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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.

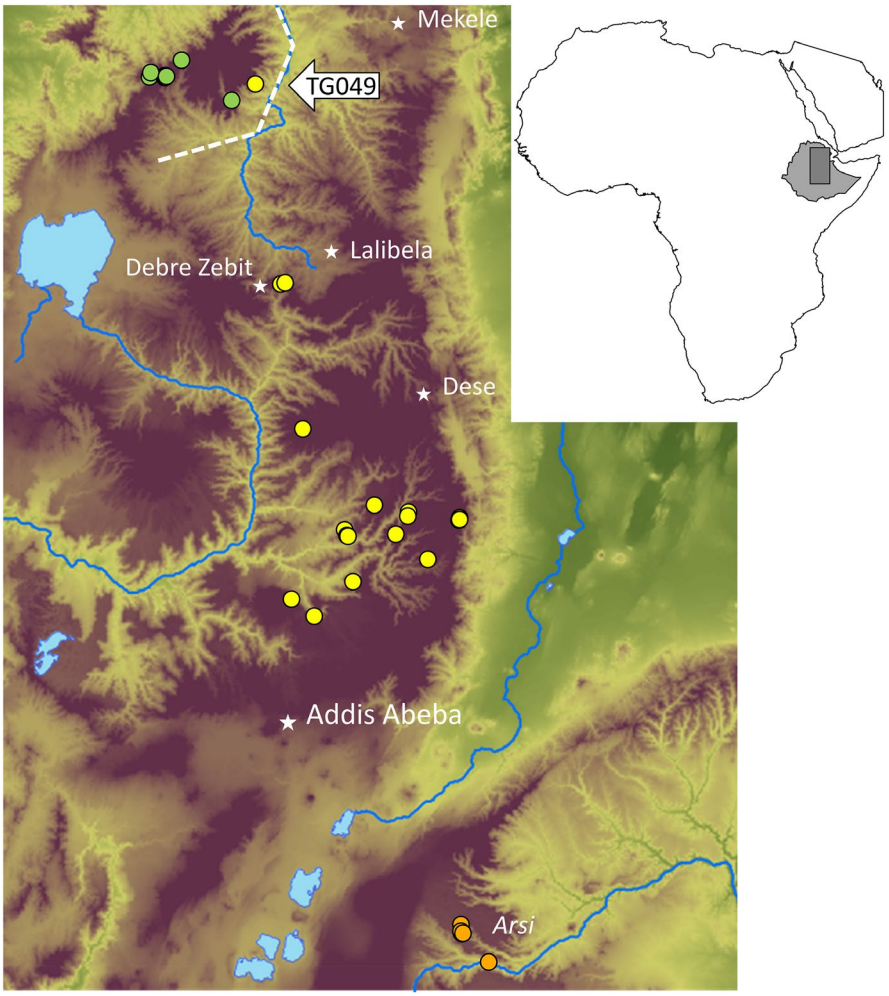

FIGURE 1 Geographic distribution of gelada sampling sites in the Ethiopian highlands. Inset map indicates the position of the area of interest within Africa and Ethiopia. Dashed line = proposed border between the northern population (*Theropithecus gelada gelada* to the north-west of the border) and 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

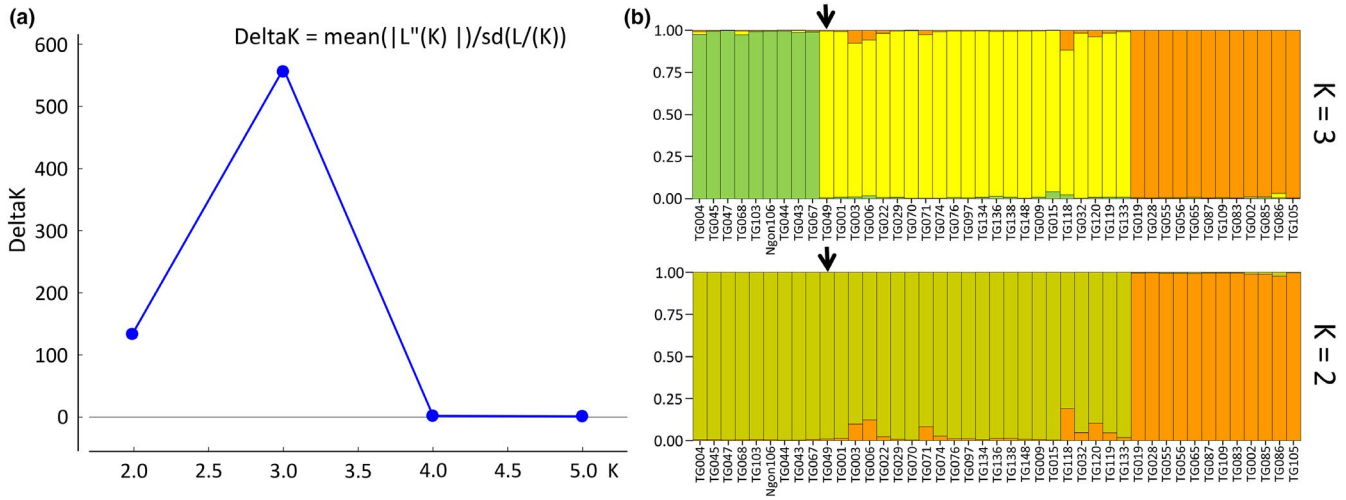

FIGURE 2 Population genetic structure of *Theropithecus gelada*, based on 13 loci with no signs of null alleles. (a) Optimal number of K clusters calculated by the Evanno method (Evanno et al., 2005). A three-cluster structure is indicated. (b) STRUCTURE results for $K = 3$ and $K = 2$ clusters. Colours 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

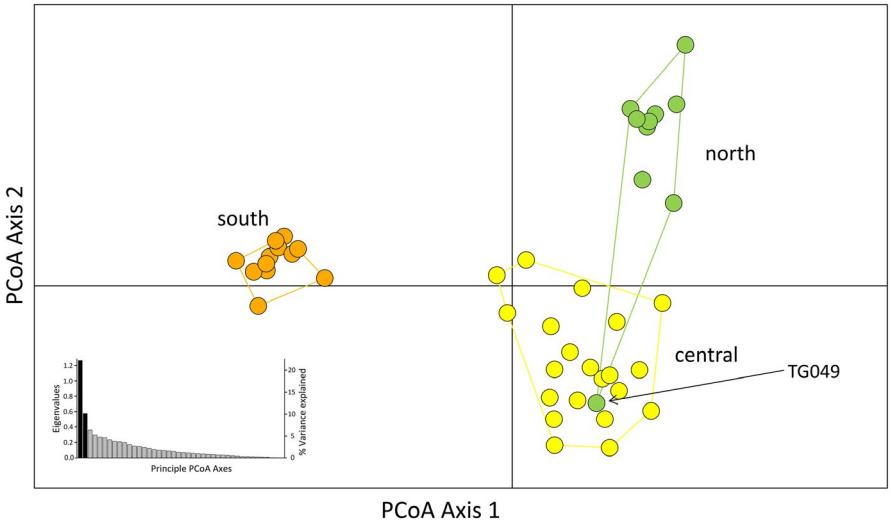

FIGURE 3 Principal coordinate analysis (PCoA) of pairwise genetic distances between individuals of the three sampling areas of *Theropithecus gelada*. Included are only loci without null alleles. Colours correspond to geographic origin of samples: black (green) = northern; grey (yellow) = central; white (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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