunocontraceptive Vaccines,” *Vaccines* 15, 17-18 (1997): 1858.
17. Julie K. Levy and others, “GnRH Immunocontraception of Male Cats,” *Theriogenology* 62, 6 (2004): 1116, http://www.sciencedirect.com/science/article/pii/S0093691X04000810.
18. Lowell A. Miller and others, “The Single-Shot GnRH Immunocontraceptive Vaccine (GonaCon™) in White-Tailed Deer: Comparison of Several GnRH Preparations,” *American Journal of Reproductive Immunology* 60, 3 (2008): 214
19. Thomas L. Sefrass and others, “Genetic Variation among Populations of River Otters in North America: Considerations for Reintroduction Projects,” *Journal of Mammalogy* 79, 3 (1998): 736.
20. Pierre Comizzoli and others, “Reproductive Biotechnologies for Endangered Mammalian Species,” *Reproduction Nutrition Development*, 40, no. 5 (2000): 493.
21. Helen L. Bateman and others, “Characterization of Basal Seminal Traits and Reproductive Endocrine Profiles in North American River Otters and Asian Small-Clawed Otters,” *Zoo Biology* 28, 2 (2009): 107.
22. Katarina Jewgenow and Nucharin Songsasen, “Reproduction and Advances in Reproductive Studies in Carnivores,” in *Reproductive Sciences in Animal Conservation*, ed. W.V. Holt (New York: Springer Science+Business Media, 2014), 205.
23. Nucharin Songsasen and Melissa D. Rodden, “The Role of the Species Survival Plan in Maned Wolf *Chrysocyon brachyurus* Conservation,” *International Zoo Yearbook* 44, 1 (2010): 136.
24. Kevin R. Kimber and George V. Kollias II, “Infectious and Parasitic Diseases and Contaminate-Related Problems of North American River Otters (*Lontra canadensis*): A Review,” *Journal of Zoo and Wildlife Medicine* 31, 4 (2000): 452.