GENETICS OF CAPTIVE NAKED MOLE-RAT POPULATIONS

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Bachelors of Science in the
School of Biology
and the Research Option

Georgia Institute of Technology
May 2015

GENETICS OF CAPTIVE NAKED MOLE-RAT POPULATIONS

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LIST OF SYMBOLS AND ABBREVIATIONS

NMR	naked mole-rat
PCR	polymerase chain reaction
HWE	Hardy-Weinberg Equilibrium

ABSTRACT

The evolution of highly social behavior (eusociality) represents one of the major transition points in evolutionary history. Naked mole-rats (NMRs), *Heterocephalus glaber*, are one of the few known eusocial mammals, meaning that they have a social caste system with a reproductive division of labor. In addition, NMRs show remarkable aging properties and tolerance to pain. Thus NMRs are important systems for studying life history traits. Surprisingly, however, very little is known about the mating systems and habits of NMRs. The goal of this study is to gain a better understanding of the population genetics and breeding habits of NMRs by creating a method for determining variation at microsatellite marker regions. Microsatellites are highly variable regions of the genome, which can act as identifiable markers for individuals. We have collaborated with Zoo Atlanta to study the population genetics of NMRs. We developed primer sets for examining variation at 54 microsatellite locations. Each of these loci were studied with up to 18 NMR individuals. We did this using traditional fluorescent primers and an M13-tailed fluorescent primer method that allows for cheaper and easier screening of samples. Six of these markers showed variability with two possible alleles. Thus, we have obtained the first estimates of genetic variation from the Zoo Atlanta NMR population. Our preliminary results also suggest that the population is in Hardy-Weinberg Equilibrium (HWE), which is unexpected because the population is not randomly mating. These methods and preliminary results provide insight into the breeding programs among captive NMR populations. In addition, the techniques developed will be useful for studying NMR biology in other contexts and help us understand the development of sociality and variation in health systems.

CHAPTER 1
INTRODUCTION

Eusociality is an important evolutionary development in animals, in which, colonies consist of a cooperative society with a reproductive caste system. Eusocial reproduction is usually defined by limited numbers of reproductive individuals including female queens (or a single queen) that mate with one or multiple males to produce offspring. There is also commonly a division of labor and tasks among non-mating colony members. This division of labor can be based on age, behavior, or morphologies of the worker individuals (1). It is most commonly seen and studied in insects, such as ants, bees, wasps, and termites. But this trait has also been seen in mammals, specifically naked mole-rats (NMRs), *Heterocephalus glaber*.

NMRs are one of the only known eusocial mammals. They are native to Kenya, Somalia, and Ethiopia and build extensive tunnel systems in which they live and forage for roots and tubers (2). The caste systems of NMRs consist of a queen, frequent workers who do most of the foraging and tunnel building, infrequent workers who work less often than the frequent workers, and nonworkers who are involved in the rearing of pups (3). The female NMRs suppress the reproduction of other females and non-breeding males through social interaction by causing physiological changes to individuals involving the ovarian cycle and proper sperm production (4).

NMRs also have many other important physiological traits that make them important for understanding the evolution of sociality as well as for medical sciences in general. They have low rates of cancer occurrence, long lives with slow aging, inability to feel certain types of pain, and the ability to survive in environments with low oxygen levels (5). Sequencing of the NMR genome has helped to provide a foundation for

studying all of these characteristics and applying them to human health and evolution. With regards to aging, it was seen that genes involved in mitochondrial proteins and telomerases also remain stable during aging and support current theories about the causes of aging in all mammals (5). NMR genome studies have also been useful in the discovery of the function of certain tumor suppressors in NMRs (5). Understanding of the NMR genome can thus be applied to human aging and cancer research.

NMRs are important for research on human health and the evolution of sociality, but we have very limited knowledge about their reproductive habits. Most of the current research involves the relatedness between natural colonies. In a recent study, twelve primer sets for microsatellite regions were used to show variation between natural populations. Very little variation was seen between colonies, suggesting extensive inbreeding and colony relatedness (6). This level of inbreeding between colonies suggests even higher rates of inbreeding within colonies. Inbreeding is very common among NMRs and is most likely a trait that allowed them to successfully maintain a eusocial system.

Inbreeding creates a high relatedness coefficient between individuals in a population, which provides more incentive for altruism, kin selection, and cooperative care for pups, which is a key aspect of the eusocial behavior (7). Kin selection is a process by which alleles of an individual affect the fitness of other individuals with the same alleles in the population. Cooperative care for pups is usually conducted by the nonworking class of NMRs and involves communal nesting although only the queen can nurse the pups (1). These behaviors are favorable to the non-breeding NMRs because

they are supporting the proliferation of very closely genetically related individuals due to the high levels of inbreeding.

Although we do know some information about NMRs' tendency to inbreed, it is not known how many males the female will reproduce with in her lifetime, the sex ratios of the offspring, or the amount of inbreeding within the colonies. Understanding the population structure and relatedness of captive NMRs, especially in relation to captive populations, can help scientists and zoos develop breeding programs to maintain healthy captive populations, and provide insight into future studies.

The purpose of this study is to develop a method using microsatellite markers to examine the genetics of Zoo Atlanta NMR populations in order to determine their breeding systems and the level of inbreeding within this colony. We expect to see very high levels of inbreeding and low variation within the captive populations. We identified microsatellite regions of the naked mole rat genome that will measure variation in the populations. Microsatellite regions are areas in the genome with repeated short nucleotide sequences and can have a highly variable number of repeats among individuals in a population. The specific number of nucleotide repeats in these regions act as identifiable markers, or alleles. These alleles are inherited by offspring and can be used to determine paternity and reproductive habits by detecting the alleles present in offspring and drawing conclusions based on the patterns observed. Here we describe the optimized methods for screening NMR samples at 54 loci. These markers will be used for variation screenings in the NMR population from our partners at Zoo Atlanta and populations from other sources in the future, such as the San Diego Zoo and the Philadelphia Zoo. If variation at the selected loci is seen between individuals from these zoo colonies, then considerations can

be made to determine the need for breeding programs and their design. Breeding programs can be used to provide more genetic variation within the colonies to help maintain a healthy genetic structure and maintenance of colonies.

CHAPTER 2
METHODS

Primer Development and Method Optimization

Sample Collection and DNA Extractions

All genotyped NMRs were received from Zoo Atlanta and deceased pups. All pups died naturally shortly after birth due to neglect from colony members. Collections were in keeping with IACUC procedures. NMRs were from two broods, one containing six NMRs, and the other containing 20 NMRs (Appendix A). These broods share the same mother, but it is unknown whether they have the same father.

A Chelex extraction method (8) and an EZNA (Omega Bio Tek) extraction method were tested on two individuals to determine the best protocol for extraction of DNA from skin and tail tissue samples. For the Chelex extraction method, about 1g of tissue was ground after freezing with liquid nitrogen, suspended in 1mL of Chelex solution, and heated at 95°C for 20 minutes. The samples were then spun down and the supernatant removed from the solid pellet. For the EZNA extraction method, about 1g of tissue was ground after freezing with liquid nitrogen, suspended in 200µL of TL buffer and 25µL of OB protease, vortexed and incubated on a heat block overnight at 55°C. The next day, the samples were centrifuged for 5min (13,000xg) and the supernatant was transferred to a new tube. We then added 220µL of ethanol (100%) and gently mixed before transferring the sample to a minicolumn and centrifuging for 1min (10,000xg). The column was placed in a new collection tube, 500µL of HB buffer was added, and the column was centrifuged for 1min (10,000xg). The column was placed in a third new collection tube, 700µL of wash buffer was added, and the column was centrifuged for 1min (10,000xg). This last wash step was repeated. The column was then centrifuged for

2min (13,000xg) to dry the column. The column was placed in a new tube, 200μL pre-warmed (~70°C) elution buffer was added, and the column was allowed to sit at room temperature before centrifuging for 1min (13,000xg). Finally, 100μL of elution buffer were added to the column and allowed to sit for 2 minutes before centrifuging for 1 minute (13,000xg).

Finding Microsatellite Regions

Microsatellite regions and flanking primer sets were determined for various loci. Microsatellite regions were found in a published NMR genome v1.1 (9) using MISA (MIcroSAtellite), a microsatellite identification tool (10). Flanking primer regions were determined using Primer 3 (11). Target parameters were set as follows: product size between 100 and 1000 bp with the optimal size of 200bp, primer size between 18 and 27 bp with an optimal length of 20bp, annealing temperature between 57 and 63 °C with an optimal annealing temperature of 60°C, and G-C content between 20 and 80%. These data are unpublished and all procedures to identify these novel microsatellite regions were performed by Linh Chau in the School of Biology at Georgia Tech.

Primer Optimization and Fluorescent Primers

Annealing temperatures of 68 primers were determined using gradient PCRs. The temperature range explored was 62-72°C. Thermal cycler programs included initial denaturation at 95°C for 5 min, 34 cycles of 95°C/30s, annealing temperature/30s, and 72°C/30s. It finished with a final elongation step at 72°C for 5 min. The complete list of primers tested can be found in appendix B. The primer sets with the least amount of multiple banding and the strongest bands were ordered with fluorescent labels.

Fluorescently tagged primers for 20 loci were used to genotype 18 individuals representing two broods (6 in one brood and 12 in the other). Amplicon sizes were determined using an ABI 3100 Genetic Analyzer.

M13 Tailed Primers

We ordered 22 of our own developed primers (including three controls also used with traditional fluorescently labeled primers) and 12 published primers (6) with M13 tails. The M13 primer has the sequence 5'-TGT AAA ACG ACG GCC AGT- 3' and is frequently used as a reliable primer region. We added this M13 sequence upstream of our forward primers to target our specific microsatellite regions while also reducing the cost of primer ordering by enabling us to buy a single fluorescent primer corresponding to the M13 tail, rather than buying a new fluorescent primer for every locus tested. We tested the M13-tailed primers at several loci to determine their ability to successfully amplify our targeted microsatellite regions and to provide us with information about allele lengths after being read on the ABI genetic analyzer. The M13-tailed primer PCR reactions can be seen in Figure 1.

Three NMR samples were studied at all 34 loci. The PCR reactions were set up with a ratio of fluorescent M13 primer, reverse primer, and M13-tailed forward primer of 4:4:1. Each reaction had the following thermal cycler program: 95°C/5min, [95°C /30s, annealing temperature/45s, 72°C /45s] for 30 cycles, [95°C /30s, 53°C /45s, 72°C /45s] for 8 cycles, 72°C/10min (12). A diagram of the mechanism for this reaction can be seen in figure 2. The primers were grouped into three annealing temperature groups according to their gradient PCR determined annealing temperatures (67°C or 70°C) or published annealing temperatures (58°C) (Appendix 2).

Product sizes were determined using an ABI 3100 Genetic Analyzer. M13-tailed primers were compared to traditional fluorescent primers based on the strength of the peaks and the amount of noise present in the readings.

Data Analysis and Statistical Methods

The data from the ABI 3100 Genetic Analyzer were interpreted using GeneMapper v4.0. Hardy Weinberg exact tests and allelic frequencies were determined using Genepop 4.0. Observed and expected heterozygosities were compared to make a prediction about HWE and F_{IS} values were determined to look at the amount of inbreeding (specifically the levels of heteozygosities) in the population. We were unable to do statistical analysis of HWE because we only tested one population and had a small sample size. Negative F_{IS} values suggest more heterozygosity and less inbreeding than expected, positive F_{IS} values suggest less heterozygosity and more inbreeding than expected, and F_{IS} values of 0 suggest expected heterozygosity and inbreeding levels. The samples from both broods were treated as one population.

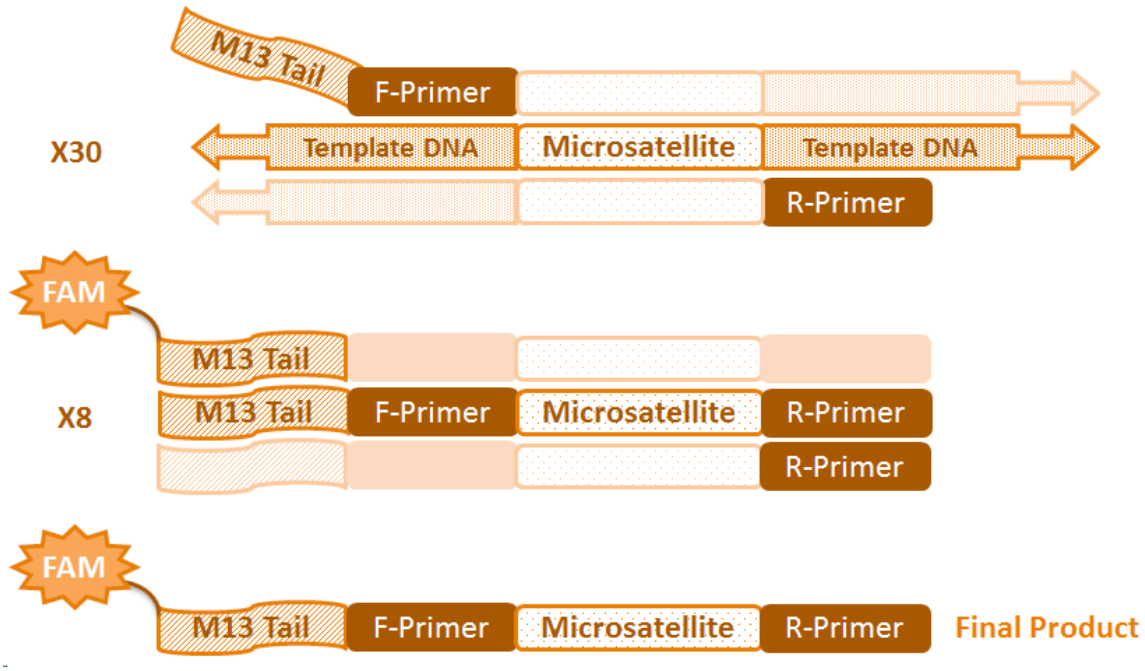

Figure 1. M13-tailed primer PCR. The are three different primers needed for the M13-tailed PCR reaction: reverse primers (normal reverse primer sequence for targeting a specific loci), forward primers with the M13 tail, and M13 primers tagged with a fluorescent dye. The first 30 cycles of the PCR reaction are conducted at using the annealing temperature of the forward primer. This allows the amplification of the target microsatellite region with the M13 sequence at the forward end. This image is based off a figure from Schuelke, M., 2000 (12).

CHAPTER 3
RESULTS

Primer Development and Method Optimization

The Chelex extraction method was used for all future extractions because it is faster, but equally effective protocol compared to the EZNA extraction method, which extracted ample DNA. Gradient PCRs allowed us to determine the annealing temperatures of primers or eliminate them from further testing based on the quality of the product bands seen in gel electrophoresis (Figure 2). A total of 42 novel primer sets exhibited the qualities described and were ordered with fluorescent tags or M13-tails along with the 12 published primers. Appropriate annealing temperatures for each primer set ranged from 63-71°C (Table 1).

Only one of the 20 traditional fluorescent primer sets did not provide consistent and reliable allele peaks after running through the ABI genetic analyzer. The M13-tailed primers products had more noise in the ABI residues than the traditional fluorescent primer products did (Figure 3). This means that there were many visible peaks that did not represent alleles and did not exhibit the traditional characteristics of alleles in GeneMapper in the M13-tailed primer residues.

Preliminary Results and Data Analysis

Three of the 20 loci studied with traditional fluorescent primers showed variation among NMR individuals (Table 2). Two of these primers had three genotypes in the population (hlg_c3591: 255/255, 255/269, 269/269 and hgl_c6757.2: 166/166, 166/168, 168/168), and one primer had two genotypes in the population (hgl_c7804: 104/109, 109/109). After variation was determined, observed and expected heterozygosities, F_{IS}

values, and allelic frequencies for the loci tested with traditional fluorescent primers were determined (Table 3). The p-value for the observed heterozygosities of the three traditional primers were not statistically significant ($p>0.05$), and the F_{IS} values were less than 0 (Table 3).

Three of the loci studied with M13 fluorescent primers showed variation among the three NMR individuals. All three of these M13 primer sets came from the 12 previously published primer sets (6). The sample size for the M13 fluorescent primer tests (3 individuals) limited our ability to do analysis.

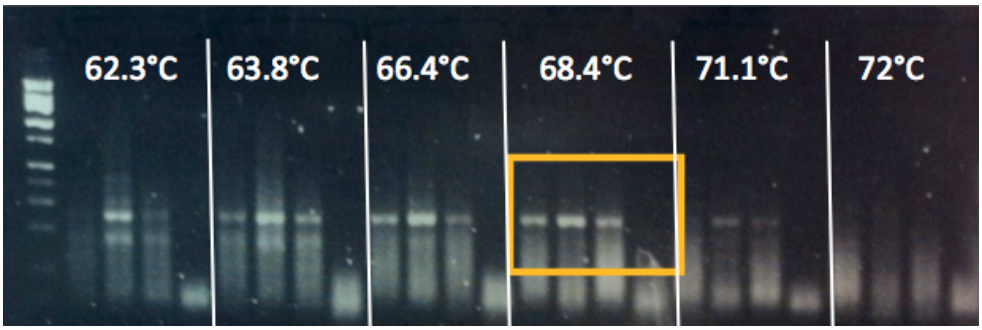

Figure 2. Gradient PCR example for loci 1610 with an expected amplicon size of 247 bp. Temperatures 62.3, 63.8, and 66.4°C are examples of multiple banding meaning that there is nonspecific primer annealing at these temperatures. Temperatures 71.1 and 72°C have weak or no banding evident, meaning that the primer was not able to anneal well at these higher temperatures and less PCR product was made. The annealing temperature 68.4°C was selected as the ideal annealing temperature because there is no multiple banding visible and the visible bands are strong, indicating a sufficient amount of PCR product was made in the reaction.

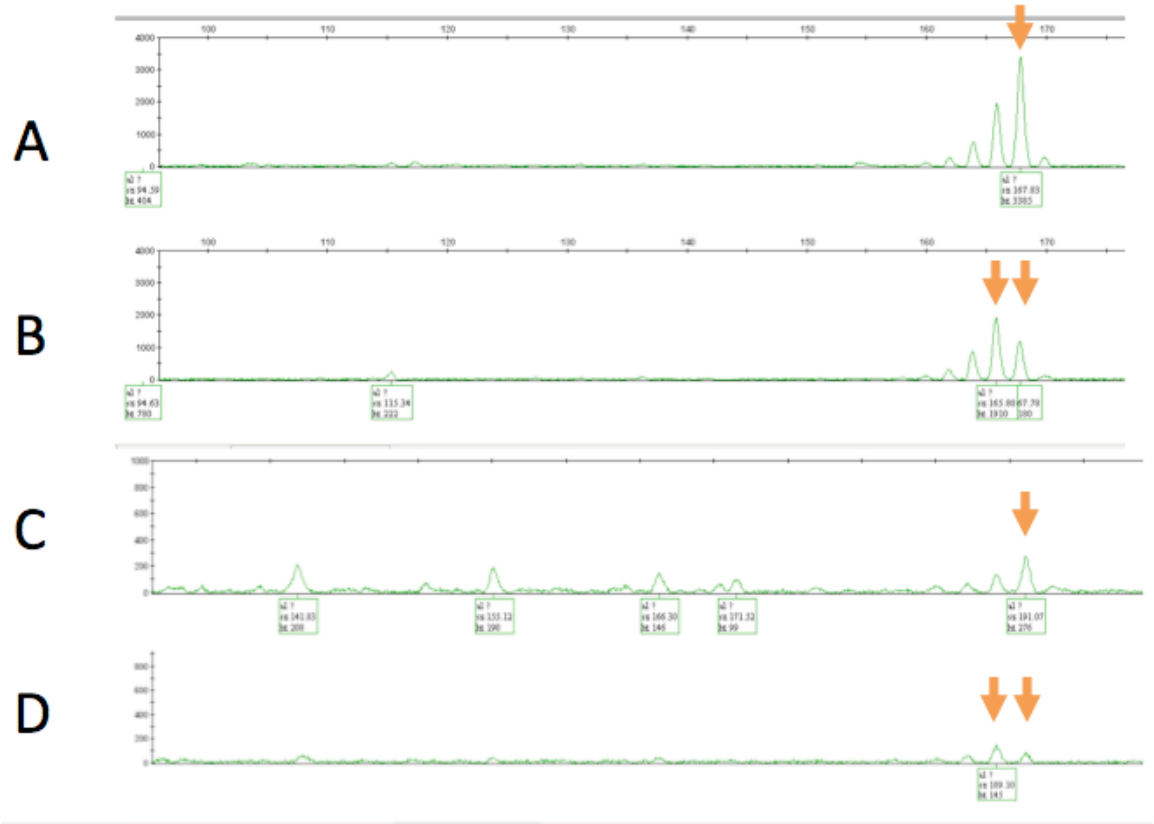

Figure 3. Examples of peak residues from ABI readings, as viewed in GeneMapper. All graphs are residues for loci nmr_c6757.2. A and C are residues from the same individual, but A is the residue from a traditional fluorescent primer, and C is a residue from an M13 fluorescent primer. This individual is a homozygote and the allele peak (orange arrows) is in the expected range, 168, based on primer length (M13-tailed primers add about 20 bp to the allele length because of the M13 sequence). The M13 reaction created more noise in the residues, which can be seen in figure C. B and D are residues from the same individual, but B is a residue from a traditional fluorescent primer, and D is a residue from an M13 fluorescent primer. The individual is a heterozygote at this locus and the two allele peaks (orange arrows) have a difference in length of 2 bp.

Table 1. Final Primer Annealing Temperatures. Annealing temperatures were determined by gradient PCRs ranging from 62-72°C.

Primer Name	Temp (°C)
hgl_c243.2	63
hgl_c3591	63
hgl_c3322.2	65
hgl_c7804	65
hgl_c7996	65
hgl_c3223	66
hgl_c4233.1	66
hgl_c6757.2	66
hgl_c7797.1	66
hgl_c7797.2	66
hgl_c8448.2	66
hgl_c9217	66
hgl_c2793	67
hgl_c3097	67
hgl_c3322	67
hgl_c3519	67
hgl_c4598	67
hgl_c7076	67
hgl_c7221.2	67
hgl_c7285	67
hgl_c9338	67
hgl_c1610	68
hgl_c2663	68
hgl_c2681	68
hgl_c330	68
hgl_c4233.2	68
hgl_c6197	68
hgl_c857	68
hgl_c10193	69
hgl_c243.1	69
hgl_c6757.1	69
hgl_c7146	69
hgl_c7269	69
hgl_c9415	69
hgl_c9776	70
hgl_c10012	71
hgl_c1976	71
hgl_c3190	71
hgl_c3519	71
hgl_c3893	71
hgl_c4642	71
hgl_c6228	71
hgl_c6655	71
hgl_c7633	71
hgl_c8448.1	71

Table 2. Characterization of Three Variant Loci. The genotypes are written as the product lengths (bp). This table describes the observed and expected number of individuals.

Loci	Genotypes	Observed	Expected
	255 , 255	5	5.2759
	269 , 255	8	7.4483
hgl_c3591	269 , 269	2	2.2759
	166 , 166	2	3
	168 , 166	11	9
hgl_c6757.2	168 , 168	5	6
	104 , 104	0	1.1613
	109 , 104	9	6.6774
hgl_c7804	109 , 109	7	8.1613

Table 3. Allelic Frequencies and Hardy-Weinberg Exact Test of Variant Loci. P-value is associated with H_o, S.E. is standard error, and W&C is Weir & Cockerham's estimate. The Chi Squared value for this test was 3.6607 with 6 degrees of freedom and a probability of 0.7225.

Loci	Allele	Sample Count	Frequency	P-value	S.E.	FIS W&C
hgl_c3591	255	18	0.6	1	0	-0.0769
	269	12	0.4			
hgl_c6757.2	166	15	0.4167	0.6251	0.0019	-0.2303
	168	21	0.5833			
hgl_c7804	104	9	0.2812	0.2565	0.002	-0.3636
	109	23	0.7188			

CHAPTER 4
DISCUSSION

Primer Development and Method Optimization

The goal of this study was to develop a method for determining the breeding system and levels of inbreeding within captive NMR populations. We did this by developing and optimizing a primer set that can be used to look at variation in lengths of microsatellite regions, and by developing a protocol for testing these primers in with the more cost-efficient M13-tailed primers. The primer set will make genotyping NMR individuals easier for zoos and other captive populations. This will make large-scale studies of NMR population genetics easier and faster, which will help us to understand their breeding habits and maintain healthy populations.

The M13 tailed primer method (described in Figure 1) allows us to buy three fluorescent primers- M13 sequence with FAM, HEX, or TAMRA fluorescent tags- that can be used for any NMR primers we order with the M13 tail, rather than ordering new fluorescent primers for every primer set we develop. This method will help save time and money by limiting the number of primers and fluorescent primers that are ordered.

Preliminary Results and Data Analysis

Our preliminary results show evidence of variation within the Zoo Atlanta NMR population. The ratios of alleles for hgl_c3591 and hgl_c6757.2 suggest that the queen and the father of the pups were both heterozygotes at each locus. Locus hgl_c7804 did not have any homozygotes for the allele of 104bp suggesting that one parent is a homozygote for the other allele, 109bp, and one parent is a heterozygote.

The evidence suggesting that the Zoo Atlanta population is in HWE was surprising because the colony does not meet the criteria for HWE involving random mating. Their eusocial system means that there is only one female mating, and it is commonly believed that the female only mates with 1-3 males (3), although it is one of the goals of this study to determine if this is true in the Zoo Atlanta population.

The negative F_{IS} values for the three variant traditional fluorescent primers suggest that there is less inbreeding than expected, but this is not statistically significant because of the P-values ($P>0.05$). We expect to see positive F_{IS} values for loci in NMRs which suggests high levels of inbreeding because the population is in captivity (no immigration or emigration), there is only one mating female, and previous evidence from wild colonies suggests this (6,7).

There were not enough individuals tested with the M13 primers to make any determinations about the parent genotypes. The M13 tailed primers often exhibited very weak signals and extraneous noise when run through the ABI Genetic Analyzer. This is probably because of nonspecific binding due to the M13-tail, non-specific binding due to the high annealing temperatures, and the two PCR cycles with widely varying annealing temperatures. More samples need to be tested with the M13 fluorescent primers to conduct HW exact tests and statistical analysis of the three variant loci.

The methods and the primers described in this study can be used to look at variation within more Zoo Atlanta NMR broods, determine variation in other zoo populations, and to develop primer sets at new loci to gain more data. Information about the variation in the populations can help us to determine the levels of inbreeding, the breeding systems, and the parentage of individuals in captive colonies. This knowledge is

very important for understanding NMR ecology and for determining if breeding programs are needed to maintain healthy captive populations. These captive populations could be important for future research on health, such as cancer and aging, or for maintaining healthy zoo colonies that are used for education of the public.

CHAPTER 5
CONCLUSIONS

In this study, we developed protocols for the use of traditional and M13 tailed primers for the study and genetic characterization of NMR populations. Using these protocols, we developed a set of 54 primer pairs that can be used to identify microsatellite variation within NMR populations. We found 6 loci with variation in the Zoo Atlanta population that can be used to understand NMR reproduction. Most microsatellite loci were monomorphic among Zoo Atlanta samples, which was expected because of previous research indicating high levels of inbreeding. The primers tested with the M13 tailed method must be studied more with more individuals to determine the genotypes of the parents and conduct statistical analysis. These methods and all 54 loci can be applied to other populations besides the Zoo Atlanta population. Future studies using these protocols should include testing individuals from the San Diego Zoo with all available primers. Determining the sex ratios for each brood of the Zoo Atlanta samples should be determined and examined to ascertain if a sex bias exists among NMRs. Although there are only six loci found to have variation so far, this study is a good starting point for future research to learn more about the population genetics of NMRs.

CHAPTER 6
ACKNOWLEDGEMENTS

Michael Goodisman, Linh Chau, and Zoo Atlanta. This research was funded by the Elizabeth Smithgall Watts Endowment.

APPENDIX A- NMR SAMPLE INFORMATION

Sample ID	Date of Birth	Date Received
NMR1	Unknown	5/29/13
NMR2	Unknown	5/29/13
NMR3	Unknown	5/29/13
NMR4	Unknown	5/29/13
NMR5	Unknown	5/29/13
NMR6	Unknown	5/29/13
NMR7	1/25/14	4/18/14
NMR8	1/25/14	4/18/14
NMR9	1/25/14	4/18/14
NMR10	1/25/14	4/18/14
NMR11	1/25/14	4/18/14
NMR12	1/25/14	4/18/14
NMR13	1/25/14	4/18/14
NMR14	1/25/14	4/18/14
NMR15	1/25/14	4/18/14
NMR16	1/25/14	4/18/14
NMR17	1/25/14	4/18/14
NMR18	1/25/14	4/18/14

APPENDIX B- COMPLETE LIST OF PRIMERS TESTED

Name	F or R	Sequence	**Product Size$_E$	***Product Size$_O$
	F	GATTTCTAGTGTGCACGCGC		
hgl_contig10012	R	GCAAGTTCAAGCCCACCATG	146	*186
	F	AGTGATAAGGGGCTGGGGAT		
hgl_contig10193	R	GTTCAAGCCCAAGCCACATG	181	182
	F	ATGGAACCCAGGGCTTCATG		
hgl_contig1252.1	R	AAGTTGGATGTGGTGGTGCA	269	
	F	CTCCATGCCAGCTCAAGTGA		
hgl_contig1252.2	R	GCCCCCTCTCTTTTGCTCAT	204	
	F	TGTGCCAGGATTTTCACCCT		
hgl_contig1581	R	TCTGTGAGGAATAGTGCTGCT	251	
	F	TGCAAGCTCAAGGTCCTGAG		
hgl_contig1843	R	CCTTGCTTTTCAACAGGGGC	199	
	F	CTGGACGTACATGCTTGGGT		
hgl_contig1976	R	GCCACCTCAAAACTCCTTGG	210	
	F	ATGGAAGCAACCTGTGTTCT		
hgl_contig2143	R	TGTTCCTCTGAAGAAATTGGAGAGA	261	
	F	CTACTGAGCTGCTTCGAGCC		
hgl_contig243.1	R	TGCAGAAGTCATCCTTGGCA	249	249
	F	TGGGGCAGGATTAGGGATGA		
hgl_contig243.2	R	ACTCCCTCATCCCCTTCCTC	196	193
	F	GTCTCTGTCTCTCTCACGCG		
hgl_contig2445	R	TGGCAAGCTGATTGTTCCCT	113	
	F	CCCACTCCATCTCTCAAGGC		
hgl_contig2663	R	TGCCTGTAATCCCAACAGCT	263	266
	F	CCCATGATCACAGCGAGACA		
hgl_contig2681	R	AGTTTGCCCTCCAGTTTCCT	254	254
	F	GATTCTCCTGGCTCCACACC		
hgl_contig3190	R	GCCGACAAGATGACCCTCAA	191	
	F	TGGAAGTTGAAAGGTCCGCA		
hgl_contig3322.1	R	ACAGCTGTTTCCCTGAGTTGT	247	246
	F	ATGATGCTTTGGGCCAGTCT		
hgl_contig3519	R	ATCCCACGTTCAAGGCTAGC	187	187
	F	TCACTGACTGCAACCATAGGT		
hgl_contig3591	R	TGCTAATGTTTAACAACTAGCTTTCCA	254	254/269
	F	GCAAATTCCCCACATGCCAG		
hgl_contig3893	R	ATCCTCTGCACAGCACTGTC	159	
	F	ATGACACAATGCAGGGGAGG		
hgl_contig4598	R	AGGCAGTGGCACAAGATGAA	231	*254
	F	TCGTTTGCTGCCTTCAGAGT		
hgl_contig5550	R	AATCCCAGCACTCAGAAGGC	204	
	F	ACTTGTGAAGGGGTCAGCAC		
hgl_contig5876	R	TCAAAGCCTCCTGCATCCTT	234	
	F	TCTGTGCACGTACCAACTCC		
hgl_contig6655	R	TGTGGACCCTGATGCATGAC	240	240
hgl_contig6757.1	F	AACCTCTTGAGTGCTGGAGC	262	265

	R	ATAGGCCCAGGGATGTAGCT		
	F	AATCTCTCTCCCCCAGCTGT		
hgl_contig6757.2	R	TATTGGATGACACCCGGCAG	168	166/168
	F	GGCTTGGCCTGAACTGTGTA		
hgl_contig7076	R	TCAGTGAGCATCTTGTACAAGTGA	157	155
	F	CCCTCATAGGAGTGTGTGCG		
hgl_contig7139	R	CTAAAGGTTCTCTCCCCCGC	170	
	F	GGCGGGAGTAATGGACACAG		
hgl_contig7146	R	CAACATGCCTGGCTGGAAAC	215	*238
	F	ACAGCCTCTGAAAAGCTCCC		
hgl_contig7221.1	R	CCCACCTTCTGGCAGTTTCA	218	
	F	TCAACTGTCTGGGATCCCCT		
hgl_contig7221.2	R	CTGTGGCCCTTGGAACAGTA	209	210
	F	CCCAGAGGACACACTGAAAGA		
hgl_contig7269	R	CCACCTGTCTCAGCCTCCTA	243	245
	F	GCTTTGCTCTTGTTGCCCAA		
hgl_contig7285	R	GCTCAGTGGTTCTGCTGAGT	205	*232
	F	TGACCTCCCTCTCCCCATTC		
hgl_contig7633	R	GCCTTCTGAAGTTCCAAGCC	195	*217
	F	AAGTGAGAACATACACCCATGT		
hgl_contig7797.1	R	GACCGGGAGAGCTAGAATGC	144	143
	F	GCATTCTAGCTCTCCCGGTC		
hgl_contig7797.2	R	TTCTGGAGGGATAGGTGGCA	277	277
	F	CGTGTCCTCTTGGTGTGACA		
hgl_contig7804	R	ACAGTCTGCCTTCACGATCG	110	104/109
	F	GAGCAAGGACCCGAGTTTGA		
hgl_contig794	R	ATGTGTGCATTGCTCCTGGA	198	
	F	TCACAAGCACAAGGTCCCAG		
hgl_contig7996	R	CTCCTCCCTTGATCCCTCCA	200	197
	F	GCAGCGAAACCACTGAGCTA		
hgl_contig80	R	CATGCGACAGGGAGGTGTAA	136	
	F	ATTCTGGAGGGCCAAGGTTG		
hgl_contig8448.1	R	TATGGTCATCAGGGCAGAGC	204	
	F	GGGCTTCTTCACCCAACAGT		
hgl_contig8448.2	R	GCCAGCCTGAGATCCTGTTT	198	196
	F	CCTCTGGGAGCAGTAGGACT		
hgl_contig8471	R	ACATTGCTGCCCACTTCCAT	275	
	F	ACAGTACAAAGGCTGGTGCA		
hgl_contig8537	R	CAGAGCCCAAGTGATCTGCA	186	
	F	ACTGTGACGTGATAAAGTGGCT		
hgl_contig9217	R	CAGTAGCAGAGCCTGAGCAT	181	182
	F	TCTGTGGTCTTTCTCACACAC		
hgl_contig9338	R	TGACAAAGTTGGACTATGCACA	217	*294
	F	TGCCGAGAAGGTGCAGAAAT		
hgl_contig9415	R	GCCTGGGCAAACTAGTGAGA	258	*282
	F	ACCTGCTCACTTTACGGTGG		
hgl_contig9916	R	GGAGTGGGGTCTAGTGGGAA	142	

	F	TCACTCCTCACAGGGTAGGG		
hgl_contig1094	R	ACCACATCAAAATCCCCGGG	194	
	F	TGAAGGTGTTTGGCCCCTTT		
hgl_contig1496	R	CCAGGAGACAGTTTTCCCCC	265	
	F	TTGGAGATCAAACCTGGGGC		
hgl_contig1610	R	AAGTGTTTCCCAACCTGCCA	247	
	F	GCAGGAGGATTGCCATGAGT		
hgl_contig2094	R	TGATTCTCTTTCTCTCTCTTTCCCT	246	
	F	ACAGAGAGAGGGAGAGAAAGAGA		
hgl_contig2793	R	TGTGTGCTGAAGATGACATCCA	220	*274
	F	AGTTCTGTATCCGTGCCAGC		
hgl_contig3097	R	AGGGGGAAATGATCTGAACAGAC	209	
	F	CCCCACCTACCCACCTATGA		
hgl_contig3223	R	TGGATTCTGGTGTGGGTTCA	196	*216
	F	ACCTGTCTGTGTGCATGTGT		
hgl_contig330	R	CAAGCACACACCTGGAGCTA	234	*258
	F	TGTTCTAACACAGTTAAGTTGACTTCA		
hgl_contig3322.2	R	ACACAGATTCACAAAACTGTTAGCA	280	-
	F	TCACTGTCATTGCTGTGCTCT		
hgl_contig3519	R	ATTGGATCTCTAGCAGCCGC	279	*302
	F	CTGGATGGGGTCTGAGGAGA		
hgl_contig4233.2	R	TATCCCTAACCCCAGACCCC	177	-
	F	AGCCGCCAACTGTGAACTAA		
hgl_contig4233.1	R	AGTAAGTACCATTTGACAAAAAGCT	236	*258
	F	GCGGGGCATTTGTTTCCTTT		
hgl_contig4642	R	AACTCAGGACCTCGTGCTTG	201	*224
	F	TTCCAAAAGGCCGTTTCCCT		
hgl_contig5619	R	AGGGGGTGGGGATATAGCTC	212	
	F	GCGGACCCTAAATCTGGCTT		
hgl_contig6197	R	ACACCATGCTCACACACACA	276	-
	F	AAATGCAGTGTTTGGCAGGG		
hgl_contig6228	R	GCACCCACTGCTTGTCTGTA	264	*285
	F	TTGTGGAATGAGGCAGTGGG		
hgl_contig6455	R	AGGGGGAAAAAGTTTCAGGCA	261	
	F	AACAGCCCGGGATTTAGCTC		
hgl_contig799	R	CTGGGGATCGAACTTGGGAC	260	
	F	GTGGGGCAGGGAGAATTGAA		
hgl_contig8024	R	GGTGCTGGGATTACAGGCTT	249	
	F	TGTCTTGGTGCCCACTTACC		
hgl_contig857	R	TCACATGATGGCAACTGGCT	252	*276
	F	CAGCCTGGGCAACTTAGTGA		
hgl_contig9045	R	CCATGCAATGGTGGCATCAC	208	
	F	CACCTTTCCTGTGCGCAAAA		
hgl_contig9776	R	AGTCTGGTTGGCCTTCATGG	205	*224

* Primers tested with M13 tailed primers (observed sequences are longer than expected because of this), **Product $Size_E$ is the expected product size, ***Product $Size_O$ is the observed product size- not all primers were tested with fluorescent markers (blank)

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