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Pedigree and Molecular Assessment of Relatedness in a Captive Chimpanzee Population

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Pedigree and molecular assessment of relatedness in a captive chimpanzee population

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STATE UNIVERSITY

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Dedication

To the ones who were with me every step of the way.

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Abstract

As a crucial component of biodiversity, genetic diversity contributes to variability among individuals, allowing populations of endangered species to be resilient in the face of changing environmental conditions. Zoological institutions have become a cornerstone of conservation efforts and a refuge for endangered species given threats imposed on wild populations by climate change, habitat fragmentation and degradation, and overexploitation. Chimpanzees (*Pan troglodytes*) are an endangered species experiencing drastic population decline in the wild, yet are common residents in zoos and wildlife sanctuaries. To sustain long-term, genetically viable populations of species of concern, zoos use studbooks and paternity testing to identify individuals, their movements, and relatedness. Lack of information or erroneous assumptions, however, can lead to mismanagement of individuals, jeopardizing the genetic integrity within *ex situ* populations. Here, we analyzed (1) studbook records to identify relatedness based on pedigree alone, then (2) evaluated eight polymorphic microsatellite loci across a captive chimpanzee population in Grand Rapids, Michigan to (3) calculate relatedness (*r*) and parentage. Our molecular-based analysis confirmed parentage and relatedness estimates from PMx pedigree analysis. While all analyses identified a mother-offspring pair, they also revealed a lack of relatedness between most individuals, an important trait for a sustainable population. Minimal relatedness in a population consisting of mainly founders is ideal, as it provides a greater genetic variability. Future research should include additional loci and individuals to gain a better understanding of this population's genetic diversity, and aid other zoos in integrating molecular-based approaches to conservation management.

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Abbreviations

AZA – Association of Zoos and Aquariums

bp – Base pairs

CI – Confidence Interval

DNA – Deoxyribonucleic acid

EAZA – European Association of Zoos and Aquariums

GD – Genetic diversity

H_e – Expected heterozygosity

H_o – Observed heterozygosity

IBD – Identical by descent

IUCN – International Union for the Conservation of Nature

JBZ – John Ball Zoo

mk – Mean Kinship

N – Population size

N_e – Effective population size

r – Relatedness

SD – Standard deviation

SE – Standard error

SSP – Special Survival Plan

WAZA – World Association of Zoos and Aquariums

Chapter 1 – Introduction

Introduction

Since zoological institutions have become a cornerstone of *ex situ* breeding programs and a reservoir of genetic diversity for endangered species (Ito *et al.,* 2016; Ochoa *et al.* 2016; Witzenberger & Hochkirch, 2011), the use of molecular genetics has become increasingly important (Norman *et al.,* 2019). Conservation efforts aim to maintain, or even increase, the genetic diversity of species of concern and do so through harnessing the genetic diversity present in captive populations (Ochoa *et al.,* 2016; Ralls & Ballou, 2004; Shan *et al.,* 2014). For example, *ex situ* breeding increased population sizes of Arabian Oryx (*Oryx leucoryx*) and California Condor (*Gymnogyps californianus*), which have become flagship success stories of conservation breeding programs (Ochoa *et al.,* 2016; Stanton *et al.,* 2015; Conrad, 2018; Ralls & Ballou, 2004).

Recent studies demonstrate that conservation breeding programs can lead to an increase in the genetic diversity of captive individuals (Norman *et al.*, 2019; Ochoa *et al.*, 2016). For instance, Ochoa *et al.* (2016) compared the genetic diversity of Arabian Oryx populations from the Phoenix Zoo and Shaumari Wildlife Reserve captive breeding programs, and determined that the captive populations had greater genetic diversity than wild population. Overall, however, dwindling population sizes lead to a decline in genetic diversity, particularly in very small populations. Small population size often leads to genetic drift, which links to random losses of genetic diversity in both captive and wild populations (Ballou & Foose, 1996; Frankham, 2015).

In addition, many populations once thriving in the wild are facing ever-escalating challenges due to habitat loss and degradation, and human encroachment (Ito *et al.* 2017, Mccarthy, Lester & Stanford, 2017; Ochoa *et al.,* 2016; Pastorini *et al.,* 2015; Schmidt *et al.,* 2015; Willis, 2001; Witzenberger & Hochkirch, 2011). Chimpanzees (*Pan troglodytes*), an endangered species relatively common in zoos, are one such species ideal to use for research in this area. Their decline in the wild stems from habitat loss and degradation, poaching, and climate change (Mccarthy *et al.*, 2017). Chimpanzee populations continue to decline, leading to their global endangered status (IUCN) and harming overall genetic diversity in the species (Ghobrial *et al.*, 2010; Sesink Clee *et al.*, 2015; Humle *et al.*, 2016; Mccarthy *et al.*, 2017). Therefore, captive populations of endangered species are of growing importance to conservation of biodiversity, and determining their genetic variability can help zoo personnel set appropriate conservation goals and management policies to maintain long-term, sustainable populations.

Specifically, as zoological institutions in the United States currently do not manage chimpanzee populations by subspecies (of which there are four), populations may (1) be prone to hybridization, (2) lose valuable genetic material, and (3) experience the negative effects of recessive deleterious alleles that resurface (Norman *et al.*, 2019; Lacy, 2019) Incorporating molecular data into management bridges a gap between molecular genetic research and zoo management that can only benefit the conservation of treasured, endangered species and ensure improved management protocols in the future.

Purpose

The purpose of this research was to address a gap in knowledge regarding the molecular genetic background of captive chimpanzees from John Ball Zoo in Grand Rapids, Michigan, USA. Typically, pedigree analysis and breeding recommendations in conservation programs use information gleaned from studbooks and visual observations. Unfortunately, these data lack specificity about the molecular genetics of each individual, which can lead to unintentionally problematic outcomes in captive-breeding programs. Therefore, the goal of this research was to fill in the gaps and provide molecular genetic data for captive individuals and determine the genetic composition and structure (i.e., measures of relatedness, parentage, sibship, etc.) of this population. By doing so, molecular data can be combined with other methods of pedigree analysis to inform future management practices to maintain long-term, sustainable captive populations.

Scope

This study focuses on a population of captive chimpanzees in a moderate-sized, public zoo in the Midwestern United States. This research specifically investigated the population of chimpanzees in John Ball Zoo, located in Grand Rapids, Michigan. Although only one small captive population was included in the study, the results reveal the need for molecular genetic integration (e.g., 10-20 microsatellite markers, representative- and whole-genome sampling) into management techniques. For instance, we used a panel of eight microsatellite loci that were able to confirm a parent-offspring relationship recorded in the studbook and low levels of relatedness between the other individuals. However, we determined that because our sample size was smaller, using a larger set of markers or a reduced representative genome sampling method (e.g., RAD sequencing) would provide a more in depth analysis of genetic diversity. This information will be helpful in guiding future research on captive populations of endangered species with traditionally small numbers.

Assumptions

The main objective of Chapter 2 was to compare pedigree- and molecular-based relatedness and parentage assignments. We used three software programs to conduct these analyses: PMx, GenAlEx, and COLONY. While the chimpanzee population at John Ball Zoo currently consists of only six individuals (as of late 2018), we included a seventh individual whose death occurred in early 2018 because we had access to their biological sample, and this individual was one of the founders of the population. Aside from the seven individuals included in this study, only one other individual had previously lived at John Ball Zoo. Unfortunately, their sample was not available. Consequently, our study included seven chimpanzees that resided at John Ball Zoo between 2018 and present, and we assumed an N = 7 for all analyses.

When calculating relatedness (*r*) and parentage/sibship for the John Ball Zoo population, we made a few assumptions. First, we assumed that the pedigree records were correct, complete, and without error. Second, COLONY parameters are delineated between monogamy v. polygamy and haploid v. diploid. For our population and microsatellite markers, we assumed polygamous mating with diploid alleles. Third, we sampled eight loci described in Hvilsom *et al.* (2013) across our seven individuals. Markers GATA129H04 and GATA50G06 were the only two loci to amplify completely for all individuals. These misamplifications were recorded as N/A, and included in calculating estimates and relatedness coefficients.

Objectives

This thesis contains a manuscript (Chapter 2) and extended literature review (Chapter 3). The objectives for my manuscript were to use studbook data to (1) conduct a pedigree analysis using PMx software (Ballou *et al.*, 2020), (2) conduct a fragment analysis of eight microsatellite loci using DNA extracted from blood samples of individuals in the John Ball Zoo chimpanzee population, (3) use GenAlEx v.6.5 (Peakall and Smouse, 2006 & 2012) and COLONY v.2.0.6.5 (Wang, 2018) to analyze microsatellite data, and (4) compare studbook- and molecular-based analyses.

Significance

This research is important for endangered species and captive population conservation as it addresses the genetic variability and structure of a Midwestern captive chimpanzee population. While pedigree analyses have been conducted in the past to determine paternity and identify parentage, few studies have used molecular genetic techniques in combination with studbook records to identify genetic and demographic records of captive chimpanzee populations in the United States. By determining the genetic variability within these populations, zoo curators and other personnel will be able to adjust current conservation goals and management policies to better maintain long-term, sustainable populations of endangered species.

Definitions

Mean kinship (*mk*): measure of relatedness with an individual and all other individuals in the population, meaning an individual's *mk* may change depending on the population in which it is located.

Coefficient of relatedness (*r*): "The probability that at a given locus, an allele sampled from one individual is identical by descent to at least one of the alleles at that locus in a second individual," (Traylor-Holzer, 2011, p. 134).

Identical by descent (IBD): "Two alleles are identical by descent if they are identical because of shared ancestral descent (in contrast to identity cause by two identical mutations)," (Nielsen & Slatkin, 2013, p. 274).

Chapter 2

Comparison of pedigree and microsatellite data of a captive chimpanzee (*Pan troglodytes*) population: Suggestions for conservation management

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Abstract

Zoological institutions have become a cornerstone of conservation efforts and a refuge for endangered species. They played a critical role in preserving populations, but climate change, habitat fragmentation, and overexploitation are continuing to exacerbate population declines for many other species, making captive populations critical to conservation efforts. Genetic diversity is associated with variability among individuals, which is commonly associated with increased resiliency in the face of changing environmental conditions. Chimpanzees (*Pan troglodytes*) are a species currently experiencing drastic population declines due to habitat fragmentation and poaching, yet are common residents of zoos. Conservation management strategies of captive endangered species traditionally consist of pairing recommendations using pedigree records alone, but recent studies have revealed the importance of incorporating molecular data with traditional parentage analysis. Here, we analyzed pedigree records and microsatellite data from a captive chimpanzee (*Pan troglodytes)* population in Grand Rapids, Michigan. The objectives of this study were to (1) compare parentage analysis from pedigree records and genetic data, and (2) understand how these findings might influence future management. We used PMx software to conduct a pedigree analysis, and two computer programs (COLONY and GenAlEx) to perform genetic analysis of microsatellite data. These analyses confirmed the presence of a mother-offspring pair recorded in the chimpanzee studbook, and revealed a lack of relatedness between founders. Therefore, for optimal management for conservation purposes, we recommend two actions, (i) combine pedigree & molecular data in future studies, and (ii) perform additional research that incorporates more molecular markers and multiple captive populations for each species studied.

HIGHLIGHTS

- Chimpanzee population (N=7) located in Grand Rapids, MI, United States of America.
- PMX and COLONY analysis both identified one mother-offspring pair, but molecular analysis identified a variety a relatedness estimates not revealed by studbook data.
- More markers and additional populations should be included in future research.

KEYWORDS

COLONY, *ex situ*, GenAlEx, Genetic Diversity, Parentage, PMx, Relatedness

1 | INTRODUCTION

Zoological institutions have become a cornerstone of *ex situ* conservation breeding programs and a reservoir of genetic diversity for endangered species given the threats imposed on wild populations (Ito *et al.*, 2016; Ochoa *et al.*, 2016; Witzenberger & Hochkirch, 2011). Along with anthropogenic disturbances like ecosystem degradation and fragmentation, climate change is one of the most significant factors impacting species and driving biodiversity decline, as it both exacerbates and accelerates biodiversity loss (Bellard *et al.*, 2012). Small populations generally have less genetic variability compared to larger populations; therefore, dwindling population sizes often lead to genetic drift (which links to random losses of genetic diversity), inbreeding, and/or a higher probability of expressing recessive deleterious alleles. This is true for both captive and wild populations (Kyriazis, Wayne & Lohmueller, 2019; Ballou & Foose, 1996; Frankham, 2015; Ochoa *et al.*, 2016).

Genetic diversity, a crucial feature of biodiversity, is addressed in zoos through conservation efforts such as captive breeding programs. Conservation efforts aim to maintain, or even increase, the genetic diversity of species of concern and do so through harnessing the genetic diversity present in captive populations (Ochoa *et al.*, 2016; Ralls & Ballou, 2004; Shan *et al.*, 2014). The use of pedigree analysis and molecular genetics has become an increasing important tool for confirming parentage and identifying genetic variation within captive populations (Constable *et al.*, 2001; Meier, Hemelruk & Martin, 2000; Norman, Putnam & Ivy 2019), such as those used in the flagship success programs with the Arabian oryx (*Oryx leucoryx*) and California condor (*Gymnogyps californianus*) (Ochoa *et al*., 2016; Stanton *et al*., 2015; Conrad, 2018; Ralls & Ballou, 2004).

For example, *ex situ* breeding increased the global population size of Arabian oryx, as well as an increase in the genetic diversity among captive individuals (Norman *et al*., 2019; Ochoa *et al*., 2016). After extirpation from the Arabian Peninsula in 1972, the remaining 11 individuals were placed in a captive breeding program at the Phoenix Zoo (Ochoa *et al*., 2016). Over time, additional captive breeding populations were established at the San Diego Wild Park and in several locations in the Middle East, and in 1982, individuals were reintroduced into the wild. By 2016, approximately 1,220 individuals lived in wild populations and an additional 6,500 were protected in captive populations (IUCN SSC Antelope, 2017).

As species become threatened, endangered, or extinct in the wild, zoos can help by breeding captive individuals to conserve the genetic integrity and diversity of species of concern, thereby facilitating their longevity. They achieve this goal through programs called Species Survival Plans (SSP). A separate SSP is developed for each species, and utilizes studbooks, a pedigree registry of each individual of a certain species, to manage that species across accredited institutions. The two prominent accreditation organizations are the Association of Zoos and Aquariums (AZA) in the United States and the European Association of Zoos and Aquariums (EAZA) for the European Union. Studbooks provide demographic information on captive individuals, such as year and location of birth, any locational changes (i.e., transfers from one zoo to another), and parentage if known or surmised (Princée, 2016; Willis, 2001).

Chimpanzees (*Pan troglodytes*), an endangered species relatively common in zoos, are an ideal study species to use for this research with their decline in the wild stemming from habitat loss and degradation, poaching, and climate change (Mccarthy, Lester & Stanford, 2017). Captive chimpanzees within the United States are managed through individual zoological institutions, as well as through the Chimpanzee Species Survival Plan (SSP) Committee. The SSP program for chimpanzees residing in the United States was created by the AZA to manage this captive metapopulation, and to develop management policies and practices for captive chimpanzees residing in AZA-accredited institutions across North America ("Species Survival Plan Programs," 2018). The goals of the Chimpanzee SSP Committee specifically focus on research, education, advocacy, conservation, and husbandry (“Chimpanzee SSP,” n.d.).

Most zoos must manage small populations with low genetic diversity and, accordingly, low prospects for long-term viability (Balmford, Mace, Leader-Williams, 1996; Norman *et al*., 2019). According to Norman *et al.* (2019), this situation is leading to “sustainability crises” for many species held in zoos and aquariums, in which captive species and populations are not thriving due to current management strategies. To help resolve this issue, zoo professionals could use information gleaned from pedigree and molecular analysis on the demographic and genetic composition of their captive populations (Ralls & Ballou, 2004; Norman *et al.*, 2019). Pedigree analysis of studbooks can be useful to identify missing information (e.g., unknown parentage), demographic framework (e.g., age structure, survivorship, breeding success), and group-living species management (Jiménez-Mena *et al.*, 2016; Farquharson *et al.*, 2017). Norman et al. (2019)

provides an excellent overview of case studies describing the benefits of using molecular technique in endangered species management.

Inclusion of molecular genetics benefits conservation and management efforts in several ways, e.g., identifying cryptic subspecies (Schmidt *et al.*, 2015) and assessing hybridization (Putnam *et al.*, 2019). Lack of genetic information may lead to unintended mismanagement of a species within which taxonomic delineations are unknown, such as hybridization or the loss of subspecies, while erroneous pedigree records with inaccurate kinship estimates could lead to inbreeding and loss of genetic diversity over time (Norman *et al*., 2019). A systematic literature review (Jensen *et al.*, 2020) of almost 8,000 papers recently revealed that, contrary to popular thought, molecular genetic resources do exist for many species found in zoos, thus, molecular analyses are possible with captive populations – and that the incorporation of molecular genetic information will be critical for *ex situ* population sustainability.

Currently, AZA-accredited zoos do not manage captive chimpanzee populations by subspecies, and most use only studbook data or paternity analysis to determine breeding recommendations (S. Ross, personal communication, May 28, 2019). While these methods provide useful background information on a population, they may lack some pedigree information and provide no explanation of genetic structure (Constable *et al*., 2001; Meier *et al.*, 2000; Norman *et al.*, 2019). This study was designed to compare pedigree- and molecular-based analysis of the captive chimpanzee population at John Ball Zoo in Grand Rapids, Michigan, USA. Initially, we used only studbook data to conduct a pedigree analysis, then we analyzed individual genotypes across eight microsatellite markers. Finally, we compared parentage and relatedness estimates calculated by both analysis methods. By doing so, we aimed to provide baseline genetic information on this population (e.g., heterozygosity and mean kinship), and propose management strategies. Given the abundance of chimpanzees in AZA-accredited institutions, the North American metapopulation of chimpanzees is critical to the long-term sustainability of this species. Determining the genetic relatedness and diversity among individuals in the population studied here is the first step in providing feedback on how current zoo management may affect conservation goals of maintaining long-term, sustainable populations. Moreover, we aim to provide a framework for future research within this and other Midwestern captive chimpanzee populations.

2 | MATERIALS AND METHODS

2.1 | Study Species

Chimpanzees (*Pan troglodytes*) have traditionally been categorized into three subspecies: Western Chimpanzees (*P. t. verus*) from west Africa, Central Chimpanzees (*P.t. troglodytes*) from central Africa, and Eastern Chimpanzees (*P. t. schweinfurthii*) from east Africa. However, in the late twentieth century a fourth subspecies was identified. First named *P. t. vellerosus,* the delineation of Nigeria-Cameroon chimpanzees from north-west Africa are now known as *P. t. ellioti* (Gonder *et al*., 1997, Gagneux *et al*., 2001, Gonder *et al*., 2011). Gonder *et al.* (2011) determined that there are three major populations of wild chimpanzees: one along Upper Guinea of West Africa (*P. t. verus*), a second along the Gulf of Guinea region (*P. t. ellioti*), and a third along equatorial Africa (*P. t. troglodytes* and *P. t. schweinfurthii*). Microsatellite genotypes revealed that the Upper Guinea population was most differentiated from the others, but the Gulf of Guinea metapopulation also differs distinctly from the other populations. Additionally, cluster analysis revealed that a subset of individuals have likely experienced hybridization (Gonder *et al*., 2011).

Chimpanzees have a unique life history and behavioral ecology. As great apes, they exhibit quadrupedal knuckle-walking on the ground, but are mostly arboreal and spend most of their time moving through trees foraging for food and nesting above ground at night. Due to their arboreal lifestyle, chimpanzees are highly frugivorous, with a majority of their diet consisting of fruit (60-80%). Chimpanzees also consume a variety of nuts, insects, plant material, and meat (Campbell, 2007; Standford, 2018).

The social structure of chimpanzees can be described as a patriarchal hierarchy, meaning that males are ranked higher than females and there exists an alpha male at the head of the group. Chimpanzee populations are known as "troops" and can vary in size from a few to over 100 individuals depending upon geographic location and other ecological factors (e.g., food availability and number of estrus females) (Ross & McNary, 2009; Fulk & Garland, 1992; Yerkes, 1939). A major identifier of their ecology is their fission-fusion social structure where individuals form smaller subgroups ("parties") that change size and composition throughout the day. For example, while all members of a community come together at night to nest, individual parties during the day may consist of mothers and infants, adults searching for or eating food, and males hunting or patrolling territory boarders (Campbell, 2007; Stanford, 2018).

Troop community structure is composed of multiple-male, multiple-female, multi-generational communities where males are philopatric and females disperse. Infants (birth to about 2.5 years old) stay close to their mothers and rarely venture far from their side, while juveniles (aged 3 to 8) interact with other youngsters and begin to find their place in the troop. Sexual maturation typically occurs between 7-12 years old (Yerkes, 1939). Interestingly, female chimpanzees may experience menopause and enter a post-reproductive period between the ages of 30-40 years old, yet some studies have recorded both wild and captive females reproducing well into their 40s and 50s without experiencing menopause before the end of their lifetime (Herndon *et al*., 2012; Walker & Herndon, 2008).

Chimpanzee populations have declined throughout the 20th and 21st centuries, leading to their global endangered (IUCN) status, harming overall genetic diversity in the species (Ghobrial *et al.,* 2010; Sesink Clee *et al*., 2015; Humle *et al.,* 2016; Mccarthy *et al*., 2017). Though behavioral studies have focused on wild chimpanzee populations since the early 1960s (Goodall, 1986), the genetic diversity and structure of wild populations was not a major research focus until the 1990s. Traditionally, genetic-based research on wild populations has focused on identification of parentage, gene flow, and subspecies (Takenaka *et al.*, 1993; Morin *et al.* 1994; Gonder *et al.*, 1997; Meier *et al.*, 2000; Constable *et al.*, 2001). These same research topics have been less prevalent in captive populations of chimpanzees and wildlife sanctuaries, however (Ely *et al.,* 2005; Ghobrial *et al.*, 2010; Hvilsom *et al.*, 2013). This discrepancy in research foci between wild and captive populations is often due to lack of access to resources such as funding, laboratory space, and properly trained personnel (Norman *et al.*, 2019). Furthermore, there often exists a "conservation genetics gap" in applying information revealed by research to specific management strategies (Britt *et al.*, 2018). This study aims to bridge the gap by investigating the genetic composition of a captive chimpanzee populations and providing recommendations for managing a sustainable population.

2.2 | Sample Collection & DNA Extraction

In order to analyze a captive population's genetic structure, as many individuals as possible ought to be included. Further, obtaining and including information about former and current resident is essential to population structure analysis, as current captive population sizes are typically small (usually less than 15 individuals). We obtained blood samples from seven captive chimpanzees of known origin from John Ball Zoo (JBZ) in Grand Rapids, Michigan, USA, which included all individuals currently living in that facility. We analyzed these seven samples for genetic diversity and relatedness.

Blood and tissue samples were collected during routine veterinary checks by zoo personnel and stored at -18°C at GVSU, Allendale, Michigan. Genomic DNA was extracted from whole blood using DNeasy Blood & Tissue Kits (Qiagen, Valencia, USA) following manufacturers recommendations. Prior to PCR amplification, DNA quality and quantity was analyzed using a NanoDrop One/One^C Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, USA) and gel electrophoresis. A subsample is 1µL of elute from the DNA extraction of each blood sample was analyzed on the NanoDrop following manufacturer's protocol. Similarly, 1-2µL of elute from the DNA extraction of each blood sample was used for gel electrophoresis.

2.3 | PCR & Fragment Analysis

A total of eight microsatellite loci were selected from Hvilsom *et al.* (2013) for amplification and analysis based on informativeness (Rosenberg *et al.*, 2003), number of alleles, and allele size range (to prevent overlapping of markers). These loci were: GATA104, GATA129H04, GATA176C01, GATA71H05, GATA43A04, GATA116B01N, GATA50G06, and UT7544. Forward primers with 5' modifications were labeled using 6FAM (Integrated DNA Technologies), NED, PET, or VIC fluorescent dye (ThermoFisher). PCR was carried out using a 25µL reaction volume containing 2µL genomic DNA, 0.5µL of 10µM forward primer, 0.5µL of 10µM reverse primer, 12.5µL One*Taq* 2X Master Mix with Standard Buffer, and 9.5µL nuclease-free water according to manufacturer recommendation (New England BioLabs). To check for contamination, we included a PCR negative control during electrophoresis. We used an

Eppendorf™ Mastercycler™ Nexus Thermal Cycler (Fisher Scientific) for PCR amplification using the follow conditions: amplification at 94°C for 3 min, annealing at 34 cycles of 95°C for 30 sec, 54°C for 30s, and 72°C for 30s, followed by 60°C for 45 min with a final hold at 4°C (based on Hvilsom *et al.*, 2013 protocol). Then, 2-5µL of each PCR product were run on 2% agarose gels, and electrophoresis results were visualized using a UVP MultiDoc-It™ Gel Imaging System UV transilluminator (Analytik Jena, Germany).

The resulting PCR product was processed by the Robert B. Annis Water Research Institute (AWRI) in Muskegon, MI, where they added a LIZ500 size standard to samples before conducting a fragment analysis using an ABI3500 Genetic Analyzer (Applied Biosystems). Fragment size was scored using Peak Scanner (Thermo Fisher Connect™, ThermoFisher Scientific), and genotypes for each individual recorded (Table 1).

2.4 | Studbook Records

We obtained the North American Regional Chimpanzee Studbook (*Pan troglodytes*) from the chimpanzee studbook coordinator (Ross, 2020) at the Lincoln Park Zoo. Zoo personnel collect studbook data using the management software PopLink v. 2.4 (Faust, 2019), which records the births, deaths, parentage, offspring, and transfer/location information of past and current residents (*see Appendix II for studbook reference*). Individuals from each population are identified in the studbook by a Studbook ID (e.g., 22), Local ID (e.g., 303500), and a House Name (e.g., Lucy). We refer to individuals using studbook Local ID and location in the current study. The John Ball Zoo population is included under AZA accreditation, which confirms their status as a Chimpanzee Species Survival Plan (SSP) population. We analyzed the studbook data (current as of 17 January, 2020) of the chimpanzee population at John Ball Zoo (JBZ) in Grand

Rapids, Michigan, USA. The seven individuals used in this study included all six living individuals (n = 6) plus an additional individual that recently died (in 2018, n = 1).

2.5 | Pedigree Analysis

Pedigree analysis software PMx v. 1.6.2 (Ballou *et al.,* 2020) was used to estimate population demographics (i.e., life table summary, mortality, survival, age distributions, and birth and death seasonality) and genetics (i.e., gene diversity, population mean kinship, inbreeding, effective population size, and kinship matrix). This software was created to determine the demographic and genetic status of pedigreed populations originating from pedigree data collection software (e.g., SPARKS developed by the International Species Information System (ISIS) or PopLink). A total of seven individuals (as described above) from JBZ were included in this analysis.

When calculating probabilities, such as individual survival, PMx follows a set of genetic assumptions detailed by Ralls and Ballou (2004) where an autosomal or Mendelian mode of inheritance is assumed during “gene drop” iterations (1000 iterations) to assign probabilities to population founders (Ballou *et al*., 2020; Ralls & Ballou, 2004). While most genetic parameters use kinship estimates, those that cannot are calculated using a simulation of allele transmission (Lacy, 1995; Ballou *et al*., in press). As a note, the initial analysis only included information obtained from the North American Regional Chimpanzee Studbook, meaning that information of parentage was recorded from visual and behavioral observations. Consequently, the “genetic data” estimated using PMx may not reflect the genetic information revealed from molecular analyses such as microsatellite, mtDNA, or genome sampling (Galla *et al*., 2020; Wang, 2017). We conducted a second analysis of population relatedness using the molecular data collected from microsatellite analysis to discern the accuracy of pedigrees generated from studbook data.

We also analyzed the relatedness (*r*), a kinship coefficient, among individuals within the small JBZ population. This process enabled us to determine kinship of individuals based on their r values. For instance, PMx software uses the assumption that an individual's kinship to itself is 0.5 (Lacy, 2012), while an *r* of 0 means that the two individuals have unrelated parents. If *r* is 0.125, then the individuals are most likely half-siblings, whereas *r* of 0.25 means that the two individuals are most likely full siblings, mother-son, or father-daughter. Lastly, individuals with *r* of 0.0625 means the relationship is first-cousins (Sun, 2017; Lacy, 2012; Jiménez-Mena *et al.,* 2016).

Regardless of method, identifying mean kinship (*mk*) of individuals within a population is an important indicator of gene diversity (expected heterozygosity). According to Putnam and Ivy (2014), if kinship (*f*) is the probability that two randomly sampled alleles are homozygous by descent, then *mk* is "the average pairwise kinship coefficients (*f*) between that individual and all living individuals in the population, including itself," (p. 303). Populations with lower mean kinship are less-closely related and may have more individuals of genetic value (i.e., individuals with high heterozygosity, low inbreeding, and founder genome representation). Conversely, populations with high mean kinship consist of individuals that are more closely related to one another (Putnam & Ivy, 2014; Frankham *et al.,* 2017).

2.6 | Microsatellite Analysis

We used GenAlEx v. 6.5 (Peakall & Smouse, 2006 & 2012) to calculate number of alleles (N_a), allele frequencies, observed (H_o), and expected (H_e) heterozygosity for each locus. We also estimated pairwise relatedness using three different estimators: RI = Ritland (1996) estimator, LRM = the Lynch and Ritland (1999) estimator, and QGM: Queller and Goodnight (1989) mean estimator. Values of each estimator can range between -1 (less than average *r*) and

1 (r of an individual to itself or between clones). Negative *r* values mean that the pair of individuals being compared are less related to one another than the average relatedness of the population, while positive values denote greater than average relatedness (Wang, 2014). Additionally, we inferred parental and siblingship relationships using COLONY 2.0.6.5 (Wang, 2018), a software designed to assign parentage and estimate relationships using microsatellite data. We chose the following parameters: female and male polygamy, diploid species, full-likelihood (FL) analysis, codominant for marker type, and set genotyping error rates to zero. The COLONY software offers four likelihood methods; we selected FL analysis because as a full-pedigree likelihood methods it is the most accurate (Wang, 2012). All calculations were conducted using a frequency-based approach, which uses empirical allele frequency data (Table 1).

3 | RESULTS

3.1 | Molecular Data

We successfully performed fragment analysis for seven chimpanzee individuals from the John Ball Zoo in Grand Rapids, Michigan, and calculated relatedness coefficients (*r* and *mk*) across eight microsatellite loci (Table 1). Of the eight loci sampled, GATA104 had the lowest heterozygosity (H_o = 0.167, H_e = 0.153), while the H_e values of the other seven loci ranged from 0.750-0.860 (Table 2). All microsatellite markers were within Hardy-Weinberg equilibrium (Table 3), and allele frequencies varied for each locus based on number of alleles: GATA176C01 had the greatest number of alleles (No. alleles = 8) and GATA104 had the least (No. alleles = 2). Further, GATA104 had the largest difference between allele frequencies (Allele 184 frequency = 0.917 and Allele 188 frequency = 0.083) and was the least polymorphic (Table 4). Alleles that failed to amplify (recorded as N/A) were present in at least one individual for all microsatellite markers except GATA129H04 and GATA50G06 (Table 1).

3.2 | Mean Kinship & Pairwise Estimates

Pedigree-based analysis using PMx characterized the John Ball Zoo chimpanzee population by the following statistics: No. of founders = 7, GD = 0.82, *mk* = 0.1837, and % Pedigree Known = 65% (Table 5). While five of the seven chimpanzees are estimated to be unrelated (*mk* = 0), individuals 302526 (Sanga) and 302527 (Kiambi) had an *mk* of 0.25 (Table 6). Our genetic-based analysis, using GenAlEx to calculate relatedness, calculated 21 pairwise comparisons and provided a mean *r* estimate averaging between -0.180 and -0.259 (Table 7-8). Most pairwise estimates between individuals were negative except between 302527 and 302526 (RI = 0.037, LRM = 0.95, QGM = 0.259), and 302526 and 303501 (RI = 0.027, LRM = 0.074, QGM = 0.119). Individuals 303500 and 303501 had a smaller degree of relatedness (RI = 0.005, LRM = 0.015, QGM = -0.075).

After estimating pairwise relatedness values, COLONY analyzed microsatellite data to estimate parentage for each of the seven individuals sampled. All seven individuals were assigned a father outside of the seven individuals sampled, and six individuals were assigned a mother outside of the population sampled. One individual (302527) was assigned a mother (302526) within the population (Table 9, Figure 1). Further, based on shared alleles, individuals were placed into five clusters: (#1) 302525 with probability of 0.6285; (#2) 302527, 302526, and 303501with probability of 0.1376; (#3) 303500 with probability of 0.5559, (#4) 302522 with probability 0.3351, and (#5) 302524 with probability of 0.8647 (Figure 2).

4 | DISCUSSION

The objectives of this study were to compare the relatedness and genetic diversity of a small population of captive chimpanzees with the intention of generating recommendations for zoo personnel. After conducting a pedigree- and molecular-based analysis on the John Ball Zoo chimpanzee population, we determined that there were major differences between the two types of methods. Our research focused on sampling seven individuals across eight microsatellite loci, however not all markers were amplified in all individuals (Table 1). PMx analysis confirmed the parent-offspring relationship of 302526 and 302527 recorded in the chimpanzee studbook, and revealed a lack of relatedness among the other individuals (Table 6). This confirmed the purported relationships among the chimpanzees at JBZ as found in the studbook: all founding individuals of the JBZ population are unrelated, a subset of founders from the Detroit Zoo population that were subsequently transferred to JBZ to start their troop (Ross, 2020).

The parentage and relatedness estimates assigned by our genetic-based analysis provided similar results. The COLONY analysis assigned the same parent-offspring relationship to individuals 302526 and 302527, as that found in our studbook analysis, and it placed the two individuals into the same cluster (Table 9, Figure 1-2). A noticeable different between PMx and COLONY analysis is that the latter placed individual 303501 into the same cluster of 302526 and 302527 (Cluster #2). It also assigned the same unknown mother (#2) to 302526 and 302501, a half-sibling relationship that is not revealed from PMx analysis (Tables 1 and 9). The COLONY software assumes that all individuals within a population (genotypes loaded into the program) must be assigned parentage and sibship, and it does so on the basis of identical by descent (IBD). This means that when two individuals have matching (identical) alleles, the assumption is made that the two individuals are related solely because they share alleles. This assumption can cause relatedness bias in small population sizes with fewer markers, which might explain why COLONY interpreted our molecular data as it did. Both analysis methods determined 302526 (mother) and 302527 (offspring) to be related, however only microsatellite analysis placed 303501 into the same cluster and assigned a half-sibling relationship between 302526 and 303501. Their cluster (#2) had the lowest probability (0.1376) when compared to the four other clusters, composed of one individual each (Figure 2).

Our study identified a difference in parentage and siblingship assignment when using studbook-only analysis versus a microsatellite-based approach. As described previously, the use of genetic methods to understand the genetic diversity within and between populations is a robust tool that should be included in wildlife conservation and captive animal management. Maintaining self-sustaining and genetically robust captive populations is no simple task, however (Ivy & Lacy, 2012). Pedigree analyses are beneficial for providing historical data on individuals (i.e., parentage, birth place, subspecies, etc.), but they also provide important estimates of genetic value, relatedness, and kindship for a particular population. These estimates become useful baseline information when incorporating molecular-based approaches (i.e., microsatellite loci, SNP markers, whole-genome sequencing) into management (Norman *et al.*, 2019). By creating a DNA profile for each individual, molecular data can complement pedigree analyses to help determine the genetic diversity and structure of captive populations, and assist in the developing sustainable management practices (Norman *et al.*, 2019).

Studies often overlook and do not address the fact that studbook pedigrees may be missing information or contain uncertainty (Ochoa *et al.*, 2016). This concept was deemed the pedigree “black hole” in the early 21st century. The pedigree black hole characterizes the gap in knowledge managers have when pedigree information from studbooks does not describe relatedness among individual accurately (Willis, 2001). Molecular genetic techniques help SSP Programs support conservation efforts by revealing gaps or errors in pedigree records (studbooks) that could negatively affect management decisions and lead to inbreeding, hybridization, or unsuccessful breeding pairs (Willis, 2001; Schmidt *et al*., 2015).

A study comparing multiple methods of estimating relatedness (pedigree, genetic, and genomic) in endangered bird species found that using a SNP-based approach was more precise than using microsatellites (Galla *et al.,* 2020). While whole-genome sequencing may not be feasible due to genome size or budget restrictions, we strongly suggest using representative genomic sampling methods for a more robust sample size and precise estimates (Narum *et al.,* 2013; Dodds *et al.,* 2015). This study demonstrates the importance of combining pedigree records with molecular approaches and how doing so can lead to better-informed decision-making and management.

While one avenue of future research is to increase the number of microsatellites used or switch to genome-based methods, a second suggestion we put forth is to focus on a multi-institution approach. Currently, captive chimpanzee populations are managed by individual institution, with the exception of individuals targeted for potential transfer from one zoo to another. Unlike their wild counterparts, captive populations may not always reflect a multi-generational or multi-male, multi-female population structure because individuals may be contracepted, sterilized, or transferred. The JBZ population, for example, includes more females than males and features only a single reproductively viable male (the other male is neutered, and thus, sterile) (Ross, 2020). While management protocols of captive chimpanzees state that populations should consist of multi-male, multi-female populations with at least three adult males and five females (in addition to pre-reproductive age individuals), due to a number of constraints, the fluid fission-fusion community structure of wild chimpanzees is not replicated in captive populations. The structure of captive populations change slowly, if at all, and very few facilities are able to support such behavioral fluidity of subgroup composition (Ross & McNary, 2009). This limits the number of individuals within a population, reducing the abundance and genetic diversity of potential mates, and the potential for behavioral fission-fusion structure of subgroups.

Thus, we suggest the importance of managing endangered species management across institutions, rather than isolating each captive subpopulation and managing them individualistically and in isolation of subpopulations. While rarely achieved in practice, such a broad-reaching, cooperative management philosophy is a founding tenant of the SSP Program, which focuses on managing “genetically diverse, demographically varied, and biologically sound population[s]” (Associations of Zoos and Aquariums, 2021) across AZA-accredited facilities, spanning across the United States and overseas. In order for zoological institutions and SSP committees to manage breeding programs and other conservation efforts, they need to take a step beyond following the Ark paradigm (retaining 90% of wild founder genes over time, proposed by Soule *et al.* 1986) and focus on population-level analysis to ensure long term, sustainable populations successfully (Lees & Wilcken, 2009; Traylor-Holzer *et al*., 2019; Caspers *et al.*, 2019; Putnam *et al.*, 2019).

5| SUGGESTIONS FOR MANAGEMENT

- In order to maximize genetic diversity, managers should select individuals with the greatest genetic value or those individuals that are less-closely related because of potentially rare deleterious alleles (Ballou & Lacy, 1995; Frankham *et al.*, 2017).

- For metapopulation management (i.e., management of multiple captive populations together), we suggest using an additional 5-10 microsatellite markers, as small sample sizes can exacerbate relatedness bias and be insufficient for assigning relatedness.
- We suggest, however, using representative sampling methods (e.g., SNP) or whole-genome sequencing to bypass the limitations of using microsatellite markers for smaller captive populations.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to note and testify that their study was performed in accordance with the GVSU Institutional Animal Care and Use Committee and Biosafety Committee regulations.

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FIGURE CAPTIONS

Figure 1. Best (ML) Configuration of seven chimpanzees at the John Ball Zoo in Grand Rapids, Michigan, USA as estimated by COLONY and drawn by Pedigree Viewer (Kinghorn & Kinghorn, 2015). Fathers are denoted as orange lines, while mothers are denoted using purple lines. Unknown parents (father and mother) are assigned a symbol (* or #) and an integer. For example, unknown father of 303500 is an orange line labeled *4.

Figure 2. Best Cluster Configuration of seven chimpanzees at the John Ball Zoo in Grand Rapids, Michigan, USA as estimated by COLONY and drawn by Pedigree Viewer (Kinghorn & Kinghorn, 2015). Individuals are placed into clusters (1-5) based on maternal (purple) and paternal (orange) assignments. Probability values for each cluster are shown on a scale from 0 to 1.

TABLES

Table 1. Microsatellite genotypes of seven chimpanzees from John Ball Zoo in Grand Rapids, Michigan, USA across eight microsatellite loci.

Sample	GATA104		GATA129H04		GATA176C01		GATA71H05		GATA43A04		GATA116B01N		GATA50G06		UTT544	
302525	184	188	225	229	212	227	262	266	131	136	N/A	N/A	207	215	151	157
302527	184	184	221	233	200	212	N/A	N/A	131	148	162	185	211	215	151	169
302526	184	184	217	221	200	230	262	266	131	140	N/A	N/A	211	211	N/A	N/A
303500	184	184	229	229	216	223	270	278	N/A	N/A	146	162	207	215	155	161
302522	N/A	N/A	221	229	N/A	N/A	274	293	131	140	181	185	203	215	151	161
303501	184	184	217	225	216	223	N/A	N/A	136	140	N/A	N/A	203	211	N/A	N/A
302524	184	184	210	221	220	239	270	274	131	143	162	166	223	251	N/A	N/A

In Table 1 above, amplified alleles were 100-300bp in size, while N/A represents alleles where no amplification occurred.

Table 2. Number of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_e) statistics calculated from microsatellite loci (GenAlEx).

Locus	No. Alleles	Ho	He
GATA104	2	0.167	0.153
GATA129H04	6	0.857	0.786
GATA176C01	8	1.000	0.861
GATA71H05	6	1.000	0.820
GATA43A04	5	1.000	0.722
GATA116B01N	5	1.000	0.750
GATA50G06	6	0.857	0.786
UTT544	5	1.000	0.750

Table 3. Summary of Chi-Square test for Hardy-Weinberg Equilibrium across eight loci.

Locus	DF	ChiSq	Prob
GATA104	1	0.050	0.824
GATA129H04	15	9.625	0.843
GATA176C01	28	33.000	0.236
GATA71H05	15	17.500	0.290
GATA43A04	10	5.200	0.877
GATA116B01N	10	6.667	0.756
GATA50G06	15	20.125	0.167
UTT544	10	6.667	0.756

KEY: Locus = microsatellite marker, DF = degrees of freedom, ChiSq = chi-square statistic, and Prob = probability (p-value). Probabilities < 0.05 are statistically significant; no probability in Table 3 was significant.

Table 4. Allele frequency of eight microsatellite markers sampled in seven chimpanzees housed at John Ball Zoo from 2018 to 2020.

Locus	Allele	Frequency
GATA104	184	0.917
	188	0.083
GATA129H04	210	0.071
	217	0.143
	221	0.286
	225	0.143
	229	0.286
	233	0.071
GATA176C01	200	0.167
	212	0.167
	216	0.167
	220	0.083
	223	0.167
	227	0.083
	230	0.083
	239	0.083
GATA71H05	262	0.200
	266	0.200
	270	0.200
	274	0.200
	278	0.100
	293	0.100
GATA43A04	131	0.417
	136	0.167
	140	0.250
	143	0.083
	148	0.083
GATA116B01N	146	0.125
	162	0.375
	166	0.125
	181	0.125
	185	0.250
GATA50G06	203	0.143
	207	0.143
	211	0.286
	215	0.286
	223	0.071
	251	0.071
UTT544	151	0.375
	155	0.125
	157	0.125
	161	0.250
	169	0.125

Table 5. Pedigree-based genetic characteristics of the John Ball Zoo chimpanzee population from 2018 to 2020 calculated using studbook-only data (PMx).

Genetic parameters	Statistic
No. of Founders	7
Gene Diversity	0.82
Mean Kinship	0.18
% Pedigree Known	65

Gene diversity ranges from low (0.0) to high (1.0) variation. Mean kinship values are calculated by taking the average of kinship values for each individual in the population/total number of individuals. The *mk* shown in Table 5 reflects population mean kinship. The % Pedigree Known is the percent of the population with known parentage (e.g., individuals with WILD parentage have unknown parents).

Table 6. Kinship matrix of seven chimpanzees housed at John Ball Zoo from 2018-2020 using PMx software.

Sample	302525	302522	303500	302526	303501	302524	302527
302525	0.5	0	0	0	0	0	0
302522	0	0.5	0	0	0	0	0
303500	0	0	0.5	0	0	0	0
302526	0	0	0	0.5	0	0	0.25
303501*	0	0	0	0	0.5	0	0
302524	0	0	0	0	0	0.5	0
302527	0	0	0	0.25	0	0	0.5

Pairwise kinship values should be interpreted as the following: If r = 0, individuals have non-related parents. An r = 0.25 denotes full-siblings or parent-offspring, while r = 0.125 is half-sibling. The underlying calculation assumes that r = 0.5 for self. *Individual 303501 is deceased (2018), however we included them in this analysis because we received a blood sample for them.

Table 7. Pairwise relatedness estimates for seven chimpanzees at John Ball Zoo using three different estimators (GenAlEx).

Sample 1	Sample 2	RI	LRM	QGM
302525	302527	-0.070	-0.163	-0.082
302525	302526	-0.087	-0.221	-0.212
302527	302526	0.037	0.095	0.259
302525	303500	-0.040	-0.094	-0.002
302527	303500	-0.130	-0.367	-0.425
302526	303500	-0.170	-0.430	-0.489
302525	302522	-0.086	-0.287	-0.187
302527	302522	-0.045	-0.141	-0.047
302526	302522	-0.080	-0.256	-0.211
303500	302522	-0.060	-0.153	-0.078
302525	303501	-0.055	-0.138	-0.241
302527	303501	-0.138	-0.369	-0.472
302526	303501	0.027	0.074	0.119
303500	303501	0.005	0.015	-0.075
302522	303501	-0.018	-0.064	-0.285
302525	302524	-0.180	-0.340	-0.429
302527	302524	-0.106	-0.220	-0.195
302526	302524	-0.127	-0.267	-0.261
303500	302524	-0.111	-0.226	-0.229
302522	302524	-0.099	-0.222	-0.223
303501	302524	-0.178	-0.368	-0.495

Pairwise relatedness estimators: RI: Ritland (1996) estimator, LRM: Lynch & Ritland (1999) mean estimator, and QGM: Queller and Goodnight (1989) mean estimator. GenAlEx calculated a total of 21 pairwise comparisons.

Table 8. Summary of average pairwise relatedness estimates for seven chimpanzees at John Ball Zoo using three different estimators (GenAlEx).

Parameters	RI	LRM	QGM
No. of Pairs (N)	21	21	21
Sum	-1.710	-4.142	-4.258
Mean	-0.081	-0.197	-0.203
SD	0.062	0.145	0.198
SE	0.014	0.032	0.0403
Range	-0.180 - 0.037	-0.430 - 0.095	-0.495 – 0.259

RI: Ritland (1996) estimator, LRM: Lynch & Ritland (1999) mean estimator, and QGM: Queller and Goodnight (1989) mean estimator.

Table 9. Best ML Configuration of seven chimpanzees housed at John Ball Zoo from 2018 to 2020 using COLONY.

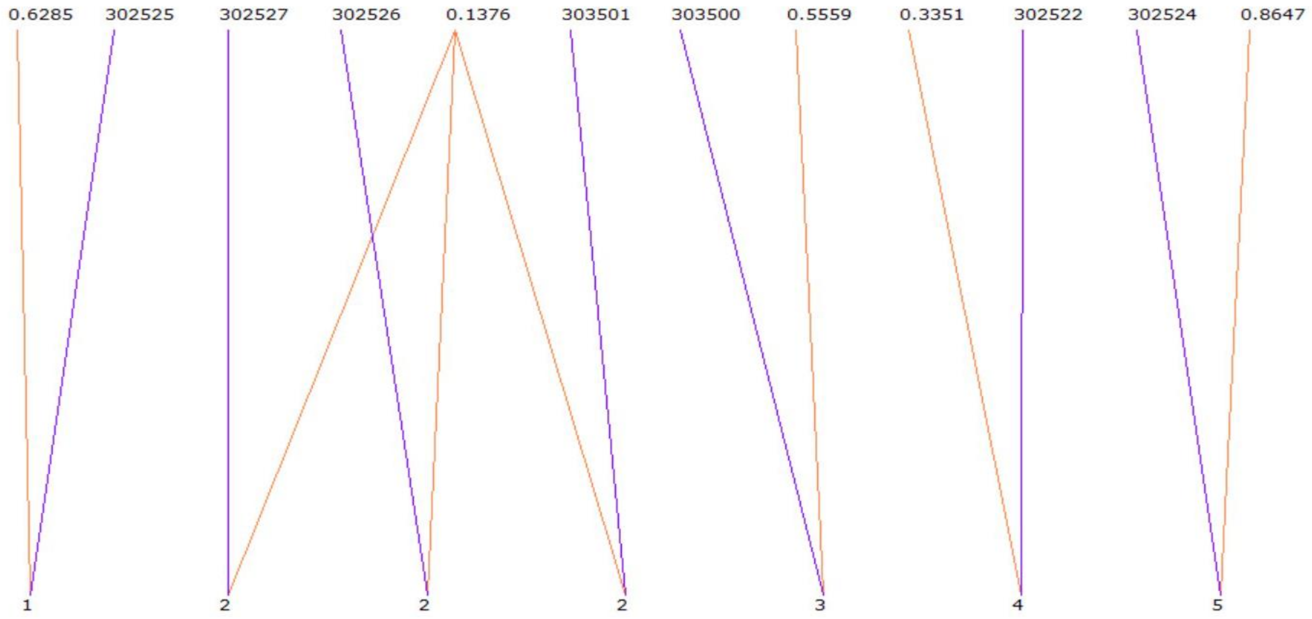
Sample	Father ID	Mother ID	Cluster Index
302525	*1	#1	1
302527	*2	302526	2
302526	*3	#2	2
303500	*4	#3	3
302522	*5	#4	4
303501	*6	#2	2
302524	*7	#5	5

COLONY defines a father outside of the population by using * followed by a number. For each new individual assigned as the father, there is a new number. This software uses a similar identification for a mother outside of the population being sampled with # instead of an asterisk, followed by a number. For example, 302525 was not paired with a father or mother in the sample population, so they were given Father ID of *1 and Mother ID of #1. The cluster index places each individual into one of four groups.

FIGURES

Figure 1.

Figure 2.



0.6285
302525
302527
302526
0.1376
303501
303500
0.5559
0.3351
302522
302524
0.8647
1
2
2
2
3
4
5

Chapter 3

Extended Review of Literature

Purpose

The purpose of this review was to address the gap in literature and lack of molecular data incorporation into *ex situ* management of endangered species within zoos accredited under the Association of Zoos and Aquariums (AZA). Specifically, I aimed to provide relevant literature highlighting the importance and availability of molecular data (e.g., measures of relatedness and kinship, genetic/genomic surveys, etc.). Studies included in this review focus on accredited zoological institutions through the Association of Zoos and Aquariums (AZA) in the United States, European Association of Zoos and Aquariums (EAZA) across Europe, and the World Association of Zoos and Aquariums (WAZA).

Background

Captive breeding programs are used by zoological institutions and aquariums to preserve the genetic diversity of captive individuals and often focus on endangered species or species of concern. The role of zoos in conservation efforts has increased from their traditional role of housing exotic animals for visitors to view to the more recent development of zoos as a cornerstone of conservation efforts through captive breeding programs and education (Rabb, 1994; Ito *et al.*, 2017; Ochoa *et al.*, 2016; Witzenberger & Hochkirch, 2011). The major goals of these breeding programs are to preserve genetic diversity, create a reserve of genetic diversity that may act as supplemental to wild populations, and to reintroduce these endangered species back into the wild (Ochoa *et al.*, 2016; Ralls and Ballou, 2004; Shan *et al.*, 2014). Additionally, species selected for captive breeding programs are typically a part of Species Survival Plan

Programs (SSP), which aim to manage captive populations and long-term sustainability across accredited institutions (“Studbooks,” 2018).

Studbook yearbooks are the most common pedigree tool used by zoo personnel to determine relatedness and origins of captive individuals. For instance, studbooks describe the birth/death year, parentage, offspring, and location (born, transfer, etc.) of each individual within a zoo population. They often describe the demographics of the zoo as a whole, and also of each individual within the population (Princée, 2016). Founders of captive breeding programs are often determined using studbooks, but a recent trend has been the use of combining studbooks and pedigrees with molecular genetic information.

Genetic analysis enables zoo personnel to determine which individuals are best suited for breeding recommendations, reintroduction as founders into the wild, etc., based on molecular genetic data and population genetic analysis (Lacy, 1989; Ochoa *et al.*, 2016; Pastorini *et al.*, 2015; Çilingir *et al.*, 2017; Ito *et al.*, 2016). The goal of this literature review was to summarize past and current research on the use of pedigrees, studbooks, and molecular genetic analysis in a format that may benefit zoo personnel, captive breeding directors, curators, and other professionals. This report provides a comprehensive understanding of the techniques used in the development of captive breeding programs and other *ex situ* conservation efforts. My objectives for this extended literature review were to (1) detect gaps in literature regarding the incorporation of molecular data in zoo management, (2) provide examples of studbook, pedigree, and molecular analysis of populations, (3) summarize the importance of combining pedigree analysis techniques, and (4) suggest recommendations for future management.

Review of Literature

Literature sources included in this review were obtained from Grand Valley State University Libraries, Google Scholar, and Google and describe research spanning thirty years (1986 - 2020). I used the following criteria when determining studies for inclusion in this review: (1) Literature addressed zoo populations and captive breeding programs for the purpose of conservation or reintroduction and may have included captive or wild individuals; (2) research focused on endangered species and species of concern noted by the International Union for the Conservation of Nature, commonly known as the IUCN, an international organization that determines a species status by examining the range, population size, habitat, ecology, and threats of global biodiversity ("Background & History," 2018). (3) Only literature describing mammals, birds, reptiles, and amphibians were included, and (4) studies used the following techniques to determine pedigree information: studbooks, pedigrees, paternity analysis, and molecular genetic analysis (e.g., mitochondrial, microsatellites, SNPs, WGS, etc.).

Techniques for Determining Relatedness

Pedigree analysis is used to determine relatedness of individuals utilized multiple techniques based on demographic of populations, species type and ecology, their origin, or other known information. Zoos typically use studbooks alone for pedigree analysis, however molecular genetic analysis is becoming more prevalent as it provides specific, descriptive genetic data on individual, population, and geographic-levels. Within the past 30 years, pedigree analysis has evolved from studbook estimates to incorporation of molecular genetic techniques (Table 1).

Table 1. Pedigree Analysis Techniques

Analysis Technique	**Species**	**Population**	**Authors**
Studboooks	California condor	Wild and Ex situ	Ralls & Ballou 2004
	Maned wolf	Ex situ	Vanstreels & Pessutti 2010
	Southern White Rhinoceros	Ex situ	Reid *et al.* 2012
	Bison	Wild and Ex situ	Willis and Willis, 2019
Existing Pedigree	Texas blind cave salamander	Ex situ	Jiménez-Mena *et al.* 2016
Genetic Analysis	Grey wolf	Wild and Ex situ	Hindrikson *et al.* 2017
	Chimpanzee	Wild and Ex situ	Hvilsom *et al.* 2013
	Black-fronted piping guan	Ex situ	Oliveira-Jr. *et al.* 2016
	Ring-tailed lemur	Wild and Ex situ	Pastorini *et al.* 2015
	Giant panda	Ex situ	Shan *et al.* 2014
	Okapi	Wild and Ex situ	Stanton *et al.* 2015
	Black-footed rock-wallaby	Wild, Ex situ, Museum	West *et al.* 2018
	Northern bald ibis	Ex situ and Museum	Wirtz *et al.* 2018
	Koala	Wild	Neaves *et al.* 2016
	Nubian ibex	Ex situ	Putnam *et al. 2019*
	Wildebeest	Wild and Ex situ	Caspers et al., 2019
Pedigree and Genetics	Burmese roofed turtle	Ex situ	Çilingir *et al.* 2017
	Visayan warty pig	Ex situ	Nuijten *et al.* 2016
Studbooks and Genetic Analysis	Zebras	Ex situ	Ito *et al.* 2017
	Swedish wolf	Wild and Ex situ	Jansson *et al.* 2015
	Arabian oryx	Wild and Ex situ	Ochoa *et al.* 2016
	African dwarf crocodile	Ex situ and Museum	Schmidt *et al.* 2015
	Asian lion	Ex situ	Atkinson *et al.* 2018
	Cheer pheasant and western tragopan	Ex situ	Mukesh *et al.* 2016

Studbook yearbooks are a pedigree technique dating back to 1932. The first known studbook record was created for the European bison, *Bison bonasus,* and later evolved into an *International Studbook Yearbook* in 1966, describing a total of eight species (Glatston, 1986). Studbook yearbooks (hereafter called studbooks) describe births, deaths, parentage, any offspring, and transfer/location information (e.g. origin, and if an individual has been transferred from the wild to a specific zoo, or from one zoo to another). They provide baseline information for zoo personnel that can be useful in determining relatedness to other residents, potential mates, or new individuals (Princée, 2016; Ralls & Ballou, 2004).

The use of ***molecular genetic analysis*** has become increasingly popular and feasible within the past 30 or so years (Norman *et al.*, 2019; Jensen *et al.*, 2020**)**. Management of captive populations using molecular genetics provides more accurate information on relatedness, genetic diversity, and parentage (Ito *et al.,* 2017; Pelletier *et al.,* 2009). Molecular genetic techniques help Species Survival Plan Programs (SSP) support conservation efforts by revealing gaps or errors in pedigree records such as studbooks. If pedigree information (e.g. parentage) is unknown, and studbooks are the sole indicator of relatedness, they may contain gaps or erroneous information that could negatively affect management decisions and lead to inbreeding, hybridization, or unsuccessful breeding pairs (Willis, 2001; Schmidt *et al.*, 2015). Molecular data can reveal such errors through creating a DNA profile using either a subset of microsatellites or genome sequencing. These data enable us to determine the genetic diversity, genetic structure, and relatedness of populations (Norman *et al.,* 2019).

Norman, Putnam, and Ivy (2019) provide an excellent overview of case studies describing the benefits of using molecular techniques in endangered species management. For instance, the authors provide case studies of microsatellites used in captive breeding programs for the Whooping Crane (*Grus Americana*) and Tasmanian devil (*Sarcophilius harissii*) in estimating kinship among individuals (Norman *et al.*, 2019). Ancestry of captive and wild chimpanzees (*Pan troglodytes*) were also determined using genetic analysis to identify subspecies diversity. In order to provide specific ancestry of captive chimpanzees from their European Endangered Species Program (EEP), microsatellite loci were chosen and DNA was amplified. Allelic frequencies and genetic diversity provided zoo personnel with population structure information to better inform captive breeding management (Hvilsom *et al.,* 2013). Similar methodology was used to determine the genetic structure of captive and wild okapi (*Okapia johnstoni*), specifically for *ex situ* captive breeding programs and potential *in situ* translocations and reintroduction (Stanton *et al.*, 2014).

Paternity testing can confirm the identity of an individual's father, but this molecular technique has constraints that limit its utility. Typically, paternity testing utilizes DNA extracted from tissue samples, specific segments of which are then copied amplified using polymerase chain reaction (PCR). The amplified DNA segments are analyzed using gel electrophoresis, which separates the different DNA segments into bands on the gel, visible when stained ("markers"). The bands for each individual are then compared to those from potential fathers, and a match made via statistical analysis in specialized software (Karcher, 2015). This technique has been used to confirm pedigrees in studbooks, but individual zoos may lack budgetary resources, facilities and expertise to conduct such testing (Norman *et al.*, 2019). Further, paternity testing cannot reveal the depth and breadth of genetic information available through microsatellite and genome sequencing techniques (Karcher, 2015; Norman *et al.*, 2019).

Published more recently are studies ***incorporating molecular genetics into pedigree analysis***. Captive breeding programs typically utilize studbook records or pedigree analysis through paternity testing. While studbooks provide demographic information on captive populations, genetic analysis tells us the genetic makeup of individuals within a population. Though these methods have been readily used in the past, a review of the literature has revealed the importance of incorporating molecular data for accuracy. Studbooks have been found to contain inaccurate and/or missing information, while genetic data alone does not provide information on when or where individuals were born. Reasons for uncertain or erroneous studbook records can be due to poor historical recordkeeping, uncertainty of parentage in group-living or private collection-originating individuals, and unknown relation to founders (Norman *et al.,* 2019).

Utilizing studbook and molecular data also provides a more complete picture of the individual and population genetic structure for zoo managers, leading to more informed management practices. For example, Schmidt *et al.* (2014) conducted molecular analysis on blood samples from African dwarf crocodiles and determined that individuals in EAZA and AZA populations were not only assignment to two subspecies, but they could be assigned to three subspecies. This prior information was unknown, thus not reflected in studbook records leading to now out-of-date records. Genetic analysis determined the correct species identification of individuals that was then recommended for correction in EAZA studbooks. Furthermore, AZA institutions did not have a studbook for this species and were recommended to create one to better manage this threatened species (Schmidt *et al.*, 2015).

A negative side effect of misidentification of subspecies within a population of group-managed individuals is hybridization (Norman *et al.,* 2019). When unpedigreed individuals are brought into a population, whether from brining in a wild individual, zoo to zoo transfer, or integration of an individual from a private collection, current management techniques may not be sufficient for preventing inbreeding (Putnam *et al.*, 2019; Willis & Willis, 2019). Once Schmidt *et al.* (2015) identified a third subspecies was present in the case of the African dwarf crocodile, they analyzed zoo populations to determine their presence or absence. By doing so, management techniques were enhanced and pedigree records updated (Schmidt *et al.,* 2015).

Similarly, Putnam *et al.* (2019) examined a group of Nubian ibex *(Capra nubiana)* that were imported from a private collection to AZA SSP institutions. Initial examination revealed that as individuals smaller in stature than Nubian ibex already in SSP population, they might be hybrid individuals. While further analysis revealed that these individuals were not hybrids, they were determined to be genetically different than the individuals originally in the SSP population, meaning they could be a source of valuable genetic information for future breeding pairs. However, without the use of molecular analysis, the genetic background of these unpedigreed individuals would still be a mystery (Putnam *et al.*, 2019).

Flagship Success Stories

Keeping accurate records of captive individuals is of vital importance, as many populations of endangered species may be extinct in the wild and many, if not all remaining individuals are found in zoos. There are two flagship species that pioneered successful captive breeding programs for endangered species. Once such program was for the California condor, *Gymnogyps californianus.* In 1987, the California condor became Extinct in the Wild, meaning that all remaining individuals were found in captivity. A total of 27 individuals were transported into captivity and the California Condor Studbook was created. Fourteen individuals were chosen as founders for the captive breeding program in 1992. Throughout the onset of the program, a genetic bottleneck was observed from an increase in the frequency of lethal dwarfism among condors. It was determined that the founding individuals contained 92% of heterozygosity from the ancestral, wild population, and had retained 99.5% of that heterozygosity in 2002 (Ralls & Ballou, 2004). As of 2018, captive individuals have thrived from extensive captive breeding programs in zoos and have been reintroduced into the wild in California, Arizona, and Mexico. This species is now considered Critically Endangered, a lower status according to the IUCN, and have an overall increasing population trend (BirdLife International, 2017; Conrad, 2018).

The Arabian Oryx possess a similar history. In 1972, the Arabian Oryx (*Oryx leucoryx)* became extirpated or locally extinct across the Arabian Peninsula, and 11 individuals were placed in a captive breeding program at the Phoenix Zoo (Ochoa *et al.*, 2016). Over time, additional captive breeding populations were established at the San Diego Wild Park and in several locations in the Middle East. This captive breeding program was extremely successful because a decade later in 1982, individuals were reintroduced back into the wild. By 2016, approximately 1,220 individuals lived in wild populations and an additional 6,500 were protected in captive populations. The Arabian Oryx species was saved from extinction by this cooperative captive breeding and reintroduction program, and current population trends are stable with around 850 mature individuals in the wild (IUCN SSC Antelope Specialist Group, 2017).

Diving into the Literature

Although the flagship success stories were successful, they reveal key limitations of using studbooks as the sole determinant of population structure. As described previously, studies may often fail to address the gap in knowledge managers have when studbook information is missing or contains uncertainty (Ochoa *et al.*, 2016). When working with missing information, a common management technique is to assume a “worst-case scenario,” whereby managers assume “unpedigreed” individuals are fully related to those in the population, thus making sure individuals closely related are not matched for breeding. On the other hand, Willis (2001) found that this often causes overestimations of relatedness and that analytical studbooks should be used that integrate current pedigree information with calculations of population size, founding genomic information, allelic frequencies, gene diversity, and kinship (Willis, 2001). By doing so, managers are more likely to avoid the “genetic cost” of over- or underestimating relatedness of individuals, which may lead to an overall loss of gene diversity within a population (Willis and Willis, 2019).

Currently, most zoos must manage small populations with low genetic variability and, accordingly, low prospects for long-term viability (Balmford, Mace, Leader-Williams, 1996; Norman *et al.*, 2019). According to Norman, Putnam, and Ivy (2019), this situation is leading to "sustainability crises" for many species held in zoos and aquariums, in which captive species and populations are not thriving due to current management strategies. To help resolve this issue, zoo professionals could utilize information gleaned from pedigree analyses on the demographic and genetic composition of their captive populations (Ralls & Ballou, 2004; Norman *et al.*, 2019). Norman et al. (2019) provides an excellent overview of case studies describing the benefits of using molecularly-based technique in endangered species management. For instance, lack of genetic information may lead to mismanagement of a species within which taxonomic delineations are unknown, such as hybridization or the loss of subspecies, while erroneous pedigree records with inaccurate kinship estimates could lead to inbreeding and loss of genetic diversity over time (Norman *et al.*, 2019).

A study published in 2017, found contrasting results between pedigree information and molecular analysis of captive individuals (Ito *et al.,* 2017). Captive populations of three types of zebras were sampled, mtDNA was amplified and sequenced, and the resulting output from 123 individuals determined that the genetic diversity of two types, the Grevy's zebra and the Hartmann's mountain zebra, contradicts what was previously believed about their genetic diversity based on studbooks. Ito *et al.* (2017) was one of the first studies to demonstrate the difference between how studbook pedigrees measure diversity compared to that done by using actual molecular genetic data. Studbooks aim to retain 90% gene diversity over 100 years, so by studbook records the Grevy’s zebra sampled could maintain this gene retention better than the Hartmann’s mountain zebra, yet contradictions arise when looking at molecular data (Ito *et al.,* 2017). According to their analysis, if the Hartmann’s mountain zebra lost 90% of its current mitochondrial genetic diversity, however, it would still display greater genetic variation than the Grevy’s zebra, posing the question of which conclusion to follow (Ito *et al.,* 2017).

Other studies likewise highlight these key limitations (Alroy, 2015; Jansson *et al.* 2015; Schmidt *et al.,* 2015; Jiménez-Mena *et al.*, 2016), which emphasizes the importance of combining molecular data with studbook pedigrees (Fernández *et al.,* 2005). Studies utilizing only genetic analysis most often focused on captive and wild populations of endangered species (Çilingir *et al.* 2017; Hindrikson *et al.,* 2017; Hvilsom *et al.,* 2013; Pastorini *et al., 2015;* Stanton *et al.*, 2015; West *et al.*, 2018), while others included the addition of museum or private collection samples to bolster their sample sizes and establish ancestry (West *et al.*, 2018; Wirtz *et al.,* 2018).

The most important theme visible from the literature, aside from the identification of genetic data of captive populations, is the role of pedigree techniques in reintroductions and future supplementation efforts (Conde *et al.,* 2011). The AZA has more than 400 captive breeding programs across accredited institutions that aim to maintain genetic diversity. In order to proliferate healthy, sustainable populations, reproduction of individuals are often managed in a cycle; for instance, a year. Within this timespan, population demographics are recorded and breeding individuals are chosen based on not only the number of offspring needed, but also on the genetic requirements to maintain genetic diversity (Ivy & Lacy, 2012). Once founding individuals are chosen, some breeding programs aim for reintroduction success, while others aim to retain genetic variation from the wild.

The threatened African dwarf crocodile, for instance, should be of special concern in captivity, as genetic analysis revealed high frequencies of hybridization between subspecies. The authors suggest separation of individuals based on subspecies to prevent this in the future and to maintain genetic diversity (Schmidt *et al.*, 2015). The Black-fronted Piping Guan (*Aburria jacutinga*), on the other hand, had been used for reintroductions, yet before Oliveria-Jr. *et al.* (2016) their genetic makeup had never been considered. While inbreeding was not detected, a recent bottleneck lead researchers to determine that should individuals be used for reintroductions, it should be done in areas where the guan had been extinct instead of as a supplement to current wild populations (Oliveria-Jr. *et al.*, 2016). Likewise, captive and wild populations of ring-tailed lemurs (Pastorini *et al.*, 2015), okapi (Stanton *et al.*, 2015), Northern Bald Ibis (Wirtz *et al.*, 2018), giant pandas (Yang *et al.*, 2017), Visayan Warty Pig (Nuijten *et al.*, 2016), and the California Condor (Meretsky *et al.*, 2000) among others, showed similar instances of how breeding programs have evolved from founding populations to reintroductions in other captive populations and into the wild.

Conclusions

The studies discussed in this literature review were selected to provide an understanding of past and current pedigree techniques and their efficacy of providing accurate information of captive individuals of concern. Studbook yearbooks may be utilized by zoo personnel individually without other pedigree information or may be combined with molecular genetic analysis. These pedigree records and techniques have been used in the past to provide a record of individuals at zoological institutions, but have become instrumental in determining population genetics for captive breeding programs and reintroduction efforts (Lacy, 1989; Ryder, 1995; Schmidt *et al.*, 2015; Schulte-Hostedde & Mastromonaco, 2015; Shan *et al.*, 2014). Each of these studies displayed the accuracy of the different pedigree techniques, most often providing some idea of population demographics. More specifically, studbook yearbooks were most effective in providing a starting point for captive breeding programs of individuals taken from the wild into captivity due to extirpation or extinction in the wild (Ralls & Ballou, 2004; Ochoa *et al.*, 2016).

Molecular genetic analysis, on the other hand, was more effective at determining the actual genetic material within individuals in a population (Hindrikson *et al.,* 2017; Hvilsom *et al.,* 2013; Neaves *et al.,* 2016; Oliveria-Jr. *et al.,* 2016; Pastorini *et al.,* 2015; Shan *et al.,* 2014; Stanton *et al.*, 2015; West *et al.*, 2018). Finally, the combination of studbooks with molecular genetics determined gaps and errors in studbooks yearbooks and provided specific genetic data of captive populations that could be used in determining which individuals should breed to maintain the greatest genetic diversity (Lacy, 1989; Ochoa *et al.*, 2016; Pastorini *et al.*, 2015; Çilingir *et al.*, 2017; Ito *et al.*, 2016).

Recommendations

Based on these conclusions, I recommend that future research include the usage of both studbook yearbooks with molecular genetic analysis because such studies will likely identify inaccuracies or gaps in our current understanding of the genetic diversity of captive populations. I was able to find a large variety of studies investigating the molecular genetics of captive and wild individuals, yet few combined pedigree techniques, and those that did were fairly recent or the first of its kind. Additionally, I would recommend that conservation geneticists and zoo research scientists collaborate in such efforts, as zoo personnel are experts on studbooks and geneticists on molecular techniques. This would enable zoos to have a better, more accurate understanding of the genetic diversity within their captive populations and would aid in gene retention and future reintroduction or translocation efforts. In turn, these efforts would provide conservationists with a reservoir of genetic material from which they might use in bolstering wild populations and reintroducing species back to where their wild extirpation occurred.

As molecular genetic analysis is being more accessible through lowered cost and partnerships with universities and research institutions (Durmaz *et al.*, 2015), the importance of genetic integration into management techniques is being revealed. Jensen *et al.* (2020) most recently unearthed the wealth of genetic resources, such as molecular markers, primers, and template genomes, available for species typically held in zoos. A systematic literature review of almost 8,000 papers revealed that not only do molecular genetic resources exists for many of the species found in zoos, but that, "Critical to the achievement of sustainable ex situ populations is the inclusion of molecular genetic information to guide population management," (Jensen *et al.,* 2020, p. 259). The authors implore scientists and zoo managers alike on the "imperative" nature of managing ex situ populations scientifically with molecular genetic resources (e.g., microsatellites, SNPs, genotype-by-sequencing, etc.) and its ability to combat the current sustainability crisis (Jensen *et al.,* 2020; Norman *et al.,* 2019).

In conclusion, the goal of this literature review was to summarize literature on the use of pedigrees in determining captive population genetics and to gain a better understanding of the pedigree techniques used in captive breeding program and *ex situ* conservation efforts. I have fulfilled these goals by detecting gaps in the literature of the combination of studbooks and genetic analysis – few published studies have truly explored this technique prior to the past five years (*see Table 1*). I have presented examples of the use of studbooks and molecular analysis, individually and combined, for a variety of endangered and threatened species, and presented recommendations on incorporating molecular genetics techniques into current and future management plans.

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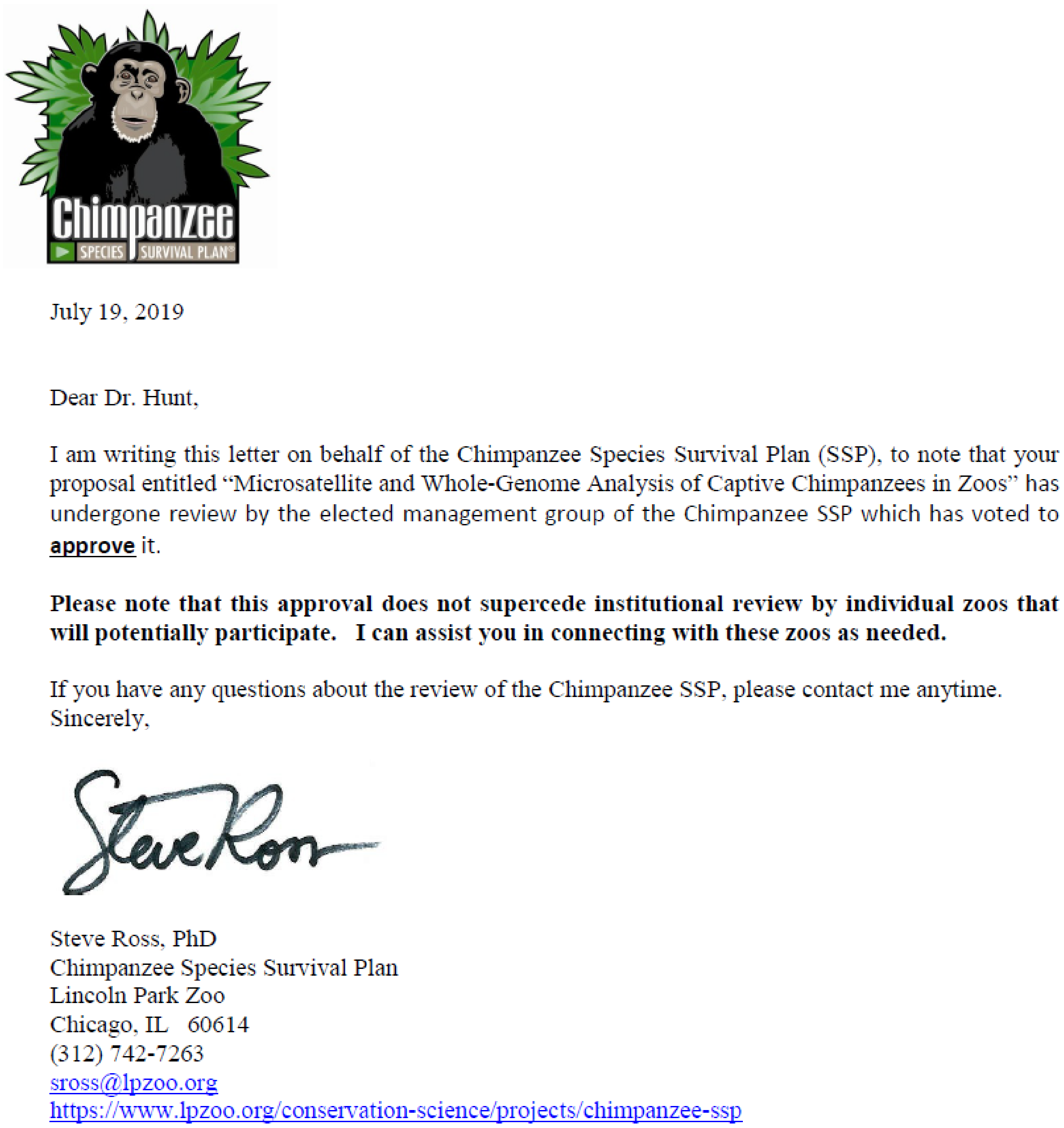
Appendix I – Permission Letter for Research from the Chimpanzee Species Survival Plan (SSP) Committee for Chapter 2



Chimpanzee
SPECIES SURVIVAL PLAN

July 19, 2019

Dear Dr. Hunt,

I am writing this letter on behalf of the Chimpanzee Species Survival Plan (SSP), to note that your proposal entitled "Microsatellite and Whole-Genome Analysis of Captive Chimpanzees in Zoos" has undergone review by the elected management group of the Chimpanzee SSP which has voted to **approve** it.

Please note that this approval does not supercede institutional review by individual zoos that will potentially participate. I can assist you in connecting with these zoos as needed.

If you have any questions about the review of the Chimpanzee SSP, please contact me anytime.
Sincerely,

Steve Ross

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Chimpanzee Species Survival Plan
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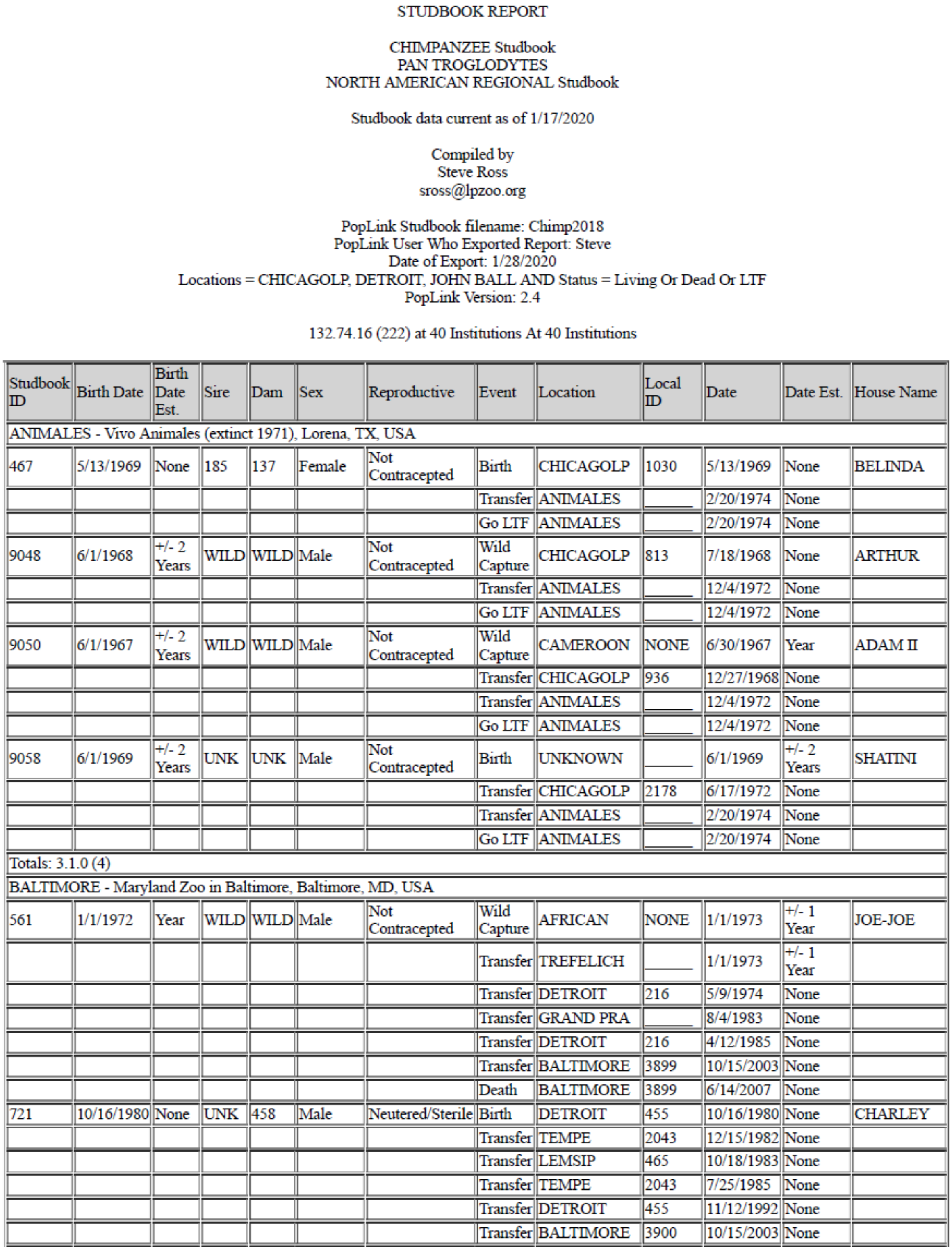
https://www.lpzoo.org/conservation-science/projects/chimpanzee-ssp

Appendix II – Excerpt from the North American Regional Chimpanzee Studbook Report for Chapter 2 Reference



STUDBOOK REPORT

CHIMPANZEE Studbook
PAN TROGLODYTES
NORTH AMERICAN REGIONAL Studbook

Studbook data current as of 1/17/2020

Compiled by
Steve Ross
sross@lpzoo.org

PopLink Studbook filename: Chimp2018
PopLink User Who Exported Report: Steve
Date of Export: 1/28/2020
Locations = CHICAGOLP, DETROIT, JOHN BALL AND Status = Living Or Dead Or LTF
PopLink Version: 2.4

132.74.16 (222) at 40 Institutions At 40 Institutions

Studbook ID	Birth Date	Birth Date Est.	Sire	Dam	Sex	Reproductive	Event	Location	Local ID	Date	Date Est.	House Name
ANIMALES - Vivo Animales (extinct 1971), Lorena, TX, USA												
467	5/13/1969	None	185	137	Female	Not Contracepted	Birth	CHICAGOLP	1030	5/13/1969	None	BELINDA
							Transfer	ANIMALES	______	2/20/1974	None	
							Go LTF	ANIMALES	______	2/20/1974	None	
9048	6/1/1968	+/- 2 Years	WILD	WILD	Male	Not Contracepted	Wild Capture	CHICAGOLP	813	7/18/1968	None	ARTHUR
							Transfer	ANIMALES	______	12/4/1972	None	
							Go LTF	ANIMALES	______	12/4/1972	None	
9050	6/1/1967	+/- 2 Years	WILD	WILD	Male	Not Contracepted	Wild Capture	CAMEROON	NONE	6/30/1967	Year	ADAM II
							Transfer	CHICAGOLP	936	12/27/1968	None	
							Transfer	ANIMALES	______	12/4/1972	None	
							Go LTF	ANIMALES	______	12/4/1972	None	
9058	6/1/1969	+/- 2 Years	UNK	UNK	Male	Not Contracepted	Birth	UNKNOWN	______	6/1/1969	+/- 2 Years	SHATINI
							Transfer	CHICAGOLP	2178	6/17/1972	None	
							Transfer	ANIMALES	______	2/20/1974	None	
							Go LTF	ANIMALES	______	2/20/1974	None	
Totals: 3.1.0 (4)												
BALTIMORE - Maryland Zoo in Baltimore, Baltimore, MD, USA												
561	1/1/1972	Year	WILD	WILD	Male	Not Contracepted	Wild Capture	AFRICAN	NONE	1/1/1973	+/- 1 Year	JOE-JOE
							Transfer	TREFELICH	______	1/1/1973	+/- 1 Year	
							Transfer	DETROIT	216	5/9/1974	None	
							Transfer	GRAND PRA	______	8/4/1983	None	
							Transfer	DETROIT	216	4/12/1985	None	
							Transfer	BALTIMORE	3899	10/15/2003	None	
							Death	BALTIMORE	3899	6/14/2007	None	
721	10/16/1980	None	UNK	458	Male	Neutered/Sterile	Birth	DETROIT	455	10/16/1980	None	CHARLEY
							Transfer	TEMPE	2043	12/15/1982	None	
							Transfer	LEMSIP	465	10/18/1983	None	
							Transfer	TEMPE	2043	7/25/1985	None	
							Transfer	DETROIT	455	11/12/1992	None	
							Transfer	BALTIMORE	3900	10/15/2003	None	

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