Microsatellites and Genetic Variation in Two Members of the African Papionini

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1. Summary

The importance of genetic variation for the fitness and viability of populations and species has been shown in many studies over the last decades. However, the way to determine such genetic variation has changed and evolved due to theoretical as well as technical advances. In wildlife studies, the current markers of choice are often still microsatellites. Microsatellites are highly abundant in eukaryote genomes, the majority is located in the non-coding parts of the genomes and therefore assumed to evolve neutrally without selection pressure. They show high levels of allelic diversity ensuring high statistical power per locus and because they are comparatively short, they can be analyzed even from samples of low quality such as feces. Further, microsatellites are often not species-specific and can be amplified across species boundaries. This allows for the fast and easy implementation of a microsatellite panel in closely related species with comparatively little effort. Therefore, they are widely applied in a wide range of studies for example for the identification of individuals, clarification of paternities, the assessment of relatedness and the population level genetic diversity as well as for the monitoring of gene flow among populations and hybridization between (sub-)species.

To test the ease of implementation for a microsatellite panel in a cross-species approach and whether the usage of microsatellites can provide a sufficient amount of data, I conducted a study in geladas to investigate their population genetic structure. This study is the first to analyze the nuclear genetic variation in geladas (*Theropithecus gelada*) using samples originating from all three populations covering their known distribution range. By using a panel of 24 microsatellite loci previously developed and adapted to the genus *Papio* I could show that the cross-species amplification of microsatellites allows for the fast and easy generation of nuclear genetic data in geladas. Further, the resulting data confirmed a three-deme population structure and therefore provided additional support for the existence of three evolutionary units (or subspecies) within geladas which is still debated based on previous studies using mitochondrial data.

The second study presented here deals with several problems that have been identified in the past for the analysis of microsatellites. These include problems due to the current standard way of analysis using capillary electrophoresis such as the high costs, the challenging data evaluation, and the underestimation of genetic variation due to undetected sequence variants in alleles. Additionally, the situation in non-human catarrhine primates is further complicated as many different microsatellite panels have been analyzed across and within species. This hinders the easy sharing, combination, and

comparison of data produced by different research groups. Therefore, I present the development and validation of a microsatellite panel that can be universally applied to all catarrhine species incorporated into a genotyping-by-sequencing approach. The resulting panel of 42 microsatellite loci can be applied to all catarrhine primates and facilitates the fast and accurate generation of nuclear genetic data from various sample sources including such with low-quality DNA. Overall, this thesis highlights the applicability of microsatellites in wildlife studies and specifically provides a new tool to analyze nuclear genetic variation in non-human catarrhine primates.

2. General Introduction

Genetic variation, or genetic diversity, enables natural populations to adapt to a changing environment (Frankham, 1996). A loss of genetic diversity, or the evolutionary potential, is therefore associated with a general decrease in fitness within populations, i.e., due to inbreeding depression and an increased risk of extinction (Frankel & Soulé, 1981; Frankham, 1996, 2005; Väli et al., 2008). On a molecular level, genetic variation is generated by mutational events. This can either be mutations of single nucleotides, e.g., by insertion, deletion, or conversion, or it can affect larger portions of the genome up to chromosomal re-arrangements. In sexually reproducing organisms another source of genetic variation is the recombination of existing genetic material during the meiosis of germ cells where genetic material can be exchanged between a pair of homologous chromosomes. Only mutational events occurring in the cells of the germline can be passed on to future generations of a population, changes in somatic cells cannot. Whether a mutational event has fitness consequences for an individual or a population (e.g., by gene alteration) therefore largely depends on the affected cell type, but also on its location in the respective genome. In primates, for example, only a small part of the genome (around 2%) is coding for genes (e.g., Wright, 2005). Most of the mutations occurring throughout the lifespan of an individual are therefore expected to be selectively neutral, i.e., they will neither result in fitness benefits nor fitness disadvantages. Due to this lack of selection pressure, neutral genetic variation is thought to be mainly influenced by, and therefore reflects, the demographic and evolutionary history of populations (Wright, 2005). Consequently, the analysis of (neutral) genetic variation is part of many biological research fields besides conservation and population genetics, e.g., phylogeny, phylogeography, and the demographic history of populations.

There are several different ways and levels on which genetic variation can be analyzed. The most informative approach is the direct sequencing of genomes (or parts thereof) to get complete genetic information. However, depending on the research question, that might be too time- and cost-intensive and generate more data than actually needed (Flanagan & Jones, 2019; Guichoux et al., 2011). Therefore, genomic markers were developed early on to analyze genetic variation on a coarser level. A genetic marker can, at a given locus, provide information about its allelic variation. In the last decades, many such markers have been developed and applied to biological fields including gene mapping, population genetics, paternity testing, phylogenetic reconstructions, and forensic applications (Schlötterer, 2004). One of the first markers were allozymes (‘allelic variants of enzymes’) which relied on differences in native gel electrophoresis due to size and charge differences caused by amino-acid substitutions. However, the

number of informative allozyme markers was mostly low and this method only indirectly hinted at underlying DNA variations. Therefore, allozymes were quickly replaced by restriction fragment length polymorphisms (RFLPs; Botstein et al., 1980) where base pair substitutions in the recognition site of restriction enzymes lead to changed patterns of restriction fragments. Next were minisatellites, also called VNTRs ('variable number of tandem repeats') (Jeffreys et al., 1985). They consist of short nucleotide sequences (up to 100 base pairs [bp]) that are tandemly repeated (Chambers & MacAvoy, 2000; Tautz, 1993). The number of such repeat units at a given locus can be highly variable among individuals resulting in length polymorphisms and high allelic diversity. Therefore, minisatellites paved the way for DNA fingerprinting, a method used for the identification of individuals (Gill et al., 1985; Jeffreys et al., 1985). However, their analyses still relied on the usage of restriction enzymes and hybridization of the resulting fragments to probes. Additionally, as for all markers mentioned before, high-molecular DNA, i.e., DNA of high quantity and quality, was needed. Therefore, these markers were rarely applied to population genetic studies in the wild where samples are often of low DNA quality and quantity (Schlötterer, 2004). A changing point in the molecular genetic analyses was the invention of the polymerase chain reaction (PCR) in 1985 (Saiki et al.). With this method, any genomic region of interest could now be amplified (i.e., multiplied) even from low amounts of DNA. Only a few years later, microsatellites began to replace most other markers (Schlötterer, 2004). Microsatellites are similar to minisatellites but are composed of much shorter tandem repeat units (2-7bp; Butler, 2012). Together with the PCR, they made samples collected in the wild accessible for genetic analyses and microsatellites became one of the most popular genetic markers in wildlife science. Although there have been other markers developed and applied, e.g., randomly amplified polymorphic DNAs (RAPDs; Williams et al., 1990), amplified fragment length polymorphisms (AFLPs; Vos et al., 1995; Zabeau & Vos, 1993), inter-retrotransposon amplified polymorphisms (IRAPs; Kalendar et al., 1999) and single nucleotide polymorphisms (SNPs), microsatellites are still the marker of choice in many wildlife studies. The reasons for their ongoing popularity will be discussed in the following section.

2.1. Microsatellites in wildlife research

Microsatellites are now used for a wide range of applications in wildlife studies. On an individual level, they are used for the identification of individuals, the clarification or confirmation of paternities, and the assessment of dyadic relatedness, often in combination. This information is for example crucial to characterize mating systems and reproductive skew as well as for studies interested in the role of kinship and relatedness in the development of various social constructs and behaviors such as the development of social bonds (e.g., Baden et al., 2020; Dal Pesco et al., 2021; De Moor et al., 2020a; De Moor et al., 2020b), but also for the captive breeding management of endangered wild species and zoos (Arandjelovic & Vigilant, 2018; Jensen et al., 2020; Norman et al., 2019). Over the last years, microsatellites were increasingly used at the population level to investigate and monitor the genetic diversity of single populations and gene flow among populations, also concerning conservation management (e.g., Baas et al., 2018; Ferreira da Silva et al., 2018; Widdig et al., 2017), or to study hybridization between (sub-)species (e.g., Charpentier et al., 2012; Godinho et al., 2015; Malukiewicz et al., 2015). Another major field of application is found in wildlife forensics were genetic analysis plays a key role in wildlife law enforcement and in monitoring the illegal wildlife trade. Here, microsatellites are used to identify the taxon or source population of an animal or its remains (e.g., ivory or meat) (e.g. Linacre & Tobe, 2011; Wasser et al., 2004) and, if possible, to assign illegally captured individuals to their source populations for targeted releases (e.g. Velo-Antón et al., 2007). To understand the ongoing popularity of microsatellites in such studies, a look at their characteristics is important.

Microsatellites are short DNA fragments (100-400 bp long) consisting of a varying number of repeat units that are 2 to 7 bp in length (Butler, 2012; Ellegren, 2004). They are highly abundant in eukaryote genomes including plants, fungi, and animals (Hamada et al., 1982; Lim et al., 2004; Tautz & Renz, 1984) where they are mostly found in non-coding parts of the genome like intergenic regions and introns (Ellegren, 2004). Only a small fraction of microsatellites (comprised mostly of trinucleotide-repeats units) is involved in gene alterations causing for example neurodegenerative diseases in humans. Therefore, the majority of microsatellites in eukaryotes is assumed to be free of selection pressure and to evolve neutrally (Schlötterer, 2000). Microsatellite mutation primarily occurs by replication slippage (Ellegren, 2000; Levinson & Gutman, 1987; Schlötterer & Tautz, 1992) leading to high levels of allelic diversity. The rapid mutation rates (10^{-3} to 10^{-5} per generation) are magnitudes higher than for example those for the mainly bi-allelic SNPs (10^{-8} to 10^{-9} per generation) (Ellegren, 2000; Nachman & Crowell, 2000). Consequently, to reach the same resolution and statistical power, many more SNP loci need to be

evaluated compared to poly-allelic microsatellites (Butler et al., 2007; Ross et al., 2014; Städele & Vigilant, 2016). Further, as the repeat regions of microsatellites are relatively short, PCR products can be generated and analyzed even from low-quality (degraded) DNA. Particularly, in the field of wildlife research this is of importance as non-invasively collected samples (e.g., hairs, feathers, urine, and feces) make up the most abundant source of genetic material. This is especially true for endangered and/or elusive species where capturing individuals to gain invasive material, like blood, is difficult or impossible (Waits & Paetkau, 2005). In addition, DNA extracted from such non-invasive material, especially from feces, is often of exogenous origin (e.g., bacteria, food items) and can contain PCR inhibitors such as complex polysaccharides (Monteiro et al., 1997; Perry et al., 2010). However, various attempts have been conducted in recent years to improve genotyping from such and other materials, including better sample preservation (Nsubuga et al., 2004; Roeder et al., 2004), refined DNA extraction methods (Perry et al., 2010; Zhang et al., 2006), endogenous DNA content quantification (Morin et al., 2001; Perry et al., 2010), improved PCR amplification as well as more accurate/reliable genotyping procedures (Arandjelovic et al., 2009; Buchan et al., 2005; Navidi et al., 1992; Sefc et al., 2003; Taberlet et al., 1996). Another advantage of microsatellites is, that they are often not species specific and hence can be amplified across closely related species. For example, microsatellite loci characterized for humans are successfully used in many other catarrhine primates (Old World monkeys and apes) (e.g., Coote & Bruford, 1996; Ely et al., 1998; Morin et al., 1998; Roeder et al., 2009), while coincident SNPs in Old World monkeys are much rarer (Malhi et al., 2011). Therefore, species-specific SNP assays need to be designed (Kraus et al., 2015) or non-target sequencing methods such as Restriction-Site Associated DNA-Sequencing (RADseq; Baird et al., 2008) or its variants (ddRAD, 2bRAD, etc.) (Peterson et al., 2012; Wang et al., 2012) need to be applied to obtain SNP information. Consequently, SNP data from different species, generated in different laboratories and using different genotyping platforms cannot be directly compared (Barbian et al., 2018).

To summarize, microsatellites are assumed to show neutral genetic variation, are highly abundant, have a high allelic diversity and therefore high statistical power per locus, are comparably easy to analyze even from samples with low DNA quality and/or quantity, and are amplifiable across species boundaries. Hence, microsatellites are still the preferred marker of choice, particularly when applied to small sample size datasets as typically found in forensic and kinship studies (Barbian et al., 2018; Guichoux et al., 2011).

Nevertheless, the analysis of microsatellites is not without criticism. Traditionally, microsatellite genotyping is conducted via fragment length analysis using acrylamide gel or capillary electrophoresis

(CE). With the latter method, amplified and fluorescent-labeled (one primer carries the dye) PCR products are size-separated via electrophoresis and the length of the allele(s) is determined by measuring the run time via laser technology in comparison to that of a size standard. However, the run time of a fragment is also influenced by the attached fluorescence dye and the conditions for the sequencing machine, e.g., the surrounding room temperature. The usage of fluorescent dyes for detection additionally limits the number of loci that can be multiplexed in one reaction, i.e., be analyzed in parallel, as typically a maximum of five different dyes can be used including one for the size standard (blue, green, yellow, black and red/orange). This drastically increases the time and money spent on a project, especially if large sample sizes need to be analyzed. Further, PCR artifacts are common during microsatellite loci amplifications leading to stutter peaks (polymerase slippage), split peaks (inconsistent Adenine overhangs), and off-target PCR products showing up in the resulting electropherogram (Ewen et al., 2000; Fernando et al., 2001; Guichoux et al., 2011; Hauge & Litt, 1993; Pompanon et al., 2005; Schuelke, 2000). Hence, a high level of experience of the researcher doing the data evaluation is required, but even if that is given, allele calling remains challenging and often shows poor inter-laboratory comparability (Barbian et al., 2018; De Barba et al., 2017; Delmotte et al., 2001; Pasqualotto et al., 2007). Additionally, as only the length information is given by CE, size homoplasies between different alleles of the same locus (same length but different sequences) remain undetected. Although this problem has been known for a long time (e.g., Garza & Freimer, 1996; Grimaldi & Crouau-Roy, 1997; Roeder et al., 2009), it was mostly ignored in the past decades due to a lack of alternatives. In summary, the traditional genotyping process is largely dependent on the experiment and/or the investigator as well as often error-prone.

However, many of the aforementioned technical problems can be mitigated by using next-generation sequencing (NGS) technologies in form of genotyping-by-sequencing (GBS) (Barbian et al., 2018; Johannesen et al., 2017; Pimentel et al., 2018; Vartia et al., 2016). Instead of only determining the fragment length, each PCR product gets sequenced providing unambiguous allele length as well as the sequence information which allows the detection of size homoplasies. Importantly, the data is now independent of the used sequencing platform and preparation protocol. With GBS, large-scale multiplexing of PCR products is possible and the whole genotyping process becomes faster and more cost-efficient due to increasing numbers of samples being analyzed at once as well as the development and improvement of new bioinformatic analysis tools. Recently, this approach was successfully applied to answer research questions involving a variety of species, for example, the Atlantic cod (*Gadus morhua*, Vartia et al., 2016), boarfish (*Capros aper*, Farrell et al., 2016), brown bear (*Ursus arctos*, De Barba et al., 2017), chimpanzees (*Pan troglodytes*, Barbian et al., 2018), Atlantic salmon (*Salmo salar*, Bradbury et al.,

2018), East African Nile tilapia (*Oreochromis niloticus*, Tibihika et al., 2018), European hedgehogs (*Erinaceaus europaeus* and *E. roumanicus*, Curto et al., 2019) and muskellunge (*Esox masquinongy*, Gruenthal & Larson, 2021).

2.2. The Subtribe Papionina (with a focus on the genera *Theropithecus* and *Papio*)

This thesis focuses on the genetic variation in two members of the Papionina: geladas (genus *Theropithecus*) and baboons (genus *Papio*). The Papionina, or African Papionini, are a subtribe of the Papionini (family: Cercopithecidae) comprising the genera *Theropithecus*, *Papio*, *Lophocebus*, *Mandrillus*, *Cercocebus,* and *Rungwecebus* which occur almost exclusively on the African continent. The only exception is the hamadryas baboon (*Papio hamadryas*) which can be found in northeastern Africa as well as on the southwestern Arabian Peninsula (Kingdon, 1997; Sarmiento, 1998). It is currently accepted that the Papionina can be further split into two clades, one containing *Mandrillus* and *Cercocebus* and the other comprised of the genera *Papio, Rungwecebus*, *Theropithecus,* and *Lophocebus* (Disotell, 1994; Liedigk et al., 2015). The genus *Rungwecebus* was shown to be related the closest to the genus *Papio* with whom it shares a complex history of hybridization (Burrell et al., 2009; Roberts et al., 2010; Zinner et al., 2009; Zinner et al., 2018b). The phylogenetic relationships between the remaining genera *Theropithecus, Papio,* and *Lophocebus* are difficult to resolve. Depending on the dataset and applied method, all possible groupings of sister taxa have been suggested, as well as an unresolved trichotomy (Guevara & Steiper, 2014 and references therein; Liedigk et al., 2014). Several possible mechanisms have been proposed to explain this pattern including ancient hybridization and incomplete lineage sorting (ILS) (Guevara & Steiper, 2014; Liedigk et al., 2014). Similarly, mitochondrial DNA (mtDNA) data revealed paraphyletic relationships between the taxa of the genera *Mandrillus* and *Cercocebus* (Liedigk et al., 2014).

Due to the complex evolutionary history of the Papionina and because most of their evolution geographically, temporally, and ecologically parallels the early hominin evolution, they have been considered a useful model to understand the complex processes that occurred in the human evolution (Holliday, 2003; Jolly, 2001). This is especially true for the genus *Theropithecus* which diverged about 4-5 million years ago (mya) from a *Papio*-like ancestor (Delson, 1993; Gilbert et al., 2018; Jablonski, 2005; Liedigk et al., 2014) and the genus *Papio* whose diversification is estimated to have begun about 2 mya (Boissinot et al., 2014; Newman, Jolly, & Rogers, 2004; Zinner et al., 2009; Zinner et al., 2013) which is thought to be comparable in age to the origin and fast development of *Homo* (Antón et al., 2014; Jolly,

2001; Wood & Collard, 1999). Additionally, a recent study based on whole genome data and *Alu* insertion polymorphisms shared by members of the genus *Papio* and a *Theropithecus gelada* individual added evidence to their close and intertwined common ancestry involving most likely admixture and ILS (Walker et al., 2019). Indeed, hybridization between members of the different genera is still possible as was shown for captive animals (*Papio hamadryas x Theropithecus gelada*; *P. anubis x T. gelada*; Jolly et al., 1997; Markarjan et al., 1974; Markarjan et al., 1972) and was suspected for wild animals in Ethiopia (*P. anubis x T. gelada*; Dunbar & Dunbar, 1974). Therefore, the genera *Theropithecus* and *Papio* represent an interesting system to study complex divergence scenarios including ancient hybridization, introgression, and reticulation among and between genera as was suggested for ancient hominin lineages including Neanderthals, Denisovans, and modern humans (Ackermann et al., 2019; Antón et al., 2014; Green et al., 2010; Prüfer et al., 2017; Prüfer et al., 2014; Reich et al., 2010).

The genus *Papio* by itself has an interesting and complex evolutionary history. Following the phylogenetic species concept (Cracraft, 1983), there are currently six species recognized: *Papio ursinus*, *P. cynocephalus*, *P. kindae*, *P. anubis*, *P. papio,* and *P. hamadryas*, all of which are phenotypically and behaviorally distinct (Jolly, 1993; Swedell, 2011; Zinner et al., 2013). However, several genetic studies based on mtDNA reported incongruences between phenotype- or taxonomy-based and mtDNA-based phylogenies, highlighting a complex evolutionary history with multiple episodes of introgression and admixture throughout their radiation (Jolly, 2001; Zinner et al., 2009; Zinner et al., 2013). Recent studies based on *Alu* insertion polymorphisms and whole genome nuclear data could further confirm the historic exchange of genetic material between the diverging lineages (Jordan et al., 2018; Rogers et al., 2019; Steely et al., 2017). In addition, there seem to be no apparent reproductive barriers between the extant *Papio* species. Where they meet in the wild, they can produce fertile offspring and form long-lasting hybrid zones, sometimes despite substantial differences in their social organization and social structure (e.g., *P. anubis* x *P. hamadryas* in the Awash National Park, Ethiopia; Bergman, Phillips-Conroy, & Jolly, 2008). This allows researchers, to empirically study large groups, to actively monitor ongoing hybridization and introgression, and to investigate the consequences of these processes for demographic developments and genomic and phenotypic variation. However, on the genus level, the analysis of genetic variation so far relied mainly on mtDNA (e.g., Zinner et al., 2009; Zinner et al., 2015; Zinner et al., 2013) and if nuclear data was included, it was often based on very few samples most of which originated from zoo animals and not wild populations (e.g., Rogers et al., 2019). Therefore, our understanding of the past evolutionary processes in the genus *Papio* might change, if future studies on the nuclear genetic variation include a higher number of individuals from all recognized species covering their respective distribution ranges.

Contrary to the wide-spread baboons, geladas are endemic to the Ethiopian Highlands (e.g., Gippoliti, 2010). Albeit being widely distributed in Africa and Eurasia during the late Pliocene to middle Pleistocene, the genus *Theropithecus* has only one extant member: the gelada (Alba et al., 2014; Beaudet et al., 2015; Belmaker, 2010; Delson, 1993; Geraads & de Bonis, 2020; Hughes et al., 2008; Jolly, 1972). Geladas were extensively studied regarding for example their social system and ecology (e.g., Dunbar, 1992; Dunbar, 1993; Fashing et al., 2014). However, their nuclear genetic diversity is largely understudied and their subspecific taxonomy is still debated (Crook, 1966; Gippoliti, 2010; Hill, 1970; Zinner et al., 2018a). Currently, two subspecies, or evolutionary units, are generally recognized: *T. gelada gelada* (Rüppell, 1835) and *T. gelada obscurus* (Heuglin, 1863). Those subspecies correspond to a northern population (north of Lake Tana, mostly in the Simien Mountains) and a central population (between the highlands east of Lake Tana and Addis Ababa), respectively. However, the geographic distribution and the validity of the subspecies ranks are still unclear (Bergman & Beehner, 2013; Groves, 2001, 2005; Grubb et al., 2003; Kingdon, 1997; Yalden et al., 1977). In addition, a third population was discovered south of the Rift Valley in the Arsi region (Akio Mori & Belay, 1990; Neumann, 1902). Due to its geographic isolation, phenotypic differences as well as variation in blood proteins and mtDNA sequences, a subspecies rank was proposed for the southern population as well (Belay & Mori, 2006; Belay & Shotake, 1998; Bergman & Beehner, 2013; Gippoliti, 2010; Mori & Belay, 1990; Shotake et al., 2016). Shotake et al. (2016) tentatively named the southern population *T. gelada arsi*. A recent study including more samples covering the distribution range of geladas and a bigger part of the mitochondrial genome could confirm these results (Zinner et al., 2018a). However, Zinner and colleagues (2018a) also concluded that mtDNA information was not sufficient to resolve the taxonomic rank of the three gelada populations. Further, as we know from baboons, the distribution of mitochondrial clades might not reflect the true population relationships and phenotypic or nuclear genetic variation. Therefore, comparable nuclear genetic data of the different populations is needed to resolve this issue and complete the population genetic picture.

As already mentioned in the beginning, one instrument to characterize the nuclear genetic diversity of populations and/or to study hybridization in the wild is the analysis of microsatellites, especially if low-quality samples, like feces, make up the majority of the sampling material. However, the microsatellite analysis in the Papionini and all other non-human catarrhine primate species has some peculiarities which will be discussed in the following section.

2.3. Microsatellites in the Papionina and other catarrhine species

The rise of microsatellites and their application in catarrhine primates began in the early 1990s. Encouraged by studies using cross-species microsatellite amplification in other mammalian and avian species, Coote and Bruford (1996) showed that human-derived primers for the microsatellite amplification are applicable for the analysis of genetic variation in a wide range of catarrhine species. Although some earlier studies were using human-derived primers in non-human primates, those were limited to one species (e.g., Altmann et al., 1996: *Papio cynocephalus*) and mostly chimpanzees (e.g., Morin et al., 1994; Takasaki & Takenaka, 1991; Washio, 1992). The study by Coote and Bruford (1996) was the first to include a wide range of different primate species (N=22) from different genera and paved the way for screening studies for human microsatellite loci in non-human primates (e.g., Ely et al., 1998; Kayser et al., 1996; Morin et al., 1998; Newman et al., 2002; Roeder et al., 2009; Smith et al., 2000). As there was no reference genome data available for most species at the time, this cross-species amplification of human-derived loci presented an easy, quick and cheap alternative to the *de novo* development of species-specific primers. Since these early studies, primers successfully applied in one species were typically tested in another (closely related) species of interest and used if successfully amplified, polymorph and in accordance with Mendelian inheritance. This led to numerous studies applying such primers to this day (e.g., Barbian et al., 2018; Dal Pesco et al., 2021; De Moor et al., 2020a; De Moor at al., 2020b; Engelhardt et al., 2017; Ferreira da Silva et al., 2018; Fischer et al., 2020; Kheng et al., 2017; Minkner et al., 2018; Städele et al., 2019; Städele et al., 2021; Widdig et al., 2017).

Although helpful, this approach of cross-amplification also has a severe drawback as it increases the risk of null-alleles and allelic dropouts. Null-alleles occur when an allele is not or less efficiently amplified due to mutations in the primer-binding site, while allelic dropout is mainly the result of a failed amplification of alleles that are too long due to poor DNA quality and low DNA quantity (Pompanon et al., 2005). In both cases, a heterozygous individual is falsely classified as homozygous. Rates of null alleles and allelic dropouts can be measured using various software packages such as MICRO-CHECKER (Van Oosterhout et al., 2004), GENEPOP (Rousset, 2008) or MicroDrop (Wang et al., 2012) to include this information in down-stream analyses. However, the most efficient way to reduce problems with null alleles and allelic dropouts is to redesign primers that bind to conserved regions and amplify shorter PCR products.

Despite the growing amount of sequencing data available for many catarrhine primates, only a few studies have reported adaptations of the used primer pairs, e.g., implemented sequence changes to

avoid mismatches or shorten the PCR product (but see Bradley et al., 2000; Engelhardt et al., 2017). On the other hand, Roeder and colleagues (2009) reported in their extensive summary of microsatellite loci applied in catarrhine species several loci with more than one primer pair available. As the authors state, some of those primers are known to be redesigned as personal communication to them (Roeder et al., 2009). Consequently, researchers not only face the difficulties resulting from technical problems that hinder data comparison (see chapter 2.1) and different loci being analyzed across and within species, but also that even the same loci might be analyzed with different primer sequences depending on the research group. Somewhat ironically, Coote and Bruford (1996) expressed their hope that the usage of human-derived microsatellite loci would allow for a better comparison of data on genetic variation derived from different studies in broad taxonomic groups. Instead, due to the characteristics of the genotyping process via CE and the, in parts, poorly documented usage and adaptations of primer sequences, researchers are now in a situation where this is clearly hindered and often impossible even for data on the same species.

A striking example of this can be seen in Guinea baboons (*Papio papio*). A first study analyzed the genetic variation in Guinea baboons living in Guinea-Bissau using 14 microsatellite loci (Ferreira da Silva et al., 2014). A year later, an article was published that looked at the genetic variation of Guinea baboons living in Senegal (Kopp et al., 2015) using 25 microsatellite loci (previously used in a study on male-male bonds in Guinea baboons; Patzelt et al., 2014). Although 13 loci were included in both studies, it proved to be difficult to combine both datasets as the data were generated using different primer sequences, fluorescent dye tags for PCR fragment detection during CE, and different sequencing platforms. As a work-around, a subset of each sample set, i.e., from Guinea-Bissau and Senegal sampling sites, had to be re-analyzed with both genotyping protocols to calibrate the allele calling process and ensure consistency between datasets (Ferreira da Silva et al., 2018). In the meantime, the aforementioned panel of 25 human-derived microsatellite loci used by Kopp et al. (2015) was further changed and adapted to the genus *Papio* using the available reference genomes of *P. hamadryas* and *P. anubis* to allow for a more efficient amplification (Dal Pesco et al., 2020) and was applied in subsequent studies on the Senegalese population (Dal Pesco et al., 2021; Fischer et al., 2020).

This example clearly shows how variable the landscape of microsatellite panels used in the past and today is in catarrhine primates. Unfortunately, this hinders a direct comparison of data sets even if more and more datasets are made openly available in the course of the open science movement. Especially in the context of conservation management, collaborators must share and combine their data easily and fast. Therefore, better microsatellite panels are needed for the catarrhine primates that allow

for an easy but accurate genotyping process taking advantage of the newest technical sequencing developments including GBS.

2.4. Study aims and approach

This Ph.D. thesis aims to investigate the current usage and usefulness of microsatellites for the analysis of nuclear genetic variation in non-human catarrhine primates, with a focus on baboons and geladas. For this, I will first explore the classic approach of cross-species amplification of microsatellite loci among two catarrhine species (Chapter 1). Specifically, I will use a panel of 24 microsatellites, previously applied in Guinea baboons (Dal Pesco et al., 2020; Fischer et al., 2020), to analyze the nuclear genetic variation in geladas. This is the first time the nuclear genetic variation, i.e., the distribution of microsatellite alleles, will be analyzed in this species with samples originating from all three different populations covering the known distribution range of geladas in the Ethiopian highlands. This study will, for one, show the efficiency of cross-species microsatellite amplification in a phylogenetically close species leading to the fast generation of nuclear genetic data. Second, it will show that a limited amount of microsatellite loci can provide enough data to get an insight into the phylogeny of geladas, i.e., that the data can either support or contradict the hypothesis of three gelada subspecies (or evolutionary units) as suggested by previous studies based on blood proteins and mtDNA (Belay & Mori, 2006; Belay & Shotake, 1998; Shotake et al., 2016; Zinner et al., 2018a). In any case, the resulting genetic information will be of value for future conservation decisions and the taxonomic ranking of geladas.

As described before, several issues have been identified for the application of microsatellites in catarrhine primates. Therefore, the second study presented here (Chapter 2) is dedicated to the development, testing, and improvement of a new microsatellite panel incorporated in a GBS framework that can be applied to all catarrhine species. For this, I will start with an extensive literature review to search for microsatellite loci that have been reported to be applied in various catarrhine species. This information will then, in combination with all available catarrhine reference genomes, be used to identify loci in common by all catarrhine species and to (re-)design primer sequences that bind in conserved genome areas close to the locus. To verify the applicability of the newly designed microsatellite panel, I will test it on a range of samples spanning all major catarrhine lineages, as well as on samples of different DNA quality and quantity, i.e., blood and fecal samples. The final microsatellite panel will provide a valuable tool to universally genotype non-human catarrhine primates time- and cost-efficiently, leading to more reliable data and higher comparability among laboratories and species.

3. Chapter 1: Study I

Geographic distribution of microsatellite alleles in geladas (Primates, Cercopithecidae): evidence for three evolutionary units

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Geographic distribution of microsatellite alleles in geladas (Primates, Cercopithecidae): Evidence for three evolutionary units

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Abstract

The subspecific taxonomy and distribution of geladas (*Theropithecus gelada* Rüppell, 1835) remains uncertain. Recent molecular studies based on mitochondrial sequence data revealed a geographically structured, three-deme population, suggesting that there are three evolutionary units of geladas. However, mitochondrial distributions do not always recover population relationships, particularly in taxa with a complex history of isolation and gene flow. We therefore analysed the nuclear genetic population structure of the global gelada population based on 20 microsatellite loci in 43 samples from across its geographic range. F_{ST} values, a STRUCTURE analysis and a principal coordinate analysis (PCoA) confirmed the three-deme population

Roos and Zinner shared senior authorship

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structure corresponding to the mitochondrial population structure. Therefore, our analyses provide additional support for three evolutionary units in geladas, corresponding to (a) a northern (north of Lake Tana, primarily in the Simien Mountains, previously classified as *Theropithecus gelada gelada* Rüppell, 1835), (b) a central (between Addis Ababa and the highlands east of Lake Tana, previously classified as *Theropithecus gelada obscurus* Heuglin, 1863) and (c) a southern (south of the Rift Valley, previously tentatively classified as *Theropithecus gelada arsi* Shotake et al., 2016, *Anthropological Science*, 124, 157) population. These results pave the way for future conservation decisions and highlight that the gelada population boundaries need more fine-grained genetic sampling and phenotypic analyses, in particular for their taxonomic ranking.

KEYWORDS

distribution, Ethiopia, nuclear markers, population genetics, taxonomy, *Theropithecus gelada*

1 | INTRODUCTION

The gelada (*Theropithecus gelada* Rüppell, 1835) is a cercopithecine primate that is endemic to the Ethiopian highlands. The gelada is the only extant member of a once diverse genus that was widely distributed in Africa and Eurasia during the late Pliocene to middle Pleistocene (Alba et al., 2014; Beaudet et al., 2015; Belmaker, 2010; Delson, 1993; Geraads & de Bonis, 2020; Hughes, Elton, & O'Regan, 2008; Jolly, 1972). The extant species probably consists of three subspecies (Bergman & Beehner, 2013), (a) *Theropithecus gelada gelada* Rüppell, 1835 from northern Ethiopia, mainly the Simien Mountains (hereafter, 'northern population'), (b) *Theropithecus gelada obscurus* Heuglin, 1863 from central Ethiopia (hereafter, 'central population'), and (c) a population from the Arsi area, south of the Rift Valley, which Shotake, Saijuntha, Agatsuma, and Kawamoto (2016) tentatively named *Theropithecus gelada arsi* (hereafter, 'southern population'; Figure 1).

Although phenotypic differences among the three populations have been reported (De Beaux, 1925; Hill, 1970; Mori & Belay, 1990), the subspecific distinction is still debated (Crook, 1966; Gippoliti, 2010; Hill, 1970; Zinner et al., 2018). Similarly, while the small southern population is clearly isolated from the central and northern populations by the Rift Valley (Mori & Belay, 1990, 1991), there is no obvious geographic barrier separating the central and northern populations (Gippoliti, 2010). Yalden et al. (1977) proposed that the deep gorges of the Beleghas (Balagas) and upper Tacazze (Tekeze) rivers correspond to the boundary between the northern (*T. g. gelada*) and central (*T. g. obscurus*) populations, but we still know little about how phenotypic, geographic and genetic variations correspond across these three populations.

Molecular studies comparing blood proteins and mitochondrial DNA (mtDNA; restriction fragment length polymorphism [RFLP] of the control region) of southern geladas from Arsi and central geladas from Shoa Province supported the subspecific rank for the Arsi population (Belay & Mori, 2006; Belay & Shotake, 1998). Further analyses of mtDNA sequence data which also included samples of the northern population confirmed these results but did not find clear evidence for a subspecific differentiation of the northern and central populations (Shotake et al., 2016; Zinner et al., 2018). Phylogenetic reconstructions revealed a monophyletic clade of the southern haplotypes, two clades among the central population and another two clades among the northern population (see also Figure S1). The distributions of the respective two northern and central clades do not show clear geographic partitioning, and one individual collected in the Simien Mountains (northern population) carried a haplotype from the central population (Zinner et al., 2018).

The phylogeny of gelada mtDNA lineages was recently resolved (Shotake et al., 2016; Zinner et al., 2018). However, because mtDNA is inherited in the matriline, the full evolutionary history of the species remains incomplete. Furthermore, mtDNA can differ substantially from phenotypic and nuclear genetic variation in its geographic distribution, particularly in taxa with a history of hybridization and introgression (e.g. baboons Rogers et al., 2019; Zinner, Groeneveld, Keller, & Roos, 2009). Therefore, to expand our understanding of gelada phylogeography, we test whether nuclear DNA markers confirm the three evolutionary unit differentiation indicated by the mtDNA markers. Specifically, we explore whether the distribution of microsatellite alleles corresponds to the geographic pattern of the three populations.

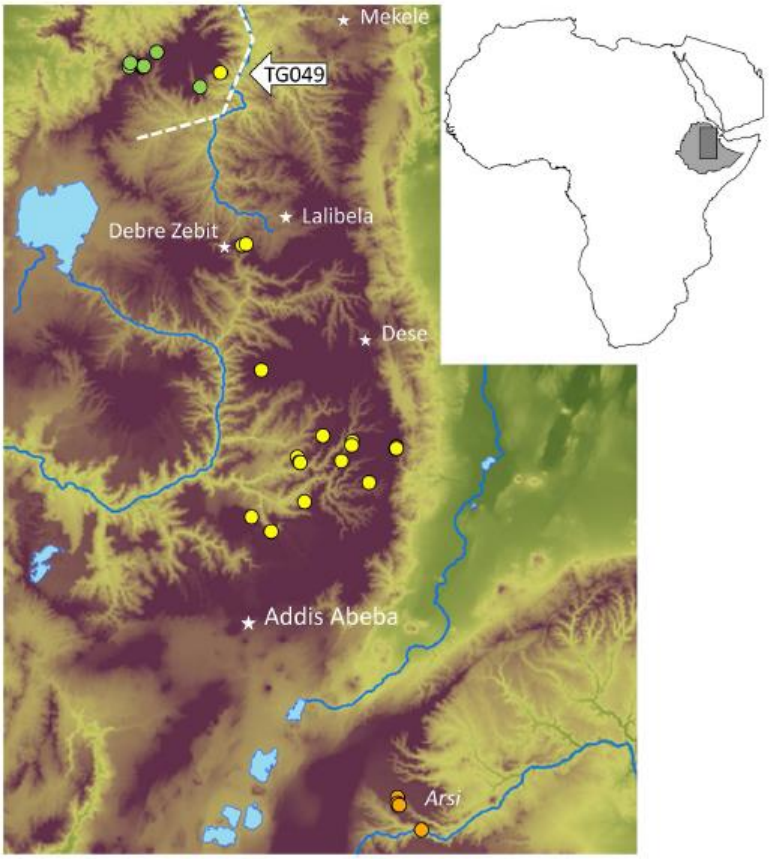

FIGURE 1 Geographic distribution of gelada sampling sites in the Ethiopian highlands. Inset map indicates the position of the area of interest within Africa and Ethiopia. Dashed line = proposed border between the northern population (*Theropithecus gelada gelada* to the north-west of the border) and the central population (*Theropithecus gelada obscurus*, according to Yalden, Largen, and Kock (1977)). Coloured circles = our sampling sites; colours indicate mitochondrial haplogroup affiliation: black (green) = northern; grey (yellow) = central; white (orange) = southern haplogroup (coloured version available online). Arrow indicates geographical provenance of sample TG049. Digital elevation model (DEM) base map (Jarvis, Reuter, Nelson, & Guevara, 2008) [Colour figure can be viewed at wileyonlinelibrary.com]

2 | METHODS

2.1 | Ethical statement

Sample collection was exclusively non-invasive and complied with the laws of Ethiopia and Germany and with the guidelines of the International Primatological Society. During sampling of faecal material, no animals were harmed or disturbed.

2.2 | Sample collection and DNA extraction

Samples for this study were collected during nationwide gelada surveys between 2014 and 2016 (Nguyen, Fashing, & Burke, 2016). All samples analysed here have been used previously in a study of gelada mtDNA phylogeny (Zinner et al., 2018). Further information on sampling and DNA extraction can be found in Zinner et al. (2018). Of the 162 samples included in the previous study, we selected those 61 that contained the highest DNA concentrations (>150 ng/µl) in our previous study (Zinner et al., 2018). Of these, only 49 contained DNA of high enough quality for microsatellite analysis. The three geographic populations (northern, central and southern) are represented by 11, 23 and 15 individuals, respectively (Figure 1). Since sample TG049 from the northern deme carried a mtDNA haplotype of the central population (Zinner et al., 2018) and since the northern and central populations contain two mitochondrial clades each (Zinner et al., 2018), the respective numbers of samples per clade were as follows: northern clade 1:7, northern clade 2:3, central clade 1:22, central clade 2:2 and southern clade 15. Further information on the geographic provenance, deme and haplogroup affiliations of the samples can be found in Table S1 and Figure S1.

2.3 | Genotyping

Genotyping was performed via analysis of microsatellite fragment length polymorphisms. Therefore, we amplified of 20 microsatellite loci in five different multiplex PCRs using the Multiplex PCR Kit (Qiagen) and fluorescent-labelled primers (Table S2). Cycling conditions for all reactions contained an initial polymerase activation step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 57°C for

TABLE 1 Descriptive statistics of the 20 microsatellite loci used for the population genetic analysis

Loci		Alleles		*F*-Statistics			Heterozygosity					
Locus ID	Locus no.	Allele range	No. of alleles	F_{IT}	F_{ST}	F_{IS}	H_E	H_O	HWE[a] North	HWE[a] Central	HWE[a] South	Signs of null alleles
D7s503	Locus 1	147–180	7	0.750	0.579	0.406	0.723	0.233	0.003	0.013	0.109	Yes
D12s375	Locus 2	169–185	5	0.224	0.167	0.069	0.695	0.581	0.048	0.282	0.695	Yes
D3s1766	Locus 3	203–231	8	0.115	0.054	0.065	0.789	0.721	0.778	0.032	0.826	No
D14s306	Locus 4	173–245	7	0.323	0.257	0.090	0.708	0.535	0.605	0.174	1.000	No
D1s533	Locus 5	179–207	7	0.195	0.099	0.107	0.825	0.698	0.998	0.817	0.307	Yes
D2s1326	Locus 6	244–304	10	0.401	0.221	0.231	0.810	0.535	0.030	0.011	0.587	No
D8s1106	Locus 7	144–160	5	0.365	0.129	0.270	0.634	0.429	0.559	0.004	0.193	Yes
D17s791	Locus 8	164–180	8	0.275	0.219	0.072	0.797	0.634	0.004	0.486	1.000	No
D6s501	Locus 9	167–191	7	0.110	0.155	−0.053	0.749	0.714	0.452	0.076	0.514	No
D17s1290	Locus 10	203–254	15	0.174	0.090	0.092	0.908	0.786	0.064	0.225	0.910	Yes
D6s311	Locus 11	231–247	9	0.512	0.412	0.171	0.800	0.465	0.761	0.075	1.000	No
D5s1457	Locus 12	120–152	8	0.326	0.252	0.099	0.805	0.605	0.895	0.913	0.187	No
D8s505	Locus 13	145–149	3	0.023	0.196	−0.215	0.285	0.302	1.000	0.856	1.000	No
D10s1432	Locus 14	159–183	7	0.295	0.252	0.057	0.740	0.581	0.278	0.549	0.042	Yes
D3s1768	Locus 15	185–217	9	0.277	0.274	0.004	0.773	0.628	0.595	0.703	1.000	No
D7s2204	Locus 16	223–255	8	0.242	0.248	−0.008	0.745	0.628	0.029	0.478	1.000	No
D1s207	Locus 17	137–151	7	0.570	0.447	0.222	0.805	0.419	0.869	0.308	1.000	Yes
D4s243	Locus 18	155–179	7	0.351	0.352	−0.002	0.772	0.581	0.167	0.439	1.000	No
D1s548	Locus 19	201–221	6	0.153	−0.003	0.155	0.749	0.643	0.488	0.013	0.398	No
D21s1142	Locus 20	215–239	12	0.174	0.102	0.080	0.803	0.698	0.223	0.203	0.257	No

Abbreviations: H_E, expected heterozygosity; H_O, observed heterozygosity; HWE, Hardy–Weinberg equilibrium.

[a]Note that the *p*-value was corrected for multiple testing with the Bonferroni adjustment, $\alpha = (0.05/60) = .00083$; loci highlighted in grey show signs of null alleles and were excluded from the population genetic analysis presented here.

40 s and 72°C for 40 s, and a final extension step at 72°C for 30 min. Negative (no-template) controls were carried along for all reactions. Each PCR multiplex reaction was repeated a minimum of four times. The amplification success was checked on 2% agarose gels. Allele determination was done using fragment length analysis on an ABI 3130XL Genetic Analyzer (Applied Biosystems®), and subsequent analyses of the data were conducted in GeneMapper™ 5 (Applied Biosystems®).

2.4 | Population genetic analysis/ data analysis

We first checked our data set for identical genotypes as a result of accidental repeated sampling of the same individual with the 'Identity Analysis' function in Cervus v.3.0.7 (Kalinowski, Taper, & Marshall, 2007). We tested for Hardy–Weinberg equilibrium (HWE) and calculated descriptive statistics, including F-statistics, using the R package PopGenReport v.3.0.0 (Adamack & Gruber, 2014). Further, we tested for the occurrence of null alleles using MICRO-CHECKER v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004).

The population structure analysis was performed with STRUCTURE v.2.3.4 (Falush, Stephens, & Pritchard, 2003, 2007; Pritchard, Stephens, & Donnelly, 2000) using 1 million MCMC runs, based on the admixture and correlated allele frequencies model, a burn-in of 100,000 and 10 replicates of each possible number of clusters K from 1 to 6. To identify the optimal number of clusters K for our data set, we applied the delta K method (Evanno, Regnaut, & Goudet, 2005) implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). STRUCTURE runs for the chosen K were combined and the results visualized using the R packages 'pophelper' v.2.3.0 and 'pophelperShiny' v.2.1.0 (Francis, 2017). Further, we performed a hierarchical analysis of molecular variance (AMOVA) and calculated pairwise F_{ST} values using Arlequin v.3.5.2.2 (Excoffier & Lischer, 2010). To visualize the pattern of genetic distance between individuals of the three populations, we performed a principal coordinate analysis (PCoA) based on pairwise Euclidean distance using the R packages 'adegenet' (Jombart, 2008) and 'ade4' (Bougeard & Dray, 2018; Chessel, Dufour, & Thioulouse, 2004; Dray & Dufour, 2007; Dray, Dufour, & Chessel, 2007). All calculations were done using RStudio v.1.1.453 and R v.3.6.2.

3 | RESULTS

After removing identical genotypes ($N = 6$), 43 unique genotypes (northern: 10; central: 21; southern: 12) remained for the population genetic analysis. The number of alleles per locus ranged from 3 to 15 (mean: 7.8). Expected (H_E) and observed heterozygosity (H_O) ranged from 0.29 to 0.91 and from 0.23 to 0.79, respectively. All loci were in HWE, but seven loci showed signs of null alleles (Table 1). To be conservative, we excluded these loci from the subsequent analyses. Results of the analyses including all loci are provided as supplementary material (Figures S2 and S3).

The population genetic analysis revealed that $K = 3$ ($\Delta K = 560.47$) is the optimal number of clusters for our data set (Figure 2a). These three clusters correspond to

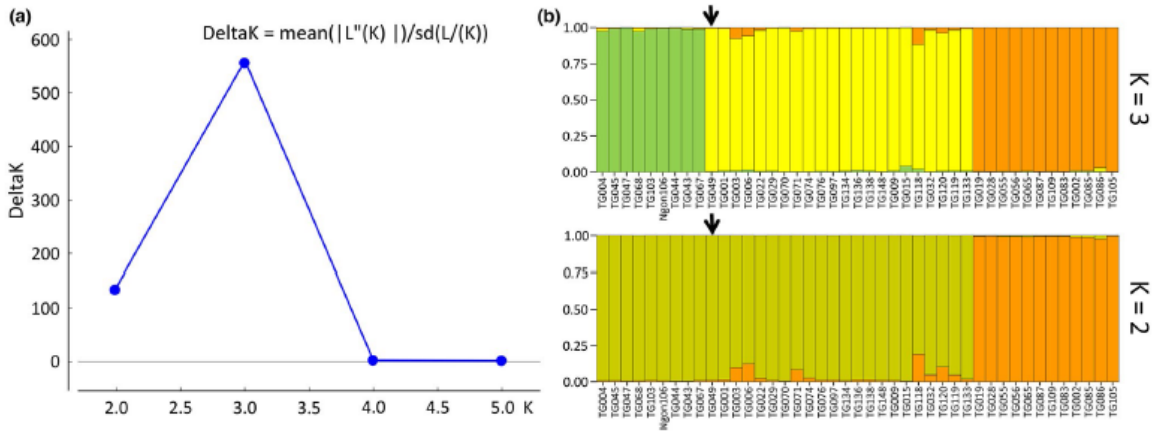

FIGURE 2 Population genetic structure of *Theropithecus gelada*, based on 13 loci with no signs of null alleles. (a) Optimal number of K clusters calculated by the Evanno method (Evanno et al., 2005). A three-cluster structure is indicated. (b) STRUCTURE results for $K = 3$ and $K = 2$ clusters. Colours correspond to mitochondrial haplogroup affiliation: black (green) = northern; light grey (yellow) = central; white (orange) = southern haplogroup; dark grey (brownish) = combined northern and central (coloured version available online). The arrows indicate sample TG049 [Colour figure can be viewed at wileyonlinelibrary.com]

the geographic sampling locations, that is to the respective demes, except for sample TG049, which was sampled in the north, but clusters with samples from the central deme (Figure 2b).

Pairwise F_{ST} values (Table 2) and the STRUCTURE analysis with $K = 2$ (Figure 2b) indicated a closer genetic relationship of northern and central samples to the exclusion of the southern samples. This was further supported by the results of the PCoA, where the southern samples were found to be separated from both other demes along the first coordinate of the PCoA. The second coordinate further separates the northern and central demes (with the exception of sample TG049; Figure 3). The first two principal coordinates of the PCoA, that is the ones with the highest eigenvalues, explained 22.2% and 10.1% of the variance, respectively. All following coordinates explained <7.0% of the variance. Individual scores on the first two principal coordinates for all individuals are provided in Table S3. A five-cluster pattern, as expected due to the five mitochondrial clades found in a previous study (Zinner et al., 2018), was not supported (Figures S2 and S4). STRUCTURE analyses of both the northern and central populations alone also did not reveal any further genetic differentiation (Figure S5).

The AMOVA revealed that most of the nuclear variance was attributed to the differences within populations (77.7%), but 22.3% could be attributed to differences among the three demes indicating overall strong genetic differentiation (Table 3).

TABLE 2 Nei's pairwise F_{ST} between all pairs of sampling areas

	North	Central	South
North	0.000		
Central	0.103	0.000	
South	0.364	0.255	0.000

Note: All F_{ST} values were significant ($p < .001$; 1,000 permutations)

4 | DISCUSSION

Despite recent mtDNA studies on geladas, their taxonomy and genetic population structure remain unclear (Gippoliti, 2010; Zinner et al., 2018). By assessing allele length polymorphisms of 20 nuclear microsatellite markers, we investigated the genetic structure among gelada populations across the Ethiopian highlands to further clarify the taxonomic status of the gelada evolutionary units.

The microsatellite data revealed a three-deme structure of the global gelada population. The three populations are geographically structured and broadly correspond to the distribution pattern of mitochondrial haplotypes (Shotake et al., 2016; Zinner et al., 2018). In our previous mtDNA study (Zinner et al., 2018; see also Figure S1), additional genetic structure became apparent: the northern and central demes contained two mtDNA clades each, dividing the global gelada population into five clades. However, we did not find a corresponding $K = 5$ cluster pattern with the nuclear DNA (Figure 2a and Figure S3). This is not surprising, given that there are no obvious geographical distribution patterns or barriers between the two respective northern and central

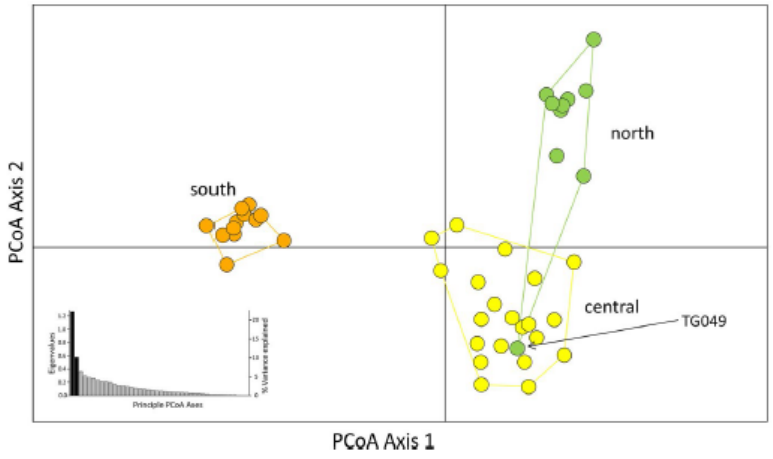

FIGURE 3 Principal coordinate analysis (PCoA) of pairwise genetic distances between individuals of the three sampling areas of *Theropithecus gelada*. Included are only loci without null alleles. Colours correspond to geographic origin of samples: black (green) = northern; grey (yellow) = central; white (orange) = southern deme and minimum convex polygons unite samples of the same geographic origin (coloured version available online). Inset: Eigenvalues of the principal coordinates indicating their corresponding variance explained by the PCoA. Black bars show the two axes represented in the main plot [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 AMOVA results for a 3-deme structure based on 13 microsatellite loci

Source of variation	*DF*	Sum of squares	Variance components	% variation
Among populations	2	70.193	1.14864	22.29
Within populations	83	332.283	4.00341	77.71
Total	85	402.477	5.15205	

Note: Fixation Index F_{ST}: 0.22295 ($p < .001$; 10,000 permutations).

mtDNA sub-clades (both are geographically mixed within their respective populations). Therefore, both the current nuclear DNA-based results and the earlier mtDNA data support three evolutionary units in gelada.

As indicated by the pairwise F_{ST} values, the PCoA and by the $K = 2$ cluster analysis, the most distinct subpopulation is the southern population from Arsi, south of the Rift Valley. Using genetic analyses of blood proteins, Belay and Shotake (1998) inferred that the southern and central populations must have been separated for several hundred thousand years with highly restricted gene flow. In addition, the divergence ages among the main mtDNA clades were estimated between 0.5 and 0.7 million years (Zinner et al., 2018). But, Belay and Shotake (1998) did not include any samples from the northern population in their study. In our previous study using mtDNA sequence data, we found weak support for the hypothesis that the northern population was a sister clade to the southern population (Zinner et al., 2018), but our microsatellite data did not support this hypothesis. By contrast, our analyses suggest a closer relationship between the northern and the central populations, resurrecting the distinctiveness of the southern population, a scenario which biogeographically is more likely. Our microsatellite data also did not suggest any further genetic structuring of both, the northern and central populations, as suggested by the occurrence of two mitochondrial clades in each of the two populations (northern 1, northern 2, central 1, central 2, see Fig. S1; Zinner et al., 2018).

Geographic provenance, haplogroup affiliations and microsatellite clusters correspond well for all gelada samples, with the exception of the single sample TG049. This sample was collected in the range of the northern deme, but contains a mtDNA haplotype identical to some found further south in the central deme (Zinner et al., 2018). According to microsatellite alleles, TG049 clusters perfectly with samples of the central deme. Therefore, both mitochondrial and nuclear data suggest that TG049 belongs genetically to the central deme. The available genetic information also does not indicate that individual TG049 is a hybrid between the northern and central populations. This could be expected if the ranges of the northern and central demes would overlap, thus providing opportunities for interbreeding. Alternatively, geladas from the central population may have been transferred to the northern population by humans. Infant geladas are often kept as pets, and when they become adult and are unable to be managed, they are in many cases released into the wild (Bergman & Beehner, 2013). For instance, one author (DZ) observed a tame gelada female in a group of hamadryas baboons in the vicinity of Asmara, the capital of Eritrea, far outside the current range of *Theropithecus*. Finally, it is also possible that the sample was wrongly labelled at some point during processing. However, until we find more individuals in the northern deme with a genetic make-up similar to that of the central deme, we regard TG049 as an artificial exception that does not contradict the three-deme population structure of geladas.

4.1 | Taxonomic and conservation considerations

Based on the correspondence of the mitochondrial and microsatellite analyses and the allopatric ranges of the three populations, we clearly show that there are three evolutionary units of geladas and, thus, solve the first major problem of a taxonomic classification, namely the grouping problem. However, a solution for the second problem, the ranking problem, largely depends on the applied species concept. Under a phylogenetic species concept (Cracraft, 1983), the three evolutionary units would probably be ranked as species, under a biological species concept (Mayr, 1942) they would most likely be classified as subspecies—the northern (*T. g. gelada*), the central (*T. g. obscura*) and the southern (tentatively *T. g. arsi*) subspecies. Nevertheless, for a thorough taxonomic decision, a comparative phenotypical description, particular of the southern population, is necessary.

Importantly, and irrespective of the taxonomic classification, all three populations need protection and should be treated as conservation units. According to IUCN, the global population of geladas is suspected to be decreasing, but in the absence of more detailed data regarding current geographic range and demographic trends, geladas are listed as 'Least Concern' (Gippoliti, Mekonnen, Burke, Nguyen, & Fashing, 2019). Similarly, *T. g. obscurus* is listed as 'Least Concern' (Fashing, Nguyen, Burke, Mekonnen, & Gippoliti, 2019a). In contrast, the conservation status of *T. g. gelada* is 'Vulnerable' owing to its more restricted range centred around the Simien Mountains (Fashing, Nguyen, Burke, Mekonnen, & Gippoliti, 2019b). The Arsi population has not yet been assessed for its conservation status. However,

due to its restricted range, small population size and a sizable human pressure (Abu, 2011), it is likely to be assessed as a 'Critically Endangered' subspecies (Bergman & Beehner, 2013). Thus, conservation measures appear most urgent for the small southern population in the Arsi Mountains.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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4. Chapter 2: Study II

A refined panel of 42 microsatellite loci to universally genotype catarrhine primates

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A refined panel of 42 microsatellite loci to universally genotype catarrhine primates

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Abstract

1. Microsatellite genotyping is an important genetic method for a number of research questions in biology. Given that the traditional fragment length analysis using polyacrylamide gel or capillary electrophoresis has several drawbacks, microsatellite genotyping-by-sequencing (GBS) has arisen as a promising alternative. Although GBS mitigates many of the problems of fragment length analysis, issues with allelic dropout and null alleles often remain due to mismatches in primer binding sites and unnecessarily long PCR products. This is also true for GBS in catarrhine primates where cross-species amplification of loci (often human derived) is common.
2. We therefore redesigned primers for 45 microsatellite loci based on 17 available catarrhine reference genomes. Next, we tested them in singleplex and different multiplex settings in a panel of species representing all major lineages of Catarrhini and further validated them in wild Guinea baboons (*Papio papio*) using fecal samples.
3. The final panel of 42 microsatellite loci can efficiently be amplified with primers distributed into three amplification pools.
4. With our microsatellite panel, we provide a tool to universally genotype catarrhine primates via GBS from different sample sources in a cost- and time-efficient way, with higher resolution, and comparability among laboratories and species.

KEYWORDS
apes, genotyping-by-sequencing, high-throughput sequencing, Old World monkeys, simple tandem repeats

1 | INTRODUCTION

Microsatellites have been and are still widely applied in various biological sciences including population genetics, kinship/pedigree analysis, human and wildlife forensics, linkage analysis, or disease association studies (e.g., Cunningham et al., 2001; Goodwin et al., 2011; Gulcher, 2012; Wasser et al., 2004). Population genetic information obtained by microsatellite genotyping is also

Franziska Trede, Niels Kil, and James Stranks shared first authorship.

important for monitoring wild populations in conservation contexts, for reintroduction programs or to refine captive breeding management (Arandjelovic & Vigilant, 2018; Norman et al., 2019). Microsatellites are also often the markers of choice to genetically characterize (wild) populations in order to determine degrees of population fragmentation and hybridization, dispersal patterns, mating systems, and reproductive success (e.g., Charpentier et al., 2012; de Moor et al., 2020; Ferreira da Silva et al., 2018; Kheng et al., 2017; McCarthy et al., 2020). The ongoing popularity of microsatellites is largely based on their high abundancy in animal genomes (Hamada et al., 1982; Tautz & Renz, 1984), the high levels of allelic diversity (Ellegren, 2000), and the possibility to amplify them across related species. Accordingly, microsatellites are preferred, for example over SNPs, because of their higher statistical power per locus and their cross-species amplifiability, particularly when applied to small sample size datasets as typically found in forensic and kinship studies (Barbian et al., 2018; Guichoux et al., 2011).

However, traditional microsatellite genotyping via fragment length analysis (FLA) using polyacrylamide gel or capillary electrophoresis has several disadvantages, such as fragment size homoplasy, allele calling difficulties (stutter and split peaks, off-target PCR products), laborious work and relatively high laboratory costs, as well as poor comparability of results among laboratories (De Barba et al., 2017; Guichoux et al., 2011; Pasqualotto et al., 2007). Even with attempts to improve PCR amplification and more accurate/reliable genotyping procedures (Arandjelovic et al., 2009; Buchan et al., 2005; Navidi et al., 1992; Sefc et al., 2003; Taberlet et al., 1996), many of the problems remained.

With microsatellite genotyping-by-sequencing (GBS) using high-throughput sequencing technologies most of the difficulties can be mitigated (Barbian et al., 2018; Johannesen et al., 2017; Pimentel et al., 2018; Vartia et al., 2016). For instance, with GBS the exact length of the microsatellite alleles can be determined, which is a typical problem of FLA genotyping, particularly when alleles differ by only one basepair (bp) (Barbian et al., 2018; Vartia et al., 2016). Moreover, the nucleotide sequence is revealed so that cryptic alleles (alleles with the same length but containing a nucleotide variant) can be detected, resulting in an increased number of alleles and consequently greater statistical power per locus.

Nevertheless, problems with null alleles due to relatively large PCR products and allelic dropout as a result of primers binding in unconserved regions remain with GBS (Pompanon et al., 2005). As many microsatellites can be cross-amplified in phylogenetically related species, primers designed for one species are often tested in related species and then applied if successfully amplified and informative (i.e., polymorph) (Barbara et al., 2007; De Barba et al., 2017). For example, various microsatellite loci characterized for humans can be successfully amplified in nonhuman catarrhine primates (Old World monkeys, apes) (Coote & Bruford, 1996; Ely et al., 1998; Kayser et al., 1996; Morin et al., 1998; Newman et al., 2002; Roeder et al., 2009; Smith et al., 2000) and have been used since then in numerous studies (e.g., Arandjelovic et al., 2014; Kopp et al., 2015; Minkner et al., 2018; Städele et al., 2019). Yet, attempts to reduce PCR product size or to adapt primers specifically to the study species have been rare (but see Bradley et al., 2000; Engelhardt et al., 2017; Inoue et al., 2016). Furthermore, various research groups use different panels of microsatellites preventing a direct comparison of results, particularly of measures such as genetic diversity and heterozygosity, which are important in a conservation context (Kolleck et al., 2013).

In our study, we aimed to establish a microsatellite panel to universally genotype catarrhine primates via GBS from different sample sources in a cost- and time-efficient way, with higher resolution, and comparability among laboratories and species. Therefore, we screened a total of 269 microsatellite loci, widely targeted in catarrhine primates, and designed conserved primers for 45 loci based on available catarrhine genomes. We then tested the new microsatellite panel in ten primate species representing all major lineages of Catarrhini and further validated their applicability to low-quality DNA samples using fecal samples of wild Guinea baboons (*Papio papio*).

2 | MATERIAL AND METHODS

2.1 | In silico selection of microsatellite loci

We screened 269 human microsatellite loci widely used in catarrhine population genetic studies. We extracted the human (GRCh38/hg38) sequence of each locus with 500 bp flanking regions from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and performed BLAT searches against the 16 available (status: 5 December 2018) nonhuman Catarrhini reference genomes (Table S1) using the UCSC (http://genome.ucsc.edu) or Ensembl (www.ensembl.org) genome browsers with standard settings. In addition, we checked the human sequence for repetitive elements (SINEs, LINES, etc.) in flanking regions using the RepeatMasker Web Server (http://www.repeatmasker.org/) with standard settings. We generated alignments for each locus containing the 16 nonhuman catarrhine species, the human, and the human repeat-masked sequences with Muscle 3.8.31 (Edgar, 2004) in SeaView 4 (Gouy et al., 2010) and added published primer sequences to the alignments.

Loci were selected for further analysis if they fulfilled the following criteria: (a) primer binding sites are not in repetitive elements thus increasing locus-specific amplifiability and reducing the risk of off-target PCR products particularly in multiplex PCR reactions; (b) primer binding sites are conserved among catarrhines so that loci can be universally amplified in this taxonomic group with >180 species (Mittermeier et al., 2013); (c) the microsatellite motif is relatively short (max. 150 bp) to allow small amplicon size (max. 250 bp) and increase locus amplification success from degraded DNA samples, such as fecal samples; and (d) loci are evenly (1–3 loci per chromosome) distributed throughout the genome (using the genomes of *Homo sapiens*, *Nomascus leucogenys*, *Macaca mulatta*, and *Chlorocebus sabaeus* as reference) to avoid potential linkage problems.

For loci which passed the selection criteria, we designed new primers using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To allow for multiplexing, primers were designed to have similar annealing temperatures. Locus specificity of primers was checked by BLAT search against the 17 available catarrhine genomes. As primer binding sites were not always fully conserved among the 17 catarrhines, primers of 21 loci were designed with wobble positions. To simplify library preparation for GBS, we added adapter nucleotide sequences to the 5′ end of the locus-specific primers (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′ to forward primers, 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′ to reverse primers; locus-specific primers are provided in Table S2).

2.2 | Laboratory work

First, we tested in singleplex reactions for the locus specificity of selected primers and their universal applicability to catarrhine species in a panel representing all major lineages of catarrhines (Table 1). High-quality DNA from a male of each of the ten species was obtained from the Gene Bank of Primates at the German Primate Center. PCRs were performed in total volumes of 25 µl containing 1× Qiagen Multiplex PCR Master Mix (Qiagen), 0.4 µM of each primer, and 50 ng genomic DNA. Cycling conditions comprised of 15 min at 95°C, 30 cycles each with denaturation at 94°C for 30 s, annealing at 57°C for 90 s and extension at 72°C for 90 s, and a final extension step of 10 min at 72°C. All reactions were run together with no-template controls (NTCs) to check for contamination. PCR performance was checked on 2% agarose gels stained with ethidium bromide (Carl Roth GmbH). Sequencing of singleplex PCR reactions was omitted.

Next, we tested for the possibility of running multiplex PCR reactions to reduce overall laboratory work and costs. Therefore, we pooled either all 45 primer pairs in a single PCR reaction (1-pool approach) or divided them into five PCR reactions each containing nine primer pairs (5-pool approach) or three PCR reactions containing 18 and 2 × 12 primer pairs (3-pool approach; for rationale of pooling and locus exclusion see Results section). Amplifications were conducted as described for the singleplex PCRs (same PCR set-up, DNA samples, cycling conditions, NTCs), but with different primer concentrations (see Tables S3–S5 for pooling schemes and concentrations of single primers within pools). To minimize PCR errors, we ran PCR reactions in two independent replicates. PCR performance was again checked on 2% agarose gels. Replicate PCR products (including the NTCs) were pooled and then cleaned with the MinElute PCR Purification Kit (QIAGEN). DNA concentrations were measured with a Qubit 3.0 (Thermo Fisher Scientific) and 100 ng were subjected to indexing PCR. Indexing PCR was performed in total volumes of 25 µl containing 1× KAPA HiFi HotStart ReadyMix (Roche), 0.4 µM of each indexing primer and 100 ng purified PCR product. Cycling conditions comprised of 45 s at 98°C, 4 cycles each with denaturation at 98°C for 15 s, annealing at 62°C for 30 s and extension at 72°C for 30 s, and a final extension step of 1 min at 72°C. Subsequently, indexed PCR products were purified with the MinElute PCR Purification Kit (Qiagen) and ran on a Bioanalyzer 2100 (Agilent) to check for PCR performance and molarity. Libraries were diluted to a final concentration of 10 nM and then pooled and sequenced with 51 cycles forward and 251 cycles reverse on Illumina's MiSeq desktop sequencer.

To check for Mendelian inheritance and whether our new microsatellite panel is also applicable to low-quality and low-quantity DNA as typically extracted from fecal samples (Monteiro et al., 1997; Perry et al., 2010), we tested our panel in 12 fecal samples of wild Guinea baboons. The samples comprised of six males and two "families" each composed of a male, a female, and their known offspring. DNA from these 12 specimens was previously genotyped via FLA at 24 microsatellite loci (Dal Pesco, 2019). The amplification procedure and follow-up steps for the applied 3-pool approach were the same as described above, but the number of cycles in the initial amplification was increased to 40, the total DNA amount was increased to 200 ng, and each PCR was performed in triplicates (Barbian et al., 2018).

2.3 | Bioinformatic analysis

The data analysis was performed using the software package CHIIMP v.3.0.0 (Barbian et al., 2018). The raw data (FASTQ files) as well as all input files (config-file, sample-file, locus-attributes-file) are available in the online supplement resources. As our microsatellite

TABLE 1 Catarrhine species used to test the new microsatellite panel

Family	Subfamily	Tribe	Species
Hominidae	Homininae		*Pan troglodytes*
			Gorilla gorilla
	Ponginae		*Pongo abelii*
Hylobatidae			*Hylobates lar*
Cercopithecidae	Colobinae	Presbytini	*Trachypithecus obscurus*
			Pygathrix nemaeus
		Colobini	*Colobus guereza*
	Cercopithecinae	Cercopithecini	*Cercopithecus diana*
		Papionini	*Papio papio*
			Macaca mulatta

TABLE 2 Number of amplified loci and alleles, as well as the level of heterozygosity per species generated in three approaches with high quality DNA (blood) and degraded DNA from fecal samples

	High-quality DNA			Degraded DNA
	5-pool approach (45 loci)	1-pool approach (45 loci)	3-pool approach (42 loci)	3-pool approach (42 loci)
Mean number of loci amplified per sample/species (range)	40.2 (37–43)	25.9 (21–32)	37.8 (33–41)	38.8 (35–41)
Mean number of alleles amplified per sample/species (range)	60.1 (53–69)	36.9 (31–45)	55.5 (46–68)	52.2 (47–56)
Mean level of heterozygosity per sample/species (range)	49.8% (23.3%–69.4%)	43.5% (22.6%–71.4%)	47.1% (19.5%–66.7%)	34.3% (23.1%–47.4%)[a] 46.3% (31.0%–64.3%)[b]

[a]Including all 42 loci.

[b]Including only the 32 loci that were polymorphic in the study species.

panel included several di-repeat loci, which stutter more frequently than tetra-repeats, we increased the stutter count ratio to 0.70 (stutter.count.ratio_max: 0.7). We further implemented a broad range of possible allele lengths in the locus attributes by setting the length buffer to 100 bp. This ensured the inclusion of all tested species even if the allele sizes at a given locus varied between species according to the available reference genomes. The minimum number of reads per locus was set to 100 (counts.min: 100). All other parameters were set to default.

With the current version of CHIIMP, wobble positions in primer sequences cannot be accounted for. Hence, for loci with a wobble position in a primer sequence, alternative nucleotides of the wobble are erroneously recognized as different alleles. Moreover, the repeat motif needs to be specified in CHIIMP, but as repeat motifs can vary in the investigated species, correct (orthologous) reads remain unrecognized for some species if CHIIMP is fed with a wrong repeat motif. Due to these reasons, the output for all loci was checked manually and corrected if needed. Additionally, we screened the processed reads for the general level of amplification per locus and the occurrence of PCR artifacts (off-target amplification, primer dimer, false primer pairings, etc.).

3 | RESULTS

3.1 | In silico selection of microsatellite loci

In total, 217 of the 269 investigated loci were not optimal for microsatellite genotyping of catarrhines. For 147 of them, one or both primer binding sites or the complete locus were located in repetitive elements. This increases the likelihood to amplify various off-target PCR products, particularly in multiplex settings when many primers that can bind multiple times in the respective genome are combined in a single PCR reaction. For an additional 32 loci, we could not find conserved primer binding sites near the microsatellite and a further 15 loci contained relatively long microsatellite repeat regions for one or more species, resulting in long PCR products (>250 bp). Longer PCR products are often difficult to generate if only degraded DNA material is available and can result in null alleles. Further problems included, for instance, the location of loci directly next to each other on the same chromosome and thus increasing the risk of linkage. Additionally, double entries of loci under different names or gaps in some of the reference genomes (especially for Y-chromosomal loci) impeded the screening process. A full list of screened loci including the respective reasons for their exclusion is provided in Table S6. Of the 52 loci which fulfilled our criteria, we selected 45 (1–3 loci per human chromosome including gonosomes) for downstream analyses. The chromosomal locations of the chosen loci in the genomes of *H. sapiens*, *N. leucogenys*, *M. mulatta*, and *C. sabaeus* are provided in the supplement (Table S2). We found no indication for the presence of linkage between any of the loci in any of the four investigated species (minimal distance between two loci 5.35 million bp).

The newly designed primers for the 45 loci (consisting of di-, tri-, and tetra-repeats) amplify PCR products between 56–215 bp (according to available genome data; Table S2). Compared to the original published primers, we were able to reduce PCR product sizes by 2–225 bp (mean 75.9 bp) in 37 loci whereas for five loci, the new primers amplify a moderately longer fragment (elongation by 2 – 15 bp; mean 7.6 bp). PCR product size for the remaining three loci did not change. As primer binding sites were not always perfectly conserved among the 17 investigated catarrhine reference genomes, primers for 21 loci contain wobble positions. Mismatches in primer binding sites, found only in a few (1–2) of the investigated species, were neglected in primer design and probably result in less efficient or no amplification of the respective locus in the given species (0–12 loci with mismatches per species, mean 3.4; Table S2).

3.2 | Singleplex PCR test

Singleplex PCR reactions of the 45 loci in ten species representing all major lineages of catarrhines were run on agarose gels and resulted, for all loci and species, in PCR products within the expected size range with no signs of amplifying any off-target PCR products (data

not shown). Thus, locus specificity and universal applicability of our primer set to catarrhine primates was indicated.

3.3 | Multiplexing approaches

Sequenced alleles ranged in size from 71 bp (D3s1768) to 211 bp (D12s372) and nine loci contained cryptic alleles in at least one species (Tables S7–S10). The level of amplification and obtained sequence reads varied across samples/species and loci in all three approaches. The amplification of all loci in one pool (1-pool approach) was least effective, resulting in the lowest number of amplified loci (mean 25.9) and alleles (mean 36.9; Table 2). In some cases, the reason for allelic dropouts could be attributed to wrong primer pairing/primer mismatches (primer dimer or off-target amplification of short products). Most loci amplified less efficiently than in the other two approaches and some (N = 11) failed to amplify at all. Only nine loci recovered the same number of alleles as in the 5-pool approach. Interestingly, even though the number of amplified alleles was reduced from a mean of 60.1 to 36.9 compared to the 5-pool approach, the level of heterozygosity was not affected to the same extent with a reduction from 49.8% to 43.5% (Table 2).

The best results, that is, the highest amplification levels for loci (mean 40.2) and alleles (mean 60.1), were generated applying the 5-pool approach (Table 2). Nevertheless, we observed again primer dimers and short off-target PCR products potentially as a result of interacting primers from different loci. Moreover, three loci (D11s1366, D12s67.2, and D15s1007) neither amplified in the 1-pool nor in the 5-pool approach and were excluded from further testing. To further improve amplification success and to reduce primer interactions among primers of different loci (based on the knowledge obtained from the 1-pool and 5-pool approaches), we distributed the 42 remaining loci into three amplification pools containing 18, 12 and 12 loci, respectively (Table S5). Using the 3-pool approach, we were able to largely minimize primer interactions, but amplification success for loci (mean 37.8) and alleles (mean 55.5) per species was slightly reduced compared to the 5-pool approach, but higher than in the 1-pool approach (Table 2). The reduced amplification success was due to allelic dropouts of single alleles or whole loci in some species (see Table S9).

3.4 | Degraded DNA samples

For the degraded DNA samples, we applied the 3-pool approach as this represented the best compromise between amplification efficiency and laboratory effort and costs (see Results Multiplex approaches). The amplification from fecal samples was successful except for four (out of 42) loci (two autosomal and two gonosomal loci; Table S10). The number of loci and alleles amplified per sample was comparable to the results obtained from high-quality DNA samples (Table 2). However, 10 of the 42 amplified loci were monomorphic in our *P. papio* population, that is, all twelve individuals showed the same allele. The remaining 32 loci showed a level of 46.3% heterozygosity (Table 2).

All autosomal loci were in accordance with Mendelian inheritance besides D7s503 and D13s1291. For D7s503, the two alleles with the highest read counts for male MRX (99/109) did not match one of the two alleles of his offspring THL (111/113; mother MMI: 103/113). A closer look at the data revealed that MRX also had many reads for allele 111 (only 23 reads less than for allele 109), indicating that the allele 109 of MRX was likely an overamplified stutter sequence. A genotype with the allele combination 99/111 also corresponds to the genotype derived from FLA for this locus (152/164; 12bp distance between alleles, based on >20 amplifications; Dal Pesco, 2019). For D13s1291, the two alleles of male MLK (130b/132b) did not match with his offspring PTC (130a/132a; mother LCY: 128/130a). MLK was the only individual with these two potential cryptic alleles (each with a G→A point mutation) and further showed reads with 130 and 132 bp length without this mutation. At this point, we can neither exclude the occurrence of a PCR artifact in this particular case, nor that this locus does not follow the rules of Mendelian inheritance.

4 | DISCUSSION

From a set of 269 microsatellite loci widely applied in catarrhine primates, we selected a total of 45 loci that can be universally applied to genotype catarrhine primates. Due to the relatively small amplicon sizes, even low-quality DNA could be genotyped and since the selected loci were evenly distributed throughout the genome (at least according to the human genome), the risk of linkage was significantly reduced. Moreover, our panel could be multiplexed to a great extent. The testing of different multiplex settings revealed that a 5-pool approach produced the best result, but that a 3-pool approach containing one pool of 18 and two of 12 loci is the best compromise between locus amplification efficiency and laboratory effort and costs.

We tested the panel with high-quality DNA samples from all major lineages of catarrhines in multiplex settings and revealed successful amplification rates of 33 to 41 (average 38) loci per species (Table 2). We additionally showed the applicability of the 3-pool approach to degraded DNA samples such as fecal samples, which is a common material in many noninvasive wildlife studies (Carroll et al., 2018; Waits & Paetkau, 2005). The results for fecal samples were similar to the results of the high-quality samples with respect to the mean number of loci and alleles amplified per sample (Table 2). All loci, besides D13s1291, were in accordance with Mendelian inheritance, demonstrating the suitability of the new microsatellite panel for kinship and relatedness analyses. To ensure high-quality genotypes from fecal samples, and depending on DNA quality, further adaptions to the protocol might be necessary. Quantifying the endogenous DNA content via quantitative PCR prior to genotyping will help to select only those samples with sufficient endogenous DNA content (e.g., >25 pg endogenous

DNA as suggested by Barbian et al., 2018). Additionally, multiple samples per individual can be analyzed or the number of PCR replicates per sample can be increased.

Through multiplexed GBS, cryptic alleles can be detected (Barbian et al., 2018; Sarhanova et al., 2018; Vartia et al., 2016), and even in our test panel of only ten catarrhine species with one individual each, we found cryptic alleles at nine loci (Tables S7–S10). Although our results are based on only two or three replicates per approach (depending on the sample type) and hence should be interpreted with caution, we are confident that these alleles are indeed cryptic alleles and not PCR artifacts. In case of PCR artifacts, we would expect mixed sequence reads showing more than two alleles or highly imbalanced sequence read counts for the "true allele" and the "artifact allele," as it is highly unlikely that the same PCR artifact occurs in all replicates. As more individuals per species get tested, the number of cryptic alleles will most likely increase and provide further accuracy and a higher statistical power of our panel.

Another advantage of GBS is that the resulting genetic data, in form of allele sequences, are independent of the used sequencing platform. Thus, data produced by different laboratories can be easily shared and compared. By applying validated bioinformatics pipelines, such as the CHIIMP pipeline (Barbian et al., 2018), one can further ensure that the resulting data are reproducible and less prone to arbitrary allele calling by different researchers while still allowing the customization of, for example, filtering parameters to fit different datasets.

Although we recommend the 3-pool approach, the amplification success of individual loci can be improved, for example, by amplifying all loci in individual reactions and then pooling before or after the indexing PCR. However, this would largely increase workload in the laboratory and costs. It is also important to check which loci are polymorphic in the species of interest, so that monomorphic loci can be excluded from large-scale population genetic investigations. Likewise, as several species exhibit mismatches in primer binding sites (0–12 loci with mismatches per species), primer design for a given species can be adjusted and optimized, which becomes easier to do with an increasing number of sequenced catarrhine genomes.

In summary, with our microsatellite panel, we provide a tool to universally genotype catarrhine primates via GBS from samples of varying DNA quality in a cost- and time-efficient way, with higher resolution, better comparability among laboratories, and largely mitigated problems of traditional FLA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Franziska Trede: Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal). **Niels Kil:** Investigation (equal); Writing-review & editing (equal). **James Stranks:** Investigation (equal); Writing-review & editing (equal). **Andrew Jesse Connell:** Software (lead); Writing-review & editing (equal). **Julia Fischer:** Funding acquisition (equal); Writing-review & editing (equal). **Julia Ostner:** Funding acquisition (equal); Writing-review & editing (equal). **Oliver Schülke:** Funding acquisition (equal); Writing-review & editing (equal). **Dietmar Zinner:** Writing-original draft (equal); Writing-review & editing (equal). **Christian Roos:** Conceptualization (equal); Investigation (supporting); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The sequencing data have been submitted to the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA672243 (http://www.ncbi.nlm.nih.gov/bioproject/672243). Input files for the bioinformatics analysis (config-, sample-, and locus-attributes-files) are available online in the supporting information (Appendix S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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5. General Discussion

5.1. Summary of results

In this thesis, I investigated the usage and usefulness of microsatellites in the analysis of genetic variation in non-human catarrhine primates, with a focus on baboons and geladas. In the first study, I showed that the cross-species amplification of microsatellites fitted for the genus *Papio* is an easy and fast approach to generating data on nuclear genetic variation in different populations of the genus *Gelada*. Further, the results confirmed a three-deme population structure and therefore provide additional support for the existence of three evolutionary units in geladas as was suggested by previous studies based on mtDNA data (Shotake et al., 2016; Zinner et al., 2018a). Although no higher resolution of the population structure was possible, these results are informative for future decisions in conservation management and the taxonomic ranking of geladas.

In the second study, I presented the development and validation process of a microsatellite panel for application in all catarrhine primates. The final 42 microsatellite loci are conserved between the species of this parvorder and produce short amplification products. In combination with a GBS approach, this panel allows for the fast and accurate generation of nuclear genetic data for catarrhines from various sample materials including low-quality DNA such as from feces. This was validated by testing the panel on blood samples spanning all major catarrhine lineages and a set of fecal samples from wild Guinea baboons. To test the statistical power of the panel in the catarrhine species, also in comparison to previously established ones, more samples per species need to be analyzed in future studies.

5.2. Advantages of microsatellites as genetic markers

The advantages of microsatellites as genetic markers can be quickly summarized: they are very short, highly polymorphic, and can often be amplified across closely related species (Guichoux et al., 2011). Because they are short, they can be analyzed from a wide range of sampling materials including samples of low DNA quality and quantity like feces, hair, feathers, and urine as often found in wildlife studies (Waits & Paetkau, 2005). Due to their high variability, a moderate number of loci is often sufficient to answer questions in a wide variety of biologic research fields (Lepais et al., 2020) whereas for example, many more di-allelic SNP loci would need to be analyzed to reach the same statistical power (Butler et al., 2007; Ross et al., 2014; Städele & Vigilant, 2016). With the implementation of NGS techniques into the workflow of microsatellite analysis, the statistical power of loci increases even more as sequencing data

can reveal additional nucleotide polymorphisms previously undetected by classic fragment length analysis (Darby et al., 2016; Sarhanova et al., 2018; Vartia et al., 2016). The proportion of newly identified, former cryptic alleles varies between study species but has been reported to be as high as 31% in a study on chimpanzees (*Pan troglodytes*; Barbian et al., 2018), 32% in Atlantic cod (*Gadus morhua*; Vartia et al., 2016), 44% in muskrats (*Ondatra zibethicus*; Darby et al., 2016), and 53% for a study in the mushroom *Armillaria ostoyae* (Lepais et al., 2020).

The ability to be amplified across species makes it easy to generate nuclear genetic data even if, for the species of interest, no reference genome data is available. As the results of the study, I show, 20 of the 24 microsatellites previously used in studies on Guinea baboons (Dal Pesco et al., 2021; Fischer et al., 2020) could be readily applied to samples of geladas without species-specific adaptations, and using the same laboratory protocols which allowed for the fast generation of nuclear genetic data. The usage of the same loci in different species additionally facilitates an easier and more direct comparison of data such as levels of heterozygosity or allelic diversity. Contrary, SNP data is often generated in species-specific assays as coincident SNPs across species are much rarer (e.g., Malhi et al., 2011: Old World monkeys) and hence cannot be directly compared (Barbian et al., 2018). However, to compare or even merge microsatellite data sets, one has to make sure that the loci were analyzed in the same way, e.g., with the same primer pairs, and/or that the datasets were calibrated. Therefore, the microsatellite panel developed in study II is a great tool to improve the data sharing and comparison in non-human catarrhine primates as it includes 42 loci that can be amplified across all catarrhine species. Again, the implementation of NGS techniques into the analysis workflow greatly improves the sharing and comparison of data as the generated data is independent of the sequencing platform and not prone to arbitrary allele calling. Further, the application of NGS increases the efficiency of microsatellite genotyping as more data can be generated in a shorter time frame with higher accuracy (Barbian et al., 2018; Bradbury et al., 2018).

5.3. Limitations of microsatellites as genetic markers

Despite their clear advantages, microsatellites also have their limitations and pitfalls. For one, the process of loci selection for a panel often introduces an ascertainment bias as only highly polymorphic loci are included (Brandstrom & Ellegren, 2008; Pardi et al., 2005). Therefore, microsatellite diversity may be less sensitive to genome-wide levels of genetic diversity (Väli et al., 2008). Indeed, by comparing the levels of genetic diversity estimated by microsatellites and multiple noncoding sequences of the genome in eight

carnivore populations, Väli and colleagues (2008) found that, although there is a positive correlation on the population level, the magnitude of variation in genetic diversity estimated by the non-coding sequences was substantially higher than for estimates based on microsatellites. Hence, two populations with the same level of microsatellite heterozygosity may significantly differ in their overall levels of genomic diversity (Väli et al., 2008). An ascertainment bias has also been recognized for studies based on other DNA markers such as SNPs (Clark et al., 2005; Nielsen, 2004). Such a bias might also have been introduced into the panel created in study II as during literature research we filtered for loci that had been reported to be polymorphic in at least one catarrhine species. However, the bias should be reduced as further selection criteria focused solely on the presence/absence of repetitive elements (LINEs, SINEs, etc.) in the primer binding sites, conserved primer binding sites among catarrhines, the length of the microsatellite repeat motif and the distribution of candidate loci throughout the genome without any knowledge on the level of diversity in all included species. In fact, ten of the 42 loci are so far monomorphic for Guinea baboons based on the sample set (N=12) analyzed in study II. This might either be due to the small samples set which also included close relatives, or it might reflect a loss of allelic diversity during the evolution history of Guinea baboons. To answer this question, more samples of (unrelated) Guinea baboons and other baboon species need to be analyzed and results compared. Our new microsatellite panel represents a perfect tool for that.

A further problem arises for the application of microsatellites in highly inbred populations as the statistical power of microsatellite analysis decreases with increasing inbreeding due to a loss of allelic diversity. In study I, for example, the mean allelic richness (AR) is roughly halved for the southern gelada population (AR=2,3) compared to the northern (AR=5,2) and central (AR=5,3) populations. This is interesting and informative in a comparative context, likely reflecting inbreeding in the southern population due to the small population size (Abu, 2011) and the geographic isolation from the other populations (Mori & Belay, 1990; Mori & Belay, 1991) preventing gene flow between them. Yet, this also drastically reduces the statistical power of the microsatellite loci to, for example, discriminate between individuals and assign parentages in the southern population. However, this problem is not limited to microsatellites and would be the same for other genetic markers too. To compensate for this loss of statistical power, one can increase the number of analyzed loci (if available). However, this can also increase costs and labor and the relation of costs and benefits should be considered when making decisions about which loci to analyze. Even with a panel of highly polymorphic loci, there is a limit to the resolution microsatellites can provide. For instance, although microsatellites are well suited for the application in paternity testing and pedigree building, they deliver less clear data in the analysis of

relatedness between individuals. Especially the estimation of dyadic relatedness values between individuals and the inference of kin categories from such values has been proven to be imprecise and erroneous (Städele & Vigilant, 2016; Van Horn et al., 2008). This problem is further elevated for samples with complex kinship structures, e.g., with co-residence of close and distant relatives within groups as well as overlapping generations, as is often the case in natural populations (Städele & Vigilant, 2016). Therefore, microsatellite data is thought to be only sufficient for the assessment of relatedness and kinship in more coarse-grained questions, e.g., to test for different levels of mean genetic relatedness within and among social groups. However, it is advised to refrain from inferring strict kin categories such as full-sibling and half-sibling (Städele & Vigilant, 2016; Van Horn et al., 2008).

Additionally, as microsatellites analyzed in population genetic studies are considered to be selectively neutral and independent of each other, each locus will experience a different level of genetic drift and mutation rate (Väli et al., 2008). Therefore, the estimated level of genetic variation can always only be an average across the selected loci. The same is true for other DNA markers, however, SNPs have been proposed to be more representative of the entire genome as normally many more SNP loci are genotyped that are randomly spread over the genome (Guichoux et al., 2011; Morin et al., 2004; Städele & Vigilant, 2016).

As mentioned in the introduction, a loss of genetic variation is often associated with a loss of evolutionary potential and therefore a decrease in population fitness and an increased extinction risk (Frankel & Soulé, 1981; Frankham, 1996, 2005; Väli et al., 2008). However, it has been questioned whether the genetic variation estimated by selectively neutral markers, such as microsatellites, correlates with fitness and viability in populations (Reed & Frankham, 2001). Indeed, some studies already showed that a low microsatellite diversity within a population is not necessarily correlated with a low viability of that population. For example, brown bears (*Ursus arctos*) on the Kodiak Island showed extremely low levels of microsatellite diversity in comparison to other brown bears of the North American range. Despite these low diversity estimates and isolation from other populations for thousands of years, the respective population was thriving and showed no signs of a population decline (Paetkau et al., 2008). On the other hand, a high microsatellite diversity does not guarantee a high adaptive potential within a population as shown by a study in the Australian tropical rainforest fly *Drosophila birchii* (Hoffmann et al., 2003). Hence, neutral genetic markers alone seem insufficient to make broad assumptions about the viability of populations and to infer their potential to adapt to possible environmental changes (Hohenlohe et al., 2021; Reed & Frankham, 2001). To estimate the adaptive potential of a population in a conservation

context, the analyses of ecological functional traits (or qualitative traits), i.e., coding genes and the influence of their variation and dynamics on fitness traits, is of great importance (Hohenlohe et al., 2021; Morin et al., 2004). For this, prior knowledge of gene functions is needed and data analysis will require extensive sequencing efforts. In the absence of such knowledge or data, microsatellites and other neutral DNA markers can only serve as substitutes to roughly estimate genetic variation, but data should be interpreted with caution. Here, the rate at which variation has been lost may be as, or even more, important than the current absolute level of variation itself (Paetkau & Strobeck, 1994). During a slow decline in genetic diversity over several generations, populations might still be able to adapt to environmental changes and purge deleterious alleles for genes, while a rapid decline increases the risk of deleterious alleles getting fixed in the population by chance, leading to reduced fitness (Hohenlohe et al., 2021). To facilitate such investigations, populations must be monitored and sampled at different time points, i.e., across generations. Alternatively, museum specimens represent a valuable source for historic levels of genetic diversity and can greatly contribute to the overall level of information (e.g. Bryant et al., 2016; Thalmann et al., 2011).

6. Conclusion and Outlook

Microsatellites will continue to play a role in the future of genetic analyses in wildlife science. Their ongoing popularity is mostly built on the ease of implementation, application, and analysis which is reflected in a still-growing number of recent publications applying microsatellites in genetic analyses of different species (e.g., Rimlinger et al., 2021 in the African plum tree [*Dacryodes edulis*]; Sarano et al., 2021 in sperm whales [*Physeter macrocephalus*]; Singh et al., 2021 in red muntjacs [*Muntiacus muntjak*]). In addition, new microsatellite panels are created even for the classic approach using CE for analysis (e.g., Fazzi-Gomes et al., 2021). Incorporating the GBS techniques into the workflow now additionally ensures more accurate and comparable data and is therefore a highly attractive and favorable alternative to the classic approach using CE. When switching to the application of GBS methods, researchers can decide to stick to the microsatellite loci that they analyzed in prior studies using CE which facilitates an easier integration of previously collected long-term genetic data (e.g., Barbian et al., 2018; Gruenthal & Larson, 2021). However, one has to keep in mind that this old long-term genetic data probably underestimates the genetic variation (due to undetected allele variants) which needs to be accounted for. Further, recent studies could show that relying on primer pairs previously developed for CE generally resulted in high levels of missing data when using GBS methods (Bradbury et al., 2018; Lepais et al., 2020; Vartia et al., 2016). Hence, a growing number of recent studies opted for the development of new microsatellite panels which allow the inclusion of many more loci than in previous studies, spanning larger parts of the genome and resulting in increased resolution and statistical power of the respective panel (e.g., Curto et al., 2019; De Barba et al., 2017; Tibihika et al., 2018). This technical update of microsatellite genotyping, which will likely become a new standard, greatly benefits from the decreasing costs of high throughput sequencing (HTS) and the growing availability of reference genomes that can be screened for suitable loci (Hohenlohe et al., 2021).

Despite these great improvements in the analyses of microsatellites, the sole use of microsatellite amplification for the analysis of genetic variation will dwindle and be limited to certain research questions and tasks in the future. As described in chapter 5.3, microsatellites do have their limitations and whether they should be applied in a particular case will be dependent on many different factors such as the available resources (e.g., money, time, equipment, reference genomes) and the amount of data required to address a specific research question (Flanagan & Jones, 2019; Guichoux et al., 2011; Hohenlohe et al., 2018). If, for example, the personal identification of individuals or the confirmation of parentage is of interest, a set of polymorphic microsatellite loci will certainly be fit for the task. As was shown in study I,

they can also be used to characterize the current population structure to a certain degree. Here, the aim was to investigate whether the nuclear genetic variation correspondents to a population structure with three gelada subspecies (or evolutionary units) that was proposed by previous analyses of mtDNA (Shotake et al., 2016; Zinner et al., 2018a). In this case, the statistical power of the data provided by microsatellites was certainly enough to answer that specific question. However, the microsatellite data alone cannot provide any additional information on the viability of the respective populations and possible adaptations to their environment which might be of interest in future studies concerned with the conservation management of this species.

Further, there is a growing agreement that fitness indicators of populations, such as the level of inbreeding and relatedness are better quantified by genomic estimates than for example by pedigrees (Kardos et al., 2015). For instance, recent studies applied hundreds to thousands of genome-wide SNPs in kinship analyses to assess levels of relatedness and inbreeding with a higher resolution and accuracy than it is possible with a moderate number of microsatellites (e.g., Andrews et al., 2018; Hoffman et al., 2014; Kleinman-Ruiz et al., 2017; Premachandra et al., 2019; Snyder-Mackler et al., 2016). Additionally, a recent study in male rhesus macaques (*Macaca mulatta*) showed that the sequencing of whole genomes, even with low coverage, enabled the accurate estimation of pairwise relatedness and the recovery of even distant relationships by analyzing genomic segments shared between individuals and identical-by-descent (Petty et al., 2021). Similarly, recent studies on gene flow, hybridization, and admixture relied on the application of genome-wide SNP loci for increased resolution. For example, Leitwein and colleagues (2018) analyzed the extant of admixture resulting from the introduction of domestic strains into wild populations of brown trouts (*Salmo trutta*). Another study, using whole genome data of all extant baboon species, investigated their complex population history which included multiple episodes of admixture and introgression based on SNP and *Alu* elements (Rogers et al., 2019). As these examples show, genome-wide sequencing data can enable more detailed and in-depth analyses of genetic variation within and among populations and species. Sequencing of whole genomes (WGS) will further be favored in the future, as the resulting data can be used for all kinds of downstream analyses (Snyder-Mackler et al., 2016). For example, the data can be filtered for SNPs (or any other DNA markers) in non-coding parts of the genome to look at the neutral genetic variation and, for instance, identify individuals, determine paternities, or estimate relatedness. However, the same data can also be filtered for alterations in protein-coding sequences that are predicted to result in functional variation. This latter data would be of special interest in studies concerned with the adaptive potential of populations which determines their future viability (Hohenlohe et al., 2021). This fine-scale view of the genome can potentially identify genomic

areas with, for example, high allelic diversity, an accumulation of deleterious alleles, or divergent adaptations. This can, in turn, inform conservation management actions, e.g., to find a potentially suitable source population for translocations of animals and to monitor the focal population after the implementation of conservation actions (Hohenlohe et al., 2021; Miller et al., 2012).

Similar to the GBS of microsatellites, WGS benefits from decreasing sequencing costs and the parallel progress in the development of analytical and bioinformatic methods for data evaluation (Flanagan & Jones, 2019; Petty et al., 2021; Snyder-Mackler et al., 2016). Further, new laboratory methods for the analysis of low-quality DNA, like fecal samples, are constantly developed and improved (e.g. Chiou & Bergey, 2018; Orkin et al., 2021; Snyder-Mackler et al., 2016; White et al., 2019) making it easier to analyze non-invasively collected samples. The analyses of such samples are further aided by a recent movement that tries to bring the lab to the field (Hohenlohe et al., 2021). The development of miniaturized and portable laboratory equipment, such as sequencing devices, thermal cyclers, and mini-centrifuges, that can be taken to the field, drastically speeds up the processing of samples and generation of data (e.g. Blanco et al., 2020; Krehenwinkel et al., 2019; Utge et al., 2020). The accelerated processing of samples after sampling greatly reduces the loss of DNA quality associated with long storage and shipping procedures (e.g., Guevara et al., 2017; Murphy et al., 2007) resulting in even more samples being accessible for the application of WGS methods.

However, to use the resulting sequencing data for the analysis of the adaptive potential of populations, an annotated reference genome of the respective species (or of a closely related one) is required to relate the data of the studied population. This highlights the need to produce such reference genomes for more and more species (Petty et al., 2021). Additionally, the analyses of WGS data require much more advanced bioinformatic skills than the analyses of microsatellites (Flanagan & Jones, 2019; Hohenlohe et al., 2021), as well as more computational power and data storage capacity. However, I am confident that these obstacles will be overcome in the next years with the ongoing progress in analytical methods and technical advances. In line with that, analyses of genetic variation will hopefully be extended to the investigation of transcriptomes, proteomes, and epigenetic variations which will improve our understanding of the adaptive potential and fitness in wildlife populations even further (Anderson et al., 2020; Hohenlohe et al., 2021) and allow for more comparative studies including our genus *Homo sapiens* (Housman & Gilad, 2020).

To summarize, I believe that, with the continuous technical and analytical advances in sequencing procedures and equipment, whole genome sequencing will eventually take over in many fields of wildlife

genetics. However, with the implementation of next-generation sequencing techniques in the analysis workflow, microsatellites will remain in the genetic toolbox for wildlife scientists as reliable markers and will be applied where appropriate in the future.

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Supervisors: Dr. Susanne Hummel and Dr. Birgit Großkopf

Bachelor of Science in Biology 10/2010 - 09/2014
University of Göttingen, Germany

Grade: 1,9

Thesis title: Morphologische und molekulargenetische Untersuchungen an drei frühneuzeitlichen Individuen aus Göttingen zur Prüfung auf Verwandtschaft.

Department: Historical Anthropology and Human Ecology

Supervisors: Dr. Susanne Hummel and Dr. Birgit Großkopf

Teaching and supervision

2017 – 2020: Teaching of two lectures per summer semester about ancient DNA and population genetics as part of the Phylogeography lecture series (M.Biodiv.415.Mp).

05/2018 - 04/2019: Teaching and supervision of the master student Anna Lemkul in the department of Cognitive Ethology at the German Primate Center, Göttingen.

03/2019 - 12/2019: Teaching and supervision of the master student Nadja Risch Ferreira in the department of Cognitive Ethology at the German Primate Center, Göttingen.

11/2021 - 12/2021: Teaching and supervision of the lab rotation student Max Zinowsky in the department of Cognitive Ethology at the German Primate Center, Göttingen.

Academic Positions

11/2017 - 03/2018: Research Assistant for the module 'Good Scientific Practice' (B.Bio.190.1), University of Göttingen

11/2018 - 03/2019: Research Assistant for the module 'Good Scientific Practice' (B.Bio.190.1), University of Göttingen

Publications

Peer-reviewed journal publications

Dal Pesco, F., **Trede, F.**, Zinner, D., & Fischer, J. (2022). Male-male social bonding, coalitionary support and reproductive success in wild Guinea baboons. *Proceedings of the Royal Society B: Biological Sciences* 289(1975): 20220347. doi:10.1098/rspb.2022.0347

Dal Pesco, F., **Trede, F.**, Zinner, D., Fischer, J. (2021). Kin bias and male pair-bond status shape male-male relationships in a multilevel primate society. *Behavioral Ecology and Sociobiology* 75(1): 24. doi:10.1007/s00265-020-02960-8

Trede, F., Kil, N., Stranks, J., Connell, A., Fischer, J., Ostner, J., Schülke, O., Zinner, D., Roos, C. (2021). A refined panel of 42 microsatellite loci to universally genotype catarrhine primates. *Ecology and Evolution* 11(1): 498-505. doi:10.1002/ece3.7069

Dal Pesco, F., **Trede, F.**, Zinner, D., Fischer, J. (2020). Analysis of genetic relatedness and paternity assignment in wild Guinea baboons (*Papio papio*) based on microsatellites. *protocols.io*: 1-4. doi:10.17504/protocols.io.bfdmji46

Fischer, J., Wegdell, F., **Trede, F.**, Dal Pesco, F., & Hammerschmidt, K. (2020). Vocal convergence in a multi-level primate society: insights into the evolution of vocal learning. *Proceedings of the Royal Society B: Biological Sciences* 287: 20202531. doi:10.1098/rspb.2020.2531

Trede, F., Lemkul, A., Atickem, A., Beehner, J. C., Bergman, T. J., Burke, B., Fashing, P. J., Knauf, S., Mekonnen, A., Moges, A., Nguyen, N., Roos, C., Zinner, D. (2020). Geographic distribution of microsatellite alleles in geladas (Primates, *Cercopithecidae*): evidence for three evolutionary units. *Zoologica Scripta* 49(6): 659-667. doi:10.1111/zsc.12451

Academic Meetings

11/2019 – Poster presentation; Phylogenetic Symposium “Reticulate Evolution”

Göttingen, Germany

04/2021 – Poster presentation; Behaviour, Ecology and Evolution Conference

Oxford, United Kingdom (online conference)