GENE FLOW, POPULATION STRUCTURE AND GENETIC SPECIATION OF GIRAFFE HAVE IMPLICATIONS FOR THEIR CONSERVATION

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“Wildlife is something which man cannot construct. Once it is gone, it is gone forever. Man can rebuild a pyramid, but he can't rebuild ecology, or a giraffe.”

Joy Adamson

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SUMMARY

BACKGROUND

The genus *Giraffa* likely evolved around seven million years ago in Indo-Asia and spread over the Arabian-African land bridge into Eastern Africa. The oldest fossil of the African lineage was found in Kenya and dated to 7-5.4 Mya. Beside modern giraffe, four additional African species have likely existed (*G. gracilis*, *G. pygmaea*, *G. stillei,* and *G. jumae*). Based on their morphological similarities, *G. gracilis* is often considered to be the closest relative of the modern giraffe. Nevertheless, the phylogeny within the genus *Giraffa* is largely unresolved.

Modern giraffe (*Giraffa* sp.) have been neglected by the scientific community for a long time and still very little is known about their biology. Traditionally, present-day giraffe have been considered a single species (*G. camelopardalis*) which is divided into six to eleven subspecies, with nine subspecies being the most accepted classification. This classification was based on morphological differences and geographic ranges. However, recent genetic analyses found hidden diversity within *Giraffa* and proposed four genetically distinct giraffe species (*G. camelopardalis, G. reticulata, G. tippelskirchi, G. giraffa*) with presumably little gene flow among them.

Gene flow on a population level is the exchange of genetic information among populations facilitated by the migration of individuals between populations. Additionally, it is an important criterion to delineate species, because many species concepts, especially the Biological Species Concept, rely on the concept of reproductive isolation. Yet, new genetic methods are identifying an increasing number of species that show signs of introgressive hybridization or gene flow among them. Therefore, strict reproductive isolation cannot always be applied to delineate species, especially in young, probably still diverging, species such as giraffe.

Therefore, giraffe are ideal study organisms to investigate the level of gene flow in recently diverged species with adjacent or potentially overlapping ranges. Furthermore, their recent classification as “Vulnerable” by the IUCN and their unreliable distribution maps require the genetic evaluation of their population structure, distribution and conservation status.

REALIZED STUDIES

In **Publication 1** (Winter et al. (2018a), *Ecological Genetics and Genomics*, *7–8*, 1–5), I studied the distribution and matrilineal population structure of Angolan giraffe (*G. giraffa angolensis*) using sequences from the cytochrome *b* gene (1,140 bp) and the mitochondrial control region for individuals from across their known range and beyond, and additionally including individuals from all known giraffe species and subspecies. The reconstruction of a phylogenetic tree and a mitochondrial haplotype network allowed to identify the most easterly known natural population of Angolan giraffe, a population that was previously assigned to their sister-subspecies South African giraffe (*G. giraffa giraffa*), indicating the limit of classification by morphology and geography. Furthermore, the analyses show that Namibia's iconic desert-dwelling giraffe population is genetically distinct, even from the nearest population at Etosha National Park, suggesting very limited, if any, natural exchange of matrilines. Yet, no geographic barriers are known for this region that would prevent genetic exchange. Therefore, the two populations are likely on different evolutionary trajectories. Limited individuals with an Etosha haplotype further suggest that translocation of Etosha giraffe into the desert population had only a minor impact on the local population. Two separate haplogroups within Etosha National Park suggest an "out of Etosha" radiation of Angolan giraffe to the East followed by a later back-migration.

In **Publication 2** (Winter et al. (2018b), *Ecology and Evolution*, *8*(20), 10156–10165), I investigated the genetic population structure of giraffe across their range (n = 137) with focus on the amount of gene flow among the proposed giraffe species with a 3-fold increased set of nuclear introns (n = 21). Limited gene flow of less than one effective migrant per generation, even between the closely related northern (*G. camelopardalis*) and reticulated giraffe (*G. reticulata*) further supports the existence of four giraffe species by a different methodology, gene flow. This is significant because most species concepts build on reproductive isolation. Furthermore, this result is corroborated by four distinct major clades in a phylogenetic tree analysis, and distinct clusters in Principal Component Analysis and STRUCTURE analysis. All these analyses suggest a low level of genetic exchange among the four giraffe species and, therefore, a high degree of reproductive isolation in accordance with the Biological Species Concept (BSC). In Addition, only a single individual in 137 was identified as being potential of natural hybrid origin, which promotes the four-species concept further.

The new findings of this study emphasize the necessity of a revision of the IUCN classification of giraffe and with this a reevaluation of their threat categories, likely resulting in higher threat categories for three of the four species.

In **Publication 3** (Winter et al. (2019), *Conservation Genetics*, *20*(3), 665–670), mitochondrial cytochrome *b* and control region sequences, seven nuclear introns, and ten microsatellites verified the taxonomic assignment of Malawi's giraffe, investigated a potential hybrid origin and elucidated their genetic diversity. A mitochondrial DNA based phylogenetic tree reconstruction identified 14 individuals (50 % of the total population) unambiguously as being South African giraffe (*G. giraffa giraffa*). Furthermore, STRUCTURE and Principal Component Analysis showed no signs of admixture with Masai giraffe, despite previous reports of translocations and migrations of Masai giraffe into Malawi. Additionally, the analysis of microsatellites revealed that Malawi's giraffe population is highly inbred and in urgent need of new introduced genetic variability to prevent increasing inbreeding depression that could lead to their extinction in the near future.

CONCLUSION

The increased dataset of 21 nuclear introns and 137 giraffe individuals from all giraffe taxa presented in this thesis allowed for the first time comprehensive gene flow analyses for giraffe, which showed limited gene flow among the four giraffe species. This complements the previous population genetic analyses and furthers the four species classification. Therefore, this thesis is a significant contribution to the ongoing taxonomic discussion within the genus *Giraffa* and is currently informing the IUCN giraffe and okapi specialist group on giraffe taxonomy for future threat assessments and the development of species-specific conservation strategies.

Furthermore, this thesis highlights the need for a clear definition of gene-flow under the Biological Species Concept and further investigation on the genetic basis of reproductive isolation.

Additionally, this thesis emphasizes the importance of genetic taxonomic assignments and genetic monitoring of small and isolated populations and pre- and post-translocation assessments to better understand giraffe distribution and for successful implementation of conservation management strategies. The findings of this thesis have already been applied to augment Malawi's giraffe population with the correct subspecies.

ZUSAMMENFASSUNG

GENFLUSS, POPULATIONSSTRUKTUR UND GENETISCHE ARTBILDUNG BEI GIRAFFEN HABEN AUSWIRKUNGEN AUF IHRE ARTERHALTUNG

HINTERGRUND

Die Gattung *Giraffa* gehört, neben der monotypischen Gattung *Okapia*, zu den zwei rezenten Gattungen innerhalb der Familie Giraffidae. Alle bekannten Arten innerhalb der Giraffidae weisen familienspezifische Merkmale wie elongierte Hälse und Extremitäten, sowie zweilappige untere Eckzähne, und Ossicone (mit Haut überzogene Knochenzapfen) auf. Die Gattung *Giraffa* entwickelte sich wahrscheinlich vor etwa sieben Millionen Jahren nach der Migration ihrer Vorfahren (Gattung *Bohlinia*) von Südosteuropa nach Indo-Asien und breitete sich über die arabisch-afrikanische Landbrücke nach Ostafrika aus. Das älteste Fossil der afrikanischen Abstammungslinie wurde in Kenia gefunden und auf ein Alter von 7-5,4 Millionen Jahren datiert. Neben den rezenten Giraffen haben wahrscheinlich vier weitere afrikanische Giraffenarten existiert (*G. gracilis*, *G. pygmaea*, *G. stillei* und *G. jumae*). Aufgrund ihrer morphologischen Ähnlichkeiten wird *G. gracilis* oft als der engste Verwandte der modernen Giraffe angesehen. Die Phylogenie innerhalb der Gattung *Giraffa* ist jedoch weitgehend ungeklärt.

Die rezenten Giraffen (*Giraffa* sp.) werden teilweise als die „vergessene Megafauna Afrikas“ bezeichnet, da sie lange Zeit von der wissenschaftlichen Gemeinschaft vernachlässigt wurden. Bis heute ist immer noch sehr wenig über ihre Biologie bekannt, obwohl sie sich seit jeher großer Beliebtheit bei Besuchern von zoologischen Gärten erfreuen. Traditionell wurden die rezenten Giraffen als eine einzige Art (*G. camelopardalis*) betrachtet, die in sechs bis elf Unterarten unterteilt wird, wobei neun Unterarten die am meisten akzeptierte Klassifikation darstellt. Diese Klassifikation basierte auf morphologischen Unterschieden wie der Fellzeichnung und der Anzahl und Ausprägung der Ossicone, sowie den geographischen Verbreitungsgebieten. Genetische Analysen fanden jedoch versteckte genetische Vielfalt innerhalb der Gattung *Giraffa* und deuten auf vier genetisch unterschiedliche Arten (*G.*

camelopardalis, *G. reticulata*, *G. tippelskirchi*, *G. giraffa*) hin, zwischen denen wahrscheinlich nur ein geringer genetischer Austausch (Genfluss) stattfindet.

Genfluss auf Populationsebene ist der Austausch genetischer Informationen zwischen Populationen, der durch die Migration von Individuen zwischen Populationen ermöglicht wird. Weiterhin ist er ein wichtiges Kriterium für die Abgrenzung von Arten, da viele Artkonzepte, insbesondere das Biologische Artkonzept, auf dem Prinzip der reproduktiven Isolation beruhen. Mit neuen genetischen Methoden werden jedoch immer mehr Arten identifiziert, die Anzeichen von Introgression (introgressive hybridization) bzw. Genfluss untereinander aufweisen. Aus diesem Grund kann vollständige reproduktive Isolation im Sinne des Biologischen Artkonzepts nicht immer als Kriterium zur Abgrenzung von Arten herangezogen werden. Insbesondere bei jungen, vermutlich noch divergierenden Arten, wie den Giraffen, sind die notwendigen reproduktiven Isolationsmechanismen noch nicht vollständig ausgebildet. Bisher fehlt jedoch eine klare Definition von Genfluss unter dem Biologischen Artkonzept.

Aufgrund der kurzen Divergenzzeiten und der bekannten Fähigkeit zur Hybridisierung in Gefangenschaft sind Giraffen ideale Untersuchungsorganismen um den Grad des Genflusses bei kürzlich divergierenden Arten mit benachbarten oder sich möglicherweise überlappenden Verbreitungsgebieten zu untersuchen. Darüber hinaus erfordert die jüngste Einstufung der Giraffe als "gefährdet" durch die International Union for Conservation of Nature and Natural Resources (IUCN) und ihre bisher unzuverlässigen Verbreitungskarten eine genetische Bewertung ihrer Populationsstruktur, Verbreitung und ihres Erhaltungsstatus.

DURCHGEFÜHRTE STUDIEN

In **Publikation 1** (Winter et al. (2018a), Ecological Genetics and Genomics, 7-8, 1-5) untersuchte ich die geographische Verbreitung und matrilineare Populationsstruktur der Angola-Giraffe (*G. giraffa angolensis*) unter Verwendung von Sequenzen des mitochondrialen Cytochrom-*b*-Gens (1.140 bp) und der Kontrollregion. Neben Individuen aus dem gesamten bekannten Verbreitungsgebiet der Angola-Giraffe und angrenzenden Gebieten, wurden zusätzlich Individuen aller bekannten Giraffenarten und -unterarten untersucht. Die Rekonstruktion eines phylogenetischen Stammbaums und eines mitochondrialen Haplotypennetzwerks ermöglichte die Identifizierung der bisher östlichsten bekannten

natürlichen Population der Angola-Giraffe. Diese Population wurde zuvor ihrer Schwester-Unterart, der Kap-Giraffe (*G. giraffa giraffa*), zugeordnet. Die Tatsache, dass diese Population bis zu diesem Zeitpunkt der falschen Unterart zugeordnet wurde, verdeutlicht die Ungenauigkeit der Klassifizierung rein durch Morphologie und Geographie. Außerdem zeigen die Analysen, dass sich Namibias ikonische Wüsten-Giraffenpopulation genetisch deutlich von der nächstgelegenen Population im Etoscha-Nationalpark unterscheidet, was auf einen sehr begrenzten, wenn überhaupt stattfindenden, natürlichen Austausch der mütterlichen Erblinie hindeutet. Für diese Region sind jedoch keine geographischen Barrieren bekannt, die einen genetischen Austausch verhindern würden. Dies lässt vermuten, dass sich die beiden Populationen auf unterschiedlichen evolutionären Entwicklungsbahnen befinden. Eine begrenzte Anzahl an Individuen mit einem Etosha-Haplotypen legt zudem nahe, dass die Aufstockung der Wüstenpopulation mit Individuen aus dem Etosha-Nationalpark in den 1990er Jahren nur geringe Auswirkungen auf die lokale Population hatte. Weiterhin lassen sich bei den untersuchten Individuen des Etoscha-Nationalparks zwei unterschiedliche Haplogruppen feststellen. Dies weist auf eine mögliche "Out-of-Etosha"-Radiation der Angola-Giraffe hin und lässt vermuten, dass sich das Verbreitungsgebiet zunächst nach Osten hin ausdehnte, gefolgt von einer späteren Rückmigration nach Westen.

In **Publikation 2** (Winter et al. (2018b), Ecology and Evolution, 8(20), 10156-10165) untersuchte ich die genetische Populationsstruktur von Giraffen in ihrem gesamten Verbreitungsgebiet mit Schwerpunkt auf dem Ausmaß an Genfluss unter den vier vorgeschlagenen Giraffenarten. Um dies zu ermöglichen erweiterte ich den vorhandenen Datensatz von sieben auf 21 nukleare Intron Sequenzen für 137 Individuen aller Arten und Unterarten. Mit diesem Datensatz konnten zum ersten Mal umfassende Genflussanalysen durchgeführt werden. Diese Analysen ermittelten einen begrenzten Genfluss von weniger als einem effektiven Migranten pro Generation zwischen den verschiedenen Giraffenarten, sogar zwischen der eng verwandten Nord-Giraffe (*G. camelopardalis*) und der Netzgiraffe (*G. reticulata*). Die Genflussrate zwischen Unterarten derselben Art war in der Regel deutlich höher als zwischen den Arten mit Ausnahme von geographisch sehr isolierten Populationen wie z.B. der Westafrikanischen Giraffe (*G. c. peralta*). Dies ist deshalb von Bedeutung, da die meisten Artkonzepte, besonders das Biologische Artkonzept, auf reproduktiver Isolation beruhen und eingeschränkter Genfluss für eine ausgeprägte, wenn auch nicht vollständige, reproduktive Isolation spricht. Darüber hinaus wird dieses Ergebnis durch die Bildung von vier

monophyletischen Gruppen in einer phylogenetischen Stammbaumanalyse und vier verschiedenen Clustern in der Hauptkomponentenanalyse (PCA) sowie einer STRUCTURE-analyse bestätigt. All diese Analysen deuten auf einen begrenzten genetischen Austausch zwischen den vier Giraffenarten und damit auf ein hohes Maß an reproduktiver Isolation, gemäß dem Biologischen Artkonzepts (BSC), hin. Zudem wurde nur ein einziges Individuum von 137 als potentiell natürlich entstandener Hybride identifiziert, obwohl drei der vier Arten angrenzende und möglicherweise überlappende Verbreitungsgebiete aufweisen und es bisher keine Anzeichen für geographische Barrieren zwischen diesen Verbreitungsgebieten gibt. Diese Erkenntnis spricht für die Existenz von vier Giraffenarten und unterstützt somit die Vier-Arten-Hypothese.

Die neuen Ergebnisse dieser Studie unterstreichen die Notwendigkeit einer Revision der Rote Liste gefährdeter Arten der IUCN bezüglich der Klassifikation der Giraffen, und damit eine Neubewertung ihrer Bedrohungskategorien. Die Bestände der Nord-, Netz und Massai-Giraffe nehmen seit ca. 30 Jahren rapide ab, nur die Bestände der Süd-Giraffe haben sich im selben Zeitraum erholt. Eine Neubewertung der Bedrohungskategorien wird daher für drei der vier Giraffenarten die Einstufung in höhere Bedrohungskategorien bedeuten und verdeutlicht die Wichtigkeit von artspezifischen Management- und Artenschutzkonzepten.

In **Publikation 3** (Winter et al. (2019), Conservation Genetics, 20(3), 665-670), verifizierte ich mittels mitochondrialen Cytochrom-*b*- und Kontrollregion-Sequenzen, sieben nuklearen Introns und zehn Mikrosatelliten die taxonomische Zugehörigkeit der Giraffen in der Republik von Malawi, untersuchte einen möglichen Hybridursprung und die genetische Vielfalt der Population. Eine auf mitochondrialer DNA basierende phylogenetische Stammbaumrekonstruktion identifizierte dabei die 14 untersuchten Individuen (50 % der Gesamtpopulation) eindeutig als Kap-Giraffe (*G. giraffa giraffa*). Darüber hinaus zeigten die STRUCTURE- und Hauptkomponentenanalysen keine Anzeichen einer Vermischung mit der Massai-Giraffe auf nuklearer Ebene, trotz früherer Berichte über Translokationen und Migrationen von Massai-Giraffen nach Malawi. Weiterhin ergab die Analyse der Mikrosatelliten, dass die Giraffenpopulation Malawis ein starkes Maß an Inzucht aufweist und dringend neu eingeführte genetische Variabilität benötigt, um eine zunehmende Inzuchtdepression zu verhindern. Inzuchtdepression zeigt sich oft in einer erhöhten Anfälligkeit für Infektionen, in der Regel verbunden mit schwererem Krankheitsverlauf, was zu einer erhöhten Sterblichkeit führt. Erste Anzeichen in Form von vermehrtem

Parasitenbefall mit z.B. Räude und Zecken ließen sich in der Giraffenpopulation Malawis bereits feststellen. Ohne genetische Auffrischung könnte die zunehmende Inzuchtdepression in naher Zukunft zum Aussterben der Giraffenpopulation in Malawi führen.

FAZIT

Diese Dissertation betont die Bedeutung von genetischen Methoden zur taxonomischen Zuordnung, dem genetischen Monitoring kleiner und isolierter Populationen, sowie der Beurteilungen der genetischen Vielfalt von Populationen vor und nach der Umsiedlung von Individuen, um die Verbreitung von Giraffen besser zu verstehen und erfolgreiche Erhaltungsmanagementstrategien zu entwickeln und umzusetzen. Die Ergebnisse dieser Arbeit wurden bereits angewandt, um die Giraffenpopulation Malawis mit der richtigen Unterart aufzustocken und somit neue genetische Vielfalt in die von Inzucht geprägte Population einzubringen.

Wie anhand der Angola Giraffe gezeigt, ist es nicht möglich sich bei der taxonomischen Zuordnung von Individuen zu einer bestimmten Art bzw. Unterart allein auf die Morphologie oder die geographische Verbreitung zu verlassen, da besonders im südlichen Afrika häufige kaum dokumentierte Translokationen, auch über Landesgrenzen hinweg, stattgefunden haben. Aus diesem Grund ist die genetische Bestimmung der taxonomischen Zugehörigkeit von Individuen/Populationen von besonderer Bedeutung.

Weiterhin ermöglichte der, in dieser Dissertation, vorgestellte erweiterte Datensatz von 21 nuklearen Introns und 137 Individuen aller Arten und Unterarten erstmals umfassende Genflussanalysen für Giraffen. Diese Analysen zeigten ein begrenztes Ausmaß an Genfluss unter den vier Giraffenarten von weniger als einem effektiven Migranten pro Generation und ergänzten damit die bisherigen populationsgenetischen Analysen. Die Ergebnisse liefern zusätzliche Unterstützung für die Existenz von vier genetisch unterschiedlichen Arten. Daher ist diese Dissertation ein bedeutender Beitrag zur laufenden taxonomischen Diskussion innerhalb der Gattung *Giraffa* und wird derzeit von der Species Survival Commission (SSC) Giraffe and Okapi Specialist Group (GOSG) der IUCN verwendet, um deren Giraffentaxonomie zu überarbeiten. Dies hat bedeutende Auswirkungen auf die zukünftige Einschätzung des Bedrohungsstatus und die Entwicklung artspezifischer Artenschutzkonzepte.

Darüber hinaus unterstreicht diese Arbeit die Notwendigkeit einer klaren Definition von Genfluss im Rahmen des Biologischen Artkonzepts und weiterer Untersuchungen zur genetischen Grundlage von reproduktiver Isolation

GENERAL INTRODUCTION

Giraffe (genus: *Giraffa*) have long been neglected by the scientific community, and many aspects of their biology are still poorly studied. This is unexpected given that the giraffe is a well-known species and a favorite animal in every zoo. Therefore, giraffe, together with their close relative the okapi (*Okapia johnstoni*), are considered Africa's forgotten megafauna (Kümpel et al., 2015). However, recent genetic studies suggest hidden diversity within the genus *Giraffa* (Bock et al., 2014; Brown et al., 2007; Fennessy et al., 2016, 2013; Hassanin et al., 2007) and decreasing population sizes (Muller et al., 2018), make it necessary to investigate their evolutionary history, speciation, gene flow, and population structure with genetic tools.

It has been suggested that giraffe may not be one species but four (Fennessy et al., 2016). This has implication for a) the taxonomic discussion, b) the species definition, and c) the understanding of biodiversity and conservation. It is important to accurately delineate species because it allows for a better understanding of evolution and the process of speciation. Additionally, it allows for improved thread assessments and consequently improved conservation measures.

Therefore, genetic diversity within giraffe characterizing new nuclear markers are important and enabled the first comprehensive gene flow analyses among giraffe. In addition to the previous findings based on population genetic tools, explicit gene flow analyses gave unequivocal support to distinguish four distinct species of giraffe.

Furthermore, the detailed analyses of the matrilineal population structure of Angolan giraffe, especially Namibia's desert-dwelling giraffe, found that the geographical distribution of giraffe populations is poorly understood. Genetic analyses identified the most easterly-distributed population of the Angolan giraffe in southern Zimbabwe, approximately 500 km further to the east than previously known. Additionally, a comprehensive dataset of mitochondrial sequences, nuclear sequences, and microsatellites (short tandem repeats, STRs) made it possible to study the genetic diversity within Malawi's giraffe, identify their (sub)species affiliation, and rule out a potential hybrid origin.

Thus, this thesis on giraffe genetics made major contributions to the understanding of the inherent biodiversity of giraffe.

In the following introduces to a) the concept of biodiversity with its three major components species, genetic, and ecological diversity, b) explains the importance of gene flow in speciation and species delimitation, and c) introduces the taxonomy/biology of the genus *Giraffa* with an overview of giraffe evolution and their current state of conservation.

SPECIES VS. GENETIC DIVERSITY

Large herbivorous vertebrates with a body mass of > 1.000 kg are referred to as megafauna or megaherbivores (Owen-Smith, 1988). These days, megafaunal species, such as elephants, rhinoceroses or giraffe, only inhabit Africa and Asia. Megaherbivores have a huge impact on ecosystem dynamics by feeding on plants and keeping landscapes heterogenous (Dirzo and Miranda, 1991). In addition, they distribute nutrition. Therefore, they are important to maintaining biodiversity.

The term "biodiversity" is frequently used in contemporary biology. But what does biodiversity refer to? The term "biodiversity" is a relatively new compound word for "biological diversity" that was introduced by Walter G. Rosen in 1985 (Harper and Hawksworth, 1994). Biological diversity in its current meaning was first proposed by Norse & McManus (1980) and includes three main components, (1) species or taxonomic diversity, (2) genetic diversity and (3) ecological diversity. The United Nations Environment Programme (2010) uses the following definition for biodiversity:

> "*Biodiversity is the variety of life on Earth, it includes all organisms, species, and populations; the genetic variation among these; and their complex assemblages of communities and ecosystems.*"

The following section will introduce to each of the main components of biodiversity, reasons for biodiversity loss, and developments in biodiversity conservation.

SPECIES DIVERSITY

Biodiversity is often used synonymously to species diversity or species richness because species are the central descriptive unit of living organisms (Swingland, 2001). Species diversity is an essential concept in biodiversity research and frequently used as a criterion to define protected areas (Volvenko, 2011) because it is the easiest component of biodiversity

to assess. Yet, species diversity is only one component of biodiversity. It describes the pure number of species in a particular area or habitat regardless of the higher taxonomic ranks they belong to and assumes that all species contribute equally to a site's biodiversity (Harper and Hawksworth, 1994).

Therefore, some authors suggest better to use the more precise "taxonomic diversity" instead (Swingland, 2001). Taxonomic diversity describes the diversity of an area regarding higher taxonomic ranks, because species that belong to different families, orders, or phyla are more diverse than species that belong to the same family or even genus. Marine habitats, for example, generally have fewer species but more phyla than terrestrial habitats and, therefore, a higher taxonomic diversity (Swingland, 2001).

Global biodiversity assessments usually state the number of species in different taxonomic groups and estimates for global species numbers (Swingland, 2001). Currently, there are approximately 1.7 million described species (Swingland, 2001). Varying estimates suggest that the total number of species on earth lies between five million and one trillion (10^{12}) (Locey and Lennon, 2016; Swingland, 2001). The latter number includes bacteria, archaea, and fungi, whose numbers, according to Locey and Lennon (2016), may be highly underestimated. More realistic estimates on the number of species have been suggested by Mora et al. (2011), who suggest a total of ~8.7 million eukaryotic species on earth, of which ~2.2 million are marine.

GENETIC DIVERSITY

In contrast to species diversity that describes the number of identified species, genetic diversity describes the diversity within and among species as the heritable genetic variation among populations but also the overall genetic variability of an ecosystem. Measurements to characterize genetic diversity are based on DNA data and are straight forward. The most common measures are heterozygosity, proportion of polymorphic loci (gene diversity), number of alleles, and often an allele or haplotype network or tree (Harper and Hawksworth, 1994).

The genetic variation originates from mutations in an individual's genome and can be spread by recombination in sexually reproductive organisms (Swingland, 2001). Genetic diversity is the prerequisite for adaptation to changing environments and for genetic isolation that leads to speciation. However, many biodiversity studies neglect the genetic diversity

because measuring the genetic diversity for the number of species of a defined area is difficult, due to sampling and sequencing constraints. (Harper and Hawksworth, 1994).

Species diversity and genetic diversity are interdependent because a loss in species diversity, for example, by the extinction of a species, results in loss of the genetic diversity that was previously present within that species. On the other hand, an increase in genetic diversity can lead to the evolution of new species, which increases species diversity.

The genetic diversity of a species is often correlated with the population size. Large populations generally have a higher genetic diversity and are more likely to maintain genetic variation (Frankham et al., 2010). On the other hand, small populations are more likely to lose genetic diversity over time by genetic drift (Frankham et al., 2010). Furthermore, inbreeding or the mating of closely related individuals with a similar genetic make-up is more likely in small populations, which often results in the fixation of a common allele, thus reducing the genetic diversity by losing alternative alleles (Crow, 2010; Frankham et al., 2010).

ECOLOGICAL DIVERSITY

Ecological diversity or ecosystem diversity is the third component of biodiversity. It describes the variety of ecosystems or habitats in a particular area (or worldwide for global assessments), the variation within ecosystems, and the accompanying abiotic and biotic processes and components that maintain them (DeLong, 1996; Swingland, 2001; United Nations Environment Programme, 2010). However, in the 1990s, it was highly debated if ecosystem diversity should be part of the definition of biodiversity because it includes abiotic components, which, by definition, are not biological (DeLong, 1996). Noss (1990) argued that ecological processes should be included in the definition of biodiversity, even though they are as much abiotic as biotic because they are crucial to maintaining biodiversity. Therefore, more recent definitions of biodiversity all seem to agree on including ecosystem diversity as an essential component (Convention on Biological Diversity, 2006; United Nations Environment Programme, 2010).

Thus, biodiversity is a multi-faceted field, and not all components have been studied equally. The genetic aspect of biodiversity has not been explored sufficiently to date, because of limited access to sufficient methods and the high costs involved. However, the current developments in genetics and genomics, for example, the drop in sequencing costs, will

enable more detailed genetic assessments of ,e.g., biodiversity hotspots, and will not only allow to detect genetic diversity but also increase the accuracy of the evaluation of species diversity based on genetic species identification.

BIODIVERSITY LOSS

Biodiversity loss or the reduction of genetic, species, or ecosystem diversity, is one of the biggest threats we are currently facing. It is not just that we lose all these species, their genetic variation, their ability to adapt, and ecosystems that evolution has created over millions of years; it also impacts humanity, e.g., by reduced crop yield due to decreased intraspecific genetic diversity (Cardinale et al., 2012). Some claim that Earth is currently facing the sixth mass extinction over the last approximately 500 Million years, and the biggest since the Cretaceous-Tertiary (K–T) extinction about 66 Mya in which ¾ of all plant and animal species went extinct including most dinosaurs (Renne et al., 2013). A mass extinction is defined as the loss of more than 75 % of species in a geologically short time frame (Barnosky et al., 2011).

Extinctions are a natural process; new species evolve, and others die-out. In fact, >99% of all species have become extinct. However, the current rate of extinction is estimated to exceed the expected background extinction rate by a factor of more than 1,000 and is assumed to increase further in the future (Ceballos et al., 2015; Vos et al., 2015). The difference to previous mass extinction events is that the current mass extinction is mainly induced by human activities (IUCN, 2019).

Overpopulation and, as a consequence, loss of habitat causes biodiversity loss and extinctions either directly by hunting, persecution, or collection, or - more importantly - indirectly by modification and destruction of habitats, e.g., trough deforestation (Swingland, 2001). The main pressures causing biodiversity loss identified by the Convention on Biological Diversity (2006) include (1) habitat loss, (2) overexploitation and unsustainable use of resources, (3) climate change, (4) invasive species, and (5) pollution.

BIODIVERSITY CONSERVATION – SPECIES VS. GENETIC CONSERVATION

The high rate in which biodiversity is lost due to human activities makes biodiversity conservation one of the biggest challenges and responsibilities of our time. Our society depends on biological resources in the form of food, organic material such as timber or natural

fibers such as cotton, wool, or silk, but biological diversity is also important for recreational activities and our well-being (Nunes and van den Bergh, 2001). Therefore, it is important to conserve as much biodiversity as possible. Furthermore, humans have an ethical obligation to conserve Earth's biodiversity, as one species does not have the right to drive other species to extinction (Frankham et al., 2010).

Global conservation strategies currently prioritize 36 so-called "biodiversity hotspots", regions where the biodiversity is especially high (Habel et al., 2019). These hotspots inhabit a high number of species, of which many are endemic to those regions (Myers et al., 2000). So do 77% of endemic plant species and 43% of all terrestrial vertebrate species, including 60% of threatened mammal and bird species, as well as 80% of threatened amphibians, occur in only 2.5% of the earth's surface (Mittermeier et al., 2004, 2011, 2007; Williams et al., 2011). Biodiversity conservation is, of course, not only needed in the hotspot regions but instead all around the globe. Yet, resources are limited, so focusing on these hotspots could help save a higher proportion of the existing biodiversity.

In many cases, conservation strategies still focus mainly on species and ecological communities, and not so much the genetic diversity within a species (Coates et al., 2018). Of course, saving intact habitats is of major importance, as this allows the survival of multiple species at once. However, genetic diversity conservation is often neglected and only comes into mind when a species is already classified as "Critically Endangered", with only a few individuals left in the wild. Then, breeding programs and translocations are considered to save as much genetic diversity within the remaining population. Yet, at this point, the majority of genetic variation which once occurred in a particular species or population may already be lost, reducing the chance of a successful conservation program due to the reduced ability to adapt to a changing environment and the increased risk of reduced fitness by inbreeding depression.

GENE FLOW - ITS ROLE IN SPECIATION AND IMPORTANCE IN SPECIES DELIMITATION

Species, such as giraffe, have so far mostly been delimited by morphological traits, and reproductive isolation, but the era of genetics has shown that reproductive isolation may not be a strict criterion and gene flow from one species into another by introgressive hybridization has been described in many cases, such as in bears and cichlids (e.g., Cahill et al., 2015; Joyce et al., 2011; Kumar et al., 2017).

Gene flow is the transfer of genetic variation from one population to another facilitated by migration. Gene flow can prevent the differentiation of fragmented populations, naturally occurring due to e.g., genetic drift if the migration rate is high enough (Frankham et al., 2010). Wright (1969) stated that in theory, *a single* migrant per generation is sufficient to prevent complete population differentiation regardless of the population size, others suggested *five*, *one to ten* or even *more than ten* migrants are needed (Lacy, 1987; Mills and Allendorf, 1996; Vucetich and Waite, 2000). However, it has to be noted that Wright's calculations are based on idealized populations. Therefore, one effective migrant, which correlates the number of migrants with the effective population size N_e, is intended (Frankham et al., 2010). Populations with an effective migration rate (N_em) of more than one likely do not exhibit fixation of alleles, whereas populations with less than one effective migrant per generation can become fixed for alternative alleles (Frankham et al., 2010). These are, however, only theoretical calculations and have not been studied in real organisms.

Limited gene flow, however, can also lead to speciation, because reproductive barriers (e.g., postzygotic barriers) might develop during the differentiation of populations, and it has been proposed that speciation is possible even with eight and up to 16 N_em (Gavrilets et al., 2000; Porter and Johnson, 2002). The recent proposal of four giraffe species (Fennessy et al., 2016) suggested limited gene flow among giraffe despite the known ability of many giraffe taxa to hybridize in captivity. Therefore, gene flow among giraffe in the wild and the taxonomic implications was studied in **Publication 2** of this thesis.

Several terms are used to describe the process of speciation with gene flow, e.g., divergence-with-gene-flow, divergence-with-genetic-exchange, and divergence-with-introgressive-hybridization (Arnold, 2016). Yet, it is not just the limitation of gene flow between populations to a certain degree that allows for speciation, but also genetic exchange between species in the form of hybridization can lead to "hybrid-species" (Abbott et al., 2013; Arnold, 2016; Meier et al., 2017). However, if these are classified as species depends on the applied species concept and how genetic exchange is handled under it. Therefore, the importance of gene flow under some of the many species concepts is detailed in the following.

GENE FLOW IN SPECIES CONCEPTS

The "Biological Species Concept" (BSC) is the most widely applied species concept, especially for sexually reproducing eukaryotes developed by Dobzhansky (1935) and Mayr

(1942). Under the BSC species are defined as "*groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups*" (Mayr, 1942). Therefore, the BSC, such as other species concepts, uses the frequency of genetic exchange or gene flow as a metric to defining species (Arnold, 2016). When the BSC is applied strictly, hybridization between species cannot occur. If gene flow is observed, the individuals hybridizing would, by definition, belong to the same species (Mayr, 1996).

However, Mayr (1963) acknowledged that interspecies hybridization occurs even among "good" species but argued that interspecies hybrids in animal taxa are rare and, therefore, evolutionary unimportant. Also, he argues that "*there is usually severe selection against introgression*". Yet, the rarity of an event, such as interspecies hybridization, cannot predict its evolutionary importance (Arnold, 2016), and many studies identified introgressive hybridization among "good" plant and animal species, such as polar and brown bear, blue and fin whale, and several species in the plant genus *Betula* (Anamthawat-Jónsson, 2019; Árnason et al., 2018; Kumar et al., 2017). Therefore, in an amended version of the BSC, Mayr (1996) stated that two species are reproductively isolated from each other and considered distinct species if they do not fuse into a single population in the presence of interspecies gene flow.

An alternative to the BSC and frequently used is the "Phylogenetic Species Concept" (PSC) that cannot only be used for sexually reproducing species but also species with asexual reproduction. The PSC defines a species as "*an irreducible cluster of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent*" (Cracraft, 1989).

The PSC recognizes species based on phylogenetic relationships classifying a species as a monophyletic cluster in a phylogenetic tree, which means that all organisms or individuals in that cluster are descendants of a common ancestor. The PSC goes back to the idea of phylogenetic systematics by Hennig (1966). In addition to monophyly, Hennig (1966) defined species also as discrete reproductive communities, thus, if applied strictly, prohibiting introgressive hybridization, because it would violate the requirements of strict monophyly (Arnold, 2016).

A third often used species concept is the "Cohesion Species Concept" (CSC) that was proposed by Templeton (1989) to consider problems with other species concepts such as the BSC. It describes species as "*the most inclusive group of organisms having the potential for*

genetic and/or demographic exchangeability" (Templeton, 1989). Under the CSC, in contrast to other species concepts such as the BSC and PSC, "*species are defined on the basis of independent evolutionary/ecological trajectories*" and can be definable on one of the criteria, but not necessarily on the other (Arnold, 2016; Templeton, 2001, 1989). Therefore, permitting interspecies gene flow and hybrid speciation to occur under the CSC (Templeton, 2001, 1989).

Under the "Genic Species Concept" (GSC) developed by Wu (2001), "*species are groups that are differentially adapted and, upon contact, are not able to share genes controlling these adaptive characters, by direct exchange or through intermediate hybrid populations. These groups may or may not be differentiated elsewhere in the genome*." Wu (2001) recognized that, as demonstrated in previous studies, some regions in the genome of related species are reproductively isolated, while other regions are exposed to genetic exchange (Harrison, 1986; Hunt and Selander, 1973; Robbins et al., 2014). He argued that the BSC should emphasize differential adaptation on the genic level rather than the isolation of the entire genome (Wu, 2001). This argument was refuted by Harrison (2012) and Mayr (2001), stating that evolutionary biologists had already recognized and incorporated the "genic" view that Wu (2001) provides.

GENE FLOW IN SPECIES DELIMITATION

The frequency of gene flow, as described above, is an essential criterion to define species in many species concepts such as the BSC. Therefore, gene flow analyses are crucial for delimiting species, yet it is often neglected, due to limited access to genomic data for non-model organisms. The more limited gene flow is between populations, the more likely it is that these populations belong to different species. Although limited gene flow under certain species concepts is permitted, there is so far no defined threshold, from which point on, does one have separated populations that still occasionally mix, or when are they different species?

Some attempts have been made to empirically delimit species based on gene flow in the past, but none of them have been widely applied (Good and Wake, 1992; Porter, 1990). To this day, species are still mainly delimited based on morphology, and if molecular methods are used, they often use a measurement of genetic distance based on the mitochondrial cytochrome oxidase I (cox1) gene with arbitrary thresholds (Câmara et al., 2019; Janzen Daniel H et al., 2005; Santos et al., 2019).

STUDY ORGANISM

The giraffe (genus *Giraffa*) are well known for their long neck and limbs and are a favorite in any zoo, yet, have long been neglected by the scientific community. Today, there is still a significant lack of understanding about giraffe behavior and ecology, and only little is known about their genetics and evolution. A recent study suggested four species of giraffe instead of the previously accepted one species hypothesis (Fennessy et al., 2016). This makes giraffe an interesting study system to investigate the genetic population structure and the role of gene flow in the speciation process.

GIRAFFE EVOLUTION

The family Giraffidae consists of two extant genera, *Okapia* and *Giraffa,* which are eutherian mammals of the Order Cetartiodactyla (Artiodactyla and Cetacea) and are placed in the Suborder Ruminantia. Cetartiodactyls likely evolved in the late Palaeocene and became noticeable ~50 Mya (Mitchell and Skinner, 2003). The oldest known fossil Cetartiodactyla assemblage likely is the Dichobunidae. A complete skeleton of a species named *Diacodexus* sp. suggests that the early cetartiodactyls were rabbit-sized forest-dwelling animals that lived in the Holarctic of Pangea that is now North America, Europe and Asia (Mitchell and Skinner, 2003). *Diacodexus* already shows the typical ankle structure called astragalus that unambiguously identifies the fossil as an early Cetartiodactyla (Mitchell and Skinner, 2003).

Extensive adaptive radiation of the taxon Cetartiodactyla in the middle Eocene resulted in twenty or more families, of which one led to the ruminants (Ruminantia) (Carroll, 1988). Within this radiation, clear signs of adaption to herbivory are evident, such as a lengthened diastema, hypsodont (higher crowned) teeth, and broader selenodont (with crescent-shaped cusps) molars are still the characteristics of modern ruminants (Mitchell and Skinner, 2003).

The family of Giraffidae likely evolved from the gelocids (Gelocidae), a group of early cetartiodactyls (Shorrocks, 2016). Gelocid fossils were found in southern Eurasia around the Black and Caspian seas, which are remnants of the Paratethys Sea. The Paratethys Sea was a large shallow inland sea spanning from Central Europe to Central Asia (McCann, 2008). *Gelocus communis* (Gentry, 1994), the archetypical fossil of Gelocidae, was described as the "*first fully progressive artiodactyl*" and likely resembled a modern gazelle (Carroll, 1988;

Mitchell and Skinner, 2003). All modern pecoran ruminants (Pecora), the Cervidae, Bovidae, Antilocapriinae, Moschidae, and Giraffidae have arisen from the gelocids (Shorrocks, 2016).

Within Pecora, Giraffidae were first thought to be part of the bovid radiation that took place approximately 18 Mya (Allard et al., 1992; Gentry, 1994) because of their appearance in the fossil record at about the same time, which was after the cervid radiation appeared in the fossil record (Gentry, 1994; Mitchell and Skinner, 2003). However, there is anatomical and early molecular evidence that suggests giraffids being more closely related to cervids (Allard et al., 1992; Colbert, 1936a; Irwin et al., 1991; Kidd, 1900). This relationship could not be confirmed by molecular studies that find giraffids as a sister group to Cervidae, Moschidae and Bovidae (Cronin et al., 1996; Hassanin and Douzery, 2003; Zurano et al., 2019) or together with the Antilocapriinae as a sister group to all other Pecorans (Chen et al., 2019; Fernández and Vrba, 2005).

Giraffidae likely originated from the gelocid subfamily Palaeomerycinae (sometimes Palaeomerycidae) (Mitchell and Skinner, 2003; Shorrocks, 2016). *Teruelia*, a small hornless gazelle-like gelocid fossil found in Spain, was potentially the first ancestral giraffid (Agustí and Moyà-Solà, 1991; Mitchell and Skinner, 2003). From there, the two giraffid families Climacoceratidae and Canthumerycidae evolved.

The Climacoceratidae is an extinct family of giraffids. The so far most primitive known climacoceratid (*Climacoceras africanus*) was described as “fossil deer” of the size of a roe deer, *Capreolus capreolus* based on fossils found at Koboko Island in Lake Victoria (MacInnes, 1936). Fossils of Climacoceras species were also found in other locations in Kenya and Namibia and were dated to approximately 14-17 Mya (Churcher, 1970; Gentry, 1994, 1970; Hendey, 1982, 1978; Thomas, 1984). While all *Climacoceras* species show features that suggest that they are closely related to giraffe, it is more likely that they are the ancestors of Sivatheriinae (Mitchell and Skinner, 2003).

Sivatheres were as large as elephants*, Loxodonta africana*, and are characterized by short legs, a short neck, and large ornamented horns (Fig. 1)(Mitchell and Skinner, 2003). Two major forms evolved, *Sivatherium giganteum* and *Sivatherium maurusium (Mitchell and Skinner, 2003)*. S. giganteum occurred mostly in Asia and likely went extinct in the early Pleistocene. *S. maurusium* occurred across Africa and became extinct, probably around 3500 years ago (Colbert, 1936b).

Canthumeryx sirtensis, a species within the second giraffid family, the Canthumerycidae, is the earliest known ancestor of Giraffinae. It was approximately the size of a fallow deer, *Dama dama* (Fig. 1) (Hamilton, 1978, 1973; Mitchell and Skinner, 2003). Its fossil was discovered around 300 km south of Tunis, Libya, an area that at the time *C. sirtensis* lived there (~14-25 Mya), was a savannah/alluvial area type habitat (Gentry, 1994; Hamilton, 1973; Morales et al., 1987). It had bilobed lower canines, which increased the breadth of the dental edge by acting as additional incisors (each spanning the width of two incisors) and are characteristic for giraffids. Furthermore, *C. sirtensis* had simple (unbranched) horns that protruded almost horizontally to the side of the skull (Churcher, 1978). *Canthumeryx* became extinct around 14 or 15 Mya (Mitchell and Skinner, 2003).

A key species in giraffe evolution is *Giraffokeryx punjabiensis*, which was a medium-sized giraffid with two pairs of ossicones (horncores) whose fossils were dated to approximately 10-15 Mya (Colbert, 1933; Matthew, 1929; Simons et al., 1971). Even though reconstructions of *Giraffokeryx* differ, they all show *Giraffokeryx* with an elongated neck, looking either like a small giraffe or more similar to okapi (Colbert, 1935; Savage and Long, 1986).

The genus *Giraffokeryx* and two additional genera *Palaeotragus* and *Samotherium* belong to the Palaeotraginae (Mitchell and Skinner, 2003). The radiation of Palaeotraginae likely coincided with the radiation of bovids and antelopes (17-12 Mya) (Allard et al., 1992; Hassanin and Douzery, 1999). The similarities between the reconstructions of *Giraffokeryx* and the okapi led to the idea that the okapi "*is a persistent, little changed palaeotragine that has survived by occupying a forest refuge niche unchallenged by other species*" (Mitchell and Skinner, 2003). This idea is consistent with a modern genomic study that estimated the divergence time between giraffe and okapi at approximately 11.5 Mya (Agaba et al., 2016).

Species within the genus *Palaeotragus* were widely distributed (from East Africa to Mongolia), medium-sized giraffids with slightly elongated limbs and neck, and usually one pair of horns (Churcher, 1970; Colbert, 1936a; Mitchell and Skinner, 2003).

Species of the genus *Samotherium* were much larger than *Palaeotragus* sp. with a typical giraffe profile (see Fig. 1) (Mitchell and Skinner, 2003). Their skulls exhibit several features characteristic for living giraffe, two horns, hypsodont teeth, and well-developed cranial sinuses (Colbert, 1938; Hamilton, 1978). Cranial sinuses allow enlarging the skull

without adding weight and are considered an important requirement for neck elongation (Mitchell and Skinner, 2003).

Samotheres became the dominant palaeotragines after *Palaetragus* sp. went extinct around 9-10 Mya, occupying their former niche (Churcher, 1970; Gentry, 1994). Several Eurasian samotheres species were described, but only the African species *Samotheres africanum* had the morphological features to be an ancestor of modern giraffe (Mitchell and Skinner, 2003).

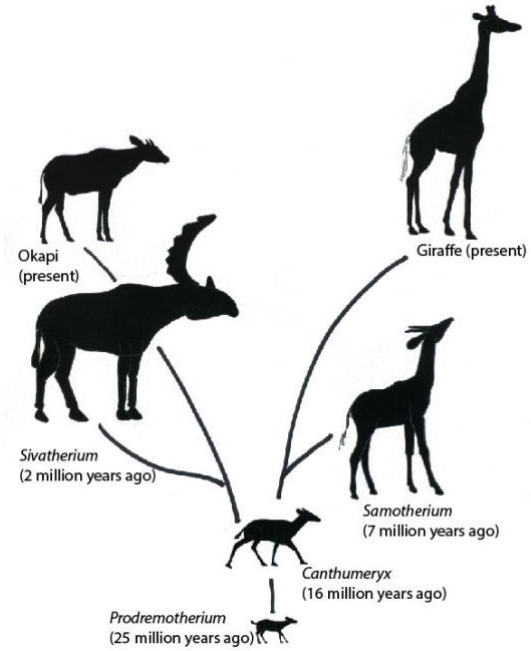

Figure 1. Schematic representation of the evolution of long limbs and neck in the family Giraffidae. With permission of Nikos Solounias.

A possible direct ancestor of giraffe is *Bohlinia attica* (found in Pikermi, Greece) that could have evolved directly from S. africanum and is so similar to giraffe that it was first described as *Giraffa attica* (Forsyth Major, 1891). *B.* attica was smaller than the early giraffe, but otherwise, with its long neck and limbs, bilobed lower canines and similar ossicones, resembled them closely (Harris, 1976).

The genus *Giraffa* likely emerged through fairly rapid radiation after migration from south-eastern Europe into Indo-Asia (Bohlin, 1926). The oldest unequivocally species within *Giraffa* and the ancestor of the later Asian giraffe is *G. punjabiensis* that lived ~7 Mya and was extinct by the end of the Pliocene (Harris, 1976, 1991).

The African lineage of *Giraffa* could have started by the migration of *G. punjabiensis* or one of its descendants into Ethiopia via the Arabian-African land bridge (Mitchell and Skinner,

2003). However, a migratory route is missing, and the anatomy of *G. punjabiensis* seems too advanced to be in the same lineage as African *Giraffa* (Churcher, 1978; Mitchell and Skinner, 2003). Therefore, Mitchell and Skinner (2003) suggested that the African lineage of *Giraffa* arose from the Pikermian *Bohlinia*. The oldest *Giraffa* fossil was found in the northern Kenya Rift valley and was dated to 7.0-5.4 Mya (Pickford, 1975). Beside extant giraffe presumably, four additional African giraffe species have existed (*G. gracilis*, *G. pygmaea*, *G. stillei,* and *G. jumae*) (Shorrocks, 2016). The phylogeny within *Giraffa*, however, is unresolved (Mitchell and Skinner, 2003).

The oldest African giraffe species is probably *G. gracilis* that has often been synonymized with *G. stillei* and *G. pygmaea* (Mitchell and Skinner, 2003). Its range was likely restricted to East Africa, and in many aspects, it resembled *Bohlinia* (Hamilton, 1978; Harris, 1976). Its fossils that have been dated to 5-2 Mya seem to be of similar stature as extant giraffe. However, *G. gracilis* appeared to be more lightly built, larger in size than *Bohlinia* and *G. pygmaea* but smaller than a modern female giraffe (Arambourg, 1947; Harris, 1976; Mitchell and Skinner, 2003).

Even though *G. gracilis* is often considered the earliest African giraffe species, it could have been preceded by a so-called “Ethiopian (Omo) form” or *G. stillei* (Arambourg, 1947; Harris, 1976). These fossils, however, were dated to 3.3 -2.7 Mya and, therefore, cannot be considered an ancestor of *G. gracilis* (Harris, 1976; Mitchell and Skinner, 2003). Harris (1976) argues that *G. stillei* and *G. gracilis* are synonymous because they can barely be separated based on fossil evidence.

G. pygmaea was even smaller than *G. stillei*, but first has also been synonymized with *G. gracilis*/*G. stillei* (Harris, 1987). Later it was suggested that *G. pygmaea* was a smaller variant that occupied a valley bottom, riverine woodland habitat rather than an *Acacia* woodland (Harris, 1991).

The largest African *Giraffa* was *G. jumae,* and it could also be one of the oldest (~6.5 My) (Pickford, 1975). Both *G. gracilis* and *G. jumae* went extinct around 1 Mya, and at the same time, modern giraffe appeared in the fossil record (Mitchell and Skinner, 2003).

It is unclear how modern giraffe have evolved, and there are several possible explanations. It has been suggested that extant giraffe could have evolved by either a reduction in size of *G. jumae* or an increase in size of *G. gracilis* or even by hybridization between *G. jumae* and *G. gracilis* (Harris, 1987, 1976; Mitchell and Skinner, 2003). However,

the most likely conclusion is that modern giraffe have evolved from *G. gracilis* because of more similar morphology (Harris, 1976; Mitchell and Skinner, 2003).

Extant giraffe are characterized especially by their height, the long neck and limbs, and their distinctive pelage pattern. The elongation of the giraffe neck is accomplished not only by the elongation of each of the cervical vertebrae but also by a "cervicalization" of the first thoracic vertebra that took over the role of articulation (Lankester, 1908).

The elongation of the limbs is enabled by an increase in bone mineralization that allows the strength to support the high body mass at a small bone diameter (Mitchell and Skinner, 2003). The amount of calcium necessary to allow such a high bone density can only be obtained by a strong dependency on legume browse, such as *Acacia* trees (Dougall et al., 1964; Mitchell and Skinner, 2003).

The elongation of neck and limbs does not only allow to exploit food resources that competitors cannot reach; the slender shape does, also, have thermoregulatory benefits by increasing the surface area for heat loss without increasing the body mass proportionally (Brownlee, 1963; Mitchell and Skinner, 2003). In addition, the tallness does also increase the ability to defend against or evade predators, which could have been the main driver in the evolution of elongated limbs and neck (Brownlee, 1963).

The pelage pattern of extant giraffe are variable, despite their uniform dark grey skin pigmentation (Mitchell and Skinner, 2003). There are anecdotal reports of albino and pure white giraffe as well as unspotted, pale brown and black giraffe and even one with a black band around the trunk (Arnold, 1940; Butler, 1912; McDougall, 1939; Mitchell and Skinner, 2003; Petzsch, 1950; Turner, 1969). These patterns contribute to the giraffe's camouflage by breaking up the body outline, which can make especially young giraffe that hide next to shrubs and trees difficult to see even if they are only a few meters away (Mitchell and Skinner, 2003). An additional function of these coat markings, first suggested by Skinner and Smithers (1990), may be thermoregulation. It was discovered that giraffe have a unique blood vessel system in the skin, with numerous vessels branching off into the darker pigmented areas (De Beaufort, 1927). These highly pigmented areas radiate more heat than the lighter colored areas, which facilitates heat loss without loss of water by sweating (Dagg, 2014; Hilsberg-Merz, 2008).

GIRAFFE SYSTEMATICS

The family Giraffidae consists of two extant genera *Okapia* (Lankester, 1902) and *Giraffa* (Brisson, 1762). *Okapia* is a monotypic genus with the okapi, *Okapia johnstoni* (Sclater, 1901), being the only extant species, without known subspecies.

The systematic within the genus *Giraffa*, however, is debated for decades. Giraffe were first scientifically described morphologically as *Cervus camelopardalis* by Linnaeus (1758). In 1762, Brisson placed the giraffe in their own genus *Giraffa* based on their ossicones, which are permanent rather than regrown every year like antlers are in cervids. Interestingly, neither Linnaeus nor Brisson had ever seen a live giraffe (Shorrocks, 2016), but based their conclusions on a 17th-century description by John Ray.

In the early 19th century, when living specimens could be studies, the idea of several species of giraffe developed. Geoffroy Saint-Hilaire was the first that studied giraffe specimens both from the northern and southern distribution and proposed two species based on morphological differences (Allin, 1998). His classification of a northern and a southern giraffe species was also favored by Swainson (1835), Owen (1839), and Lesson (1842) based on differences in cranial characters (Dagg and Foster, 1976). At the same time, Ogilby (1836) and Sundevall (1842) disagreed and either argued that there were no differences or that the differences were only variations within a species. At the end of the 19th century, de Winton (1897) acknowledged that the lack of specimens could cause the uncertainties in giraffe taxonomy but still proposed two species, *Giraffa camelopardalis,* and *Giraffa capensis*. He also described *G. c. reticulatus* as subspecies of *G. camelopardalis*.

At the beginning of the 20th century, Richard Lydekker (1904) conducted a complete revision of the genus *Giraffa* using all available information, including drawings, photographs, skins, and live animals. He concluded that there are two separate species, the “netted” giraffe *Giraffa reticulata* and the “blotched” giraffe *Giraffa camelopardalis*. Also, he divided the blotched giraffe into ten subspecies: The Nubian giraffe (*G. c. typica*), the Kordofan giraffe (*G. c. antiquorum*), the Nigerian giraffe (*G. c. peralta*), the South Lado giraffe (*G. c. cottoni*), the Kilimanjaro giraffe (*G. c. tippelskirchi*), the Baringo giraffe (*G. c. rothschildi*), the Congo giraffe (*G. c. congoensis*), the Angolan giraffe (*G. c. angolensis*), the northern Transvaal giraffe (*G. c. wardi*), and the Cape giraffe (*G. c. capensis*) (Seymour, 2001). Later, the Thornicroft’s giraffe (*G. c. thornicrofti*) was added to the list of “blotched” giraffe subspecies, as well as a second subspecies of the “netted” giraffe *G. c. nigrescens (Lydekker, 1911)*.

The classification of Lydekker was modified several times (Ansell, 1968; Dagg, 1968, 1962a; Haltenorth, 1962; Krumbiegel, 1939). For the last five decades, the most accepted classification was the amended classification of Dagg (1971) that recognized one giraffe species *Giraffa camelopardalis* with the following nine subspecies: The Nubian giraffe (*G. c. camelopardalis*, Linnaeus, 1758), the Kordofan giraffe (*G. c. antiquorum*, Jardine, 1835), the West African giraffe (*G. c. peralta*, Thomas, 1898), the reticulated giraffe (*G. c. reticulata*, Winton, 1899), the Rothschild's giraffe (*G. c. rothschildi*, Lydekker, 1903), the Masai giraffe (*G. c. tippelkirchi*, (Matschie, 1898)), the Thornicroft's giraffe (*G. c. thornicrofti*, Lydekker, 1911), the Angolan giraffe (*G. c. angolensis*, Lydekker, 1903), and the Cape giraffe (*G. c. giraffa*, Schreber, 1784).

The first molecular studies based on mitochondrial DNA (mtDNA) sequences (Hassanin et al., 2007) or mtDNA sequences and microsatellite genotypes (Brown et al., 2007) showed that mtDNA can distinguish between 6-7 giraffe clades. However, both studies did not include samples of all the nine subspecies that were recognized at that point and mostly focused on mtDNA that is maternally inherited. Yet, Brown et al. (2007) concluded that "*the seven lineages that are reciprocally monophyletic in the mtDNA tree need to be considered evolutionary significant units if not species*", despite showing that "*11 of the 18 sampling localities resolved as distinct genetic clusters at K=13,*" based on 14 microsatellites.

The most comprehensive genetic analyses of giraffe and the first that included all of the recognized nine subspecies and analyzed seven nuclear loci, identified four major genetic clusters in a nuclear phylogeny and additional population genetic analyses (Fennessy et al., 2016). They proposed that these four clusters represent four distinct giraffe species (see Fig. 2) and five subspecies resulting in the following taxonomy: (1) Northern giraffe (*G. camelopardalis*) with three subspecies, the West African giraffe (*G. c. peralta*) the Kordofan giraffe (*G. c. antiquorum*), the Nubian giraffe (*G. c. camelopardalis*) that, after the new taxonomy, includes Rothschild's giraffe (former *G. c. rothschildi*); (2) the reticulated giraffe (*G. reticulata*); (3) the Masai giraffe (G*. tippelkirchi*), that includes the Thornicroft's giraffe (former *G. c. thornicrofti*); and (4) the southern giraffe (*G. giraffa*) with two subspecies, the Angolan giraffe (*G. g. angolensis*) and the South African or Cape giraffe (*G. g. giraffa*). The systematic within *Giraffa* was further studied in **Publication 2** as part of this thesis.

Figure 2. The four giraffe species (Fennessy et al. 2017).

From left to right: (1) northern giraffe (*G. camelopardalis*), (2) reticulated giraffe (*G. reticulata*), (3) Masai giraffe (*G. tippelskirchi*), and (4) southern giraffe (*G. giraffa*)

GIRAFFE DISTRIBUTIONS

Historically, giraffe have likely occurred from desert to woodland/savannah habitats all across Africa, but their range has rapidly declined since then (O'Connor et al., 2019, see Fig. 3). Giraffe went extinct in at least seven countries over the last 300 years such as Mali, Eritrea, and Nigeria and have been reintroduced only recently into some countries (e.g., Angola and Malawi) (Giraffe Conservation Foundation, 2015; Marais et al., 2018; Muller et al., 2018).

Currently, giraffe occur in 21 African countries, with major populations in East and southern Africa and smaller, more fragmented populations in West and Central Africa (Muller et al., 2018).

The distribution maps of the different giraffe taxa were for a long time based mostly on pelage patterns and were, therefore, as uncertain as the systematics and still are in some regions. Lydekker (1904) suggested very narrow distributions for the taxa he described, which circled around the type locality of each taxon. His distribution map was clearly not the range of giraffe at that time and was likely the result of very limited information (e.g., single specimens). Krumbiegel (1939) on the contrary, even though, giving a more realistic distribution map, likely overestimated the range of some giraffe taxa at the time, especially the West African giraffe, which was suggested to occur from the African West coast in Senegal to as far to the East as Chad.

Over time, the range maps became more accurate with an increasing amount of data (Dagg, 1971, 1962b; East, 1999). However, the assignment of an individual to a particular subspecies was solely based on pelage pattern and ossicone morphology, which are not always reliable characters to identify giraffe taxa (Dagg, 2014).

It was not until 2007 when molecular studies based on mitochondrial cytochrome *b* and control region sequences were able to group individuals into distinct clades that corresponded to described subspecies (Brown et al., 2007; Hassanin et al., 2007). It was demonstrated later on that these clades allow assigning an individual to a certain taxon (Bock et al., 2014; Fennessy et al., 2016, 2013).

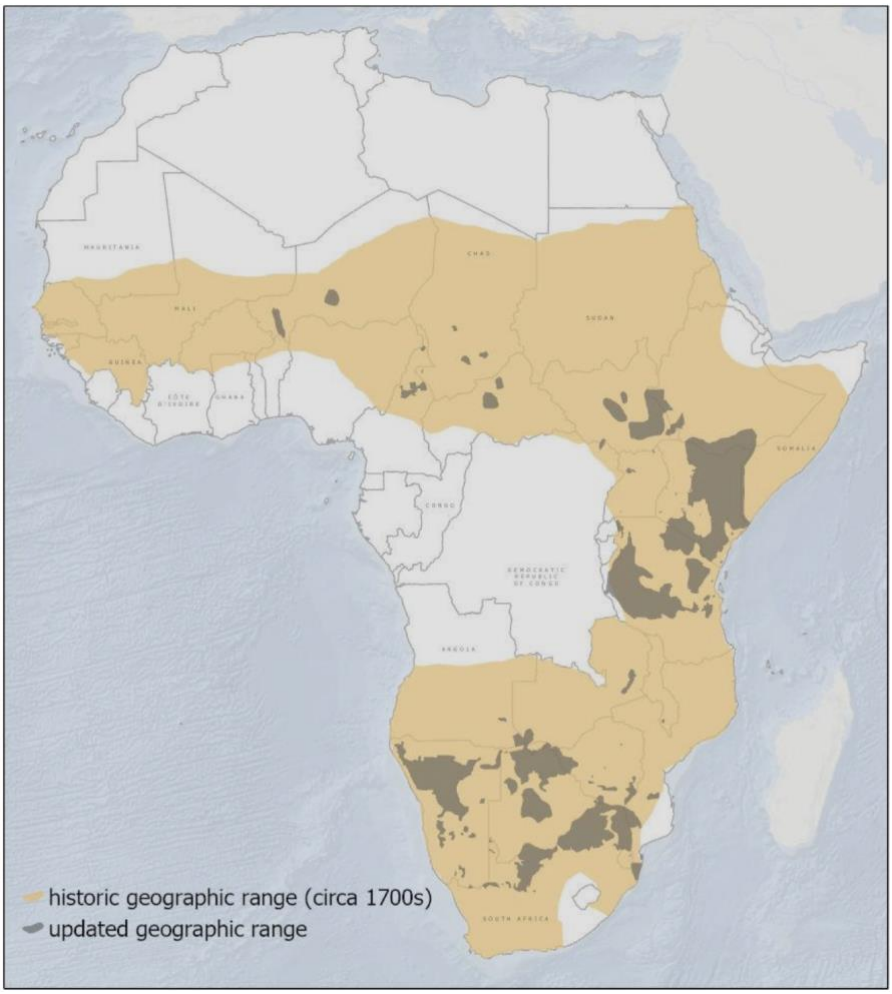

Figure 3. Giraffe distribution map with potential historic range (circa 1700s) compiled by the Giraffe Conservation Foundation (O'Connor et al., 2019).

The recent study by Fennessy et al. (2016) was the first that included samples of all formerly recognized subspecies, and in addition to mtDNA sequences also used nuclear sequences to update giraffe taxonomy. They also published the most accurate distribution map of all giraffe taxons (species and subspecies) at that time. It was based on giraffe distribution data from the Giraffe Conservation Foundation and their genetic taxon assignments. An updated distribution map for the four giraffe species based on the taxonomy proposed by Fennessy et al. (2016) was published recently (O'Connor et al., 2019).

However, this does not suggest that the distribution map, especially for subspecies, is entirely accurate. Many populations are still assigned to a taxon based solely on morphological characters, and some are likely assigned to the wrong taxon. Therefore, the distribution of giraffe taxons and the assignment of populations to the correct taxon was further studied in **Publication 1** and **3** of this thesis.

CONSERVATION STATUS OF GIRAFFE

Giraffe, still classified as a single species *(G. camelopardalis)*, are since 2016 listed as "Vulnerable" on the IUCN RED-list of threatened species due to a population decrease of 36-40% over 30 years or three generations (Muller et al., 2018). A recent subspecies assessment of eight of the nine currently by the IUCN recognized subspecies ranked several into even higher threat categories (Bercovitch et al., 2018; Bolger et al., 2019; J. Fennessy et al., 2018; Fennessy and Marais, 2018; S. Fennessy et al., 2018; Marais et al., 2018; Muneza et al., 2018; Wube et al., 2018). For an overview of the current assessments, see Table 1. Both the Kordofan and the Nubian giraffe are listed as "Critically Endangered", because of a drastic population decline of >80% and >95%, respectively, over the last three decades and still decreasing population numbers (Fennessy and Marais, 2018; S. Fennessy et al., 2018). However, it has to be noted that several studies, including the newest molecular study, suggest that Nubian and Rothschild's giraffe (classified as "Near Threatened") should be subsumed, which would potentially result in a reduced level of threat for the Nubian giraffe (East, 1999; Fennessy et al., 2016; Groves and Grubb, 2011). The reticulated giraffe faced a population decline of ~56% and is, therefore, classified as "Endangered" (Muneza et al., 2018). The recent assessment of the Masai giraffe, classified them as "Endangered", due to an estimated decline of ~49-51% listing habitat loss and illegal hunting as likely causes (Bolger et al., 2019). The isolated population of Thornicroft's giraffe, which was recently subsumed into

the Masai giraffe by Fennessy et al. (2016), is currently listed as "Vulnerable" even though being a stable population. However, their restricted range to a single location, the small population size (n=600), and the limited number of mature individuals (n=420) justify this classification (Bercovitch et al., 2018).

Even though giraffe numbers, in general, are decreasing, there are populations with a positive trend. The West African giraffe, for example, was previously listed as "Endangered", and the only existing population was down to approximately 50 Individuals in 1996 located close to Niamey, the capital of Niger (J. Fennessy et al., 2018). Due to the successful implementation of the first "National Strategy for Giraffe Conservation", the population increased to approximately 600 individuals in 2017 and is now listed as "Vulnerable" (J. Fennessy et al., 2018; Giraffe Conservation Foundation, 2019). The translocation of eight giraffe just recently established a satellite population of West African giraffe in their former more eastern range by the Niger Government, the Giraffe Conservation Foundation, and additional partners (Giraffe Conservation Foundation, 2019b).

Giraffe in southern Africa are generally doing very well recently. The Angolan giraffe population has, against the overall trend for giraffe, constantly increased over the last three decades and is now listed as "Least Concern" (Marais et al., 2018). The IUCN has not adequately assessed the South African giraffe yet, but recent studies and population estimates suggest a constant population growth, with total numbers being the highest of all giraffe taxa (Deacon and Tutchings, 2018; Giraffe Conservation Foundation, 2019a).

Table 1. A comparison of the traditional and newly proposed systematics of giraffe and their respective threat categories according to the IUCN Red List of Threatened Species. Taxa with species rank are highlighted in bold.

Dagg (1971)	Fennessy et al. (2016)	Threat category
Giraffa camelopardalis	***Giraffa camelopardalis***	Vulnerable
G. c. camelopardalis	*G. c. camelopardalis*	Critically Endangered
G. c. rothschildi	*	Near Threatened
G. c. antiquorum	*G. c. antiquorum*	Critically Endangered
G. c. peralta	*G. c. peralta*	Vulnerable
G. c. reticulata	***Giraffa reticulata***	Endangered
G. c. tippelskirchi	***Giraffa tippelskirchi***	Endangered
G. c. thornicrofti	*	Vulnerable
	Giraffa giraffa	Not assessed
G. c. giraffa	*G. g. giraffa*	Not assessed
G. c. angolensis	*G. g. angolensis*	Least Concern

* subspecies was subsumed into above-mentioned taxon

However, all giraffe populations face threats that currently contribute or will potentially contribute to a population decline. The main risks for giraffe listed by the IUCN (Muller et al., 2018) are:

- Habitat loss (e.g., through deforestation, human population growth, and expansion of agricultural activities)
- Civil unrest (e.g., rebel militias, paramilitary and military operations or ethnic violence)
- Illegal hunting (poaching)
- Ecological changes (e.g., climate-induced processes, habitat conversion to agriculture or mining activities)

The severity of these threats vary between giraffe populations, but habitat loss and fragmentation is the biggest threat for most of them (Muller et al., 2018). Especially giraffe populations in East and Central Africa are facing multiple threats. Habitat is lost through rapid conversion into farmland by and increasing human population (Muller et al., 2018). Droughts are becoming more frequent and more severe, which often results in human population movements, and often cause armed conflicts and increases poaching (Muller et al., 2018).

THESIS OBJECTIVES

The common thread of this thesis is the application of various genetic marker systems to study the population structure, taxonomy, and distribution of giraffe throughout Africa. This is to contribute to the understanding of speciation in general, to provide a solid taxonomy, and to understand giraffe biology, and to inform future conservation strategies.

The main research approach was to develop a comprehensive nuclear sequence dataset that allowed for the first wide-ranging sequence-based gene flow analyses among the previously proposed four giraffe species. Gene flow analyses are essential for delimiting species under several species concepts such as the BSC that is based on reproductive isolation. It is expected that limited gene flow between the four proposed giraffe species but higher gene flow rates among subspecies within the different species. Thus, further supporting the proposed four species taxonomy, because limited gene flow will allow for the differentiation of populations and consequently speciation. Yet, sequence-based gene flow analyses have been neglected in species delimitation of giraffe so far, making the approaches used in my work unique in this taxonomic group. Furthermore, my thesis aimed to assign giraffe

populations to their respective (sub)species and study their genetic composition to identify necessary conservation measures.

To achieve the objectives mentioned above, appropriate nuclear intron sequences were identified from a list of potentially suitable loci derived from the genomes of sheep and cow and tested for their variability among populations. In addition, a set of suitable microsatellite loci was compiled from either published studies or developed from a list of potential microsatellite loci derived from a Kordofan giraffe draft genome (Fennessy et al., 2016).

The inclusion of 14 new and variable nuclear intron loci increased the available dataset to 21 loci and made it for the first time possible to specifically analyze gene flow among all giraffe taxa allowing for deeper insights into giraffe evolution and taxonomy (Winter et al., 2018, see Publications, Publication 2). This analysis was not possible before because of the limited data available.

Species are the essential unit in conservation biology and are often identified by morphological differences, geographic origin, or reproductive isolation according to the BSC, yet many species can hybridize to some extent. Therefore, it is crucial to develop and apply a gene flow-based species delimitation approach to identify genetically distinct species despite their phenotypical resemblance. This enables us to estimate the conservation status of these species better and allows for developing necessary conservation strategies.

Moreover, the maternal lineages of giraffe from the desert-dwelling population in Namibia and a giraffe population from southern Zimbabwe were analyzed and compared to a wide range of southern giraffe populations using mitochondrial cytochrome *b* and control region sequences. It is expected that the desert-dwelling giraffe is not genetically isolated from the geographically close Etosha National Park, and they maintain genetic variability by genetic exchange because there are no obvious geographic barriers. However, they have a paler pelage pattern and a unique habitat. A previous translocation of Etosha giraffe into the desert-dwelling giraffe population may have enhanced their genetic variability (Winter et al., 2018a, see Publications, Publication 1).

Furthermore, a set of microsatellites was compiled to, in combination with mtDNA and seven nuclear introns, identify the (sub)species of giraffe occurring in Malawi, to rule out apotential hybrid origin, and to infer the degree of inbreeding (Winter et al., 2019, see Publications, Publication 3). In 1993, five giraffe were reintroduced into Malawi and were

expected to be South African giraffe. However, no genetic evaluation of the source population was conducted, and reports of possible additional translocations of Masai giraffe from Zambia could have resulted in a hybrid population. The limited number of founder individuals likely results in the current population being highly inbred. Detailed genetic information about giraffe populations can identify necessary conservation measures and are essential to inform conservation management strategies.

Overall, this thesis contributes to a better understanding of giraffe systematics and evolution, population structure, and aids in making informed decisions in future conservation measures.

GENERAL DISCUSSION

GENE FLOW AND GIRAFFE SYSTEMATICS

Species delineation does not only have academic value, such as for taxonomy or to describe nature. Most of the biodiversity concepts, but more so conservation efforts depend on a reliable upstanding of a species and biodiversity. Last but not least, Last but not least, understanding speciation, the development of different biological entities is the central topic in biology and made Darwin's fundamental work on "Origin of the Species" (Darwin, 1859) one of the pillars of the Age of Enlightenment.

Therefore, the study and the claim of four giraffe species have relevance for a broad field in biology. However, today the understanding of a species depends on its definition and increasingly on the understanding of gene flow.

GENE FLOW UNDER THE BIOLOGICAL SPECIES CONCEPT

Several attempts have been made to define species, yet a definite consensus has not been reached so far (Coyne and Orr, 2004; De Queiroz, 2007). Reproductive isolation or the absence or limitation of gene flow among taxa, however, is the key to define species under many species concepts such as the BSC, the most widely applied species concept (Arnold, 2016; Avise and Ball, 1990). Reproductive isolation and thus limited gene flow allow populations to differentiate and potentially develop reproductive barriers (Frankham et al., 2010; Gavrilets et al., 2000; Porter and Johnson, 2002; Wright, 1969). Therefore, it is of major importance to study gene flow among potential species to gain additional insights into their species status.

Yet, there is no clear consensus of gene flow or the frequency of gene flow that is allowed between species under the BSC. Mayr and Dobzhansky, the founders of the BSC, acknowledged that gene flow or introgressive hybridization can occur, but they argued that it would be of minor importance, because either the parental individuals belong to subspecies of the same species or the vast majority of interspecies hybrid genotypes would have reduced fitness (Dobzhansky, 1951; Mayr, 1963, 1942). With mounting evidence for the frequent occurrence of interspecies hybrids in plants as well as some animals (e.g., Alston and Turner, 1963; Hubbs, 1955; Johnsgard, 1960), Mayr revised his definition of isolating mechanisms,

stating that isolating mechanisms are "*biological properties of individuals which prevent the interbreeding of populations*" (Mayr, 1970).

Thus, two populations are distinct biological species as long as gene flow does not result in the fusion of the two populations into one, which would make them indistinguishable. Yet, as there is no objective measure for the threshold for the amount of gene flow so far that is allowed under the BSC, it is nearly impossible to state if two populations fuse into one or not, especially in phenotypically cryptic species.

Defining a threshold for species delimitation

The lack of a defined threshold for the allowed frequency of gene flow among species allowed under the BSC makes it challenging to interpret the results of the gene flow analyses among giraffe. I, therefore, adopted a conservative approach to delineate species based on the additional information coming from gene flow analyses. These findings were supported by a combination of additional population genetic analyses (Winter et al., 2018b). Based on the available literature, a gene flow rate of less than one effective migrant per generation (N_em) enables the complete differentiation of populations, thus allowing for the development of reproduction barriers, and consequently, speciation (Wright, 1969). Some authors even suggest that populations can completely differentiate over time with five, ten or even more than ten effective migrants per generation (Lacy, 1987; Mills and Allendorf, 1996; Vucetich and Waite, 2000). It was even proposed that up to 16 effective migrants per generation allow speciation of parapatric populations (Gavrilets et al., 2000; Porter and Johnson, 2002).

Therefore, a conservative threshold of one N_em, similar to the proposed method developed by Porter (1990), was chosen. Porter suggested delineating species based on gene flow if the gene flow rate is less than 0.5 N_em, and with corroborating support, if the gene flow rate is less than 1 N_em (Porter, 1990). Thus, a gene flow rate of < 1 N_em in combination with additional support from population genetic analyses, such as distinct clusters in PCA (Principal Component Analysis) and STRUCTURE analyses and high F_{st} values, suggest separate species.

However, the conservative threshold of one N_em to allow for speciation should not be seen as a fixed value. It should instead be taken as a starting point for further theoretical and experimental studies to develop an expanded understanding of the threshold definition of gene flow under the BSC and allowing for the development of future methods for species

delineation. Future studies will show, if a fixed threshold value to delineate species will be developed, or if a range of thresholds would better describe the fluidity of biological phenomena, such as becoming distinct evolutionary entities such as species.

LIMITED GENE FLOW SUPPORTS FOUR SPECIES OF GIRAFFE

As outlined above, the species definition and gene flow are central concepts for the understanding and delineation of a species. In the case of the giraffe, it makes a difference if one considers one or four species.

For decades, the genus *Giraffa* was considered to be monotypic, consisting of only a single extant species *Giraffa camelopardalis* with varying numbers of subspecies being recognized (6-11) (Butchart et al., 2010; Dagg and Foster, 1976; Lydekker, 1904). Even though, it has been suggested that multiple species of giraffe might exist (Brown et al., 2007; Groves and Grubb, 2011; Lydekker, 1904), the initial finding of four distinct giraffe clades in the first nuclear sequence analyses by Fennessy et al. (2016) was unexpected. The reason for this was mainly the ability of nearly all giraffe taxa to interbreed in captivity (Gray, 1972; Lackey, 2011; Lönnig, 2011). In addition, giraffe are highly mobile in the wild (Flanagan et al., 2016), which should allow for frequent admixture.

The proposal of four giraffe species was not unequivocally accepted and criticized for ignoring "*admixture in the wild, and hybridization in captivity*" (Bercovitch et al., 2017). Even though, Fennessy et al. (2016) did not ignore hybridization, as we clarified in our response to the criticism (Fennessy et al., 2017), This study explicitly focused on analyzing gene flow among giraffe in this thesis (see Publications, Publication 2).

Many species can hybridize, especially in captivity, where the choice of a mating partner is limited or even absent. Also, in nature, hybridization often occurs in the absence of a conspecific mating partner (Randler, 2002). As such, hybrids between lions and tigers, zebras and donkeys, fin whales and blue whales, and brown and polar bears have been observed, and in some cases, these hybrids are fertile (Gray, 1972; Kelly et al., 2010; Spilliaert et al., 1991). Therefore, it is not the existence of hybrids that is important to delineate species, but the frequency of introgressive hybridization (Arnold, 2016).

The first comprehensive gene flow analyses based on a three-fold increased nuclear sequence dataset compared to Fennessy et al. (2016), as part of this thesis, identify the frequency of gene flow among all four putative giraffe species to be limited to less than the

conservative threshold of one effective migrant per generation (N_em), as expected (Winter et al., 2018b). Gene flow among most of the four species is limited to < 0.2 N_em, with only one exception. A migration rate of 0.95 N_em is estimated for northern and reticulated giraffe in the direction of reticulated giraffe. In the opposite direction, with value is 0.18 N_em, thus much lower and below the conservative threshold. Furthermore, the gene flow rates among subspecies and populations within each species are generally higher. Yet, gene flow is also limited between some subspecies, especially for geographically isolated populations such as the West African giraffe. This was expected; however, it potentially allows for additional differentiation of these subspecies and may lead to the formation of new species in the future.

Additionally, PCA and STRUCTURE analyses, identify four distinct genetic clusters without high levels of admixture, which are also evident as four major clades in a multi-locus nuclear phylogeny, corroborating the findings of limited gene flow and, therefore, further supporting four distinct giraffe species. Petzold and Hassanin (2020) recently suggested three species instead, based on the same dataset and a phylogenetic species concept. Even if he did not explicitly name this concept, a fundamental argument was the lack of homoplasious sites (a cladistic/parsimony concept) between northern and reticulated giraffe. Thus, the three-species classification is based mainly on cladistic methods and questionable parameter settings in STRUCTURE.

Both PCA and STRUCTURE analyses are widely used in population genetics to detect genetic population structure and to identify the optimal number of populations (K) based on genotype data. Yet, depending on the dataset, the identified population structure does not necessarily correspond to species but instead can determine genetic structure on different taxonomic levels such as populations, subspecies, and species. Therefore, these analyses have to be interpreted regarding the results of additional analyses such as gene flow analyses, especially when used for species delimitation.

One such analysis tool is STRUCTURE, which assigns individuals to a fixed number of populations (K) based on their allele frequencies, requiring several runs with varying values of K. To identify the optimal number of populations, additional statistics such as the delta(K) method (Evanno et al., 2005) are required. This method uses the rate of increase of likelihood in the data with increasing values of K to infer the value of K that best suits the data (Hahn, 2019). Even though frequently used, delta(K) should be interpreted with caution because it can only infer the optimal value of K if there are two or more populations. Even for a single

panmictic population, the optimal number of K inferred by delta(K) will be K=2 (Janes et al., 2017). The inferred optimal number of populations can vary depending on the parameters set in STRUCTURE (Petzold and Hassanin, 2020).

PCA, on the other hand, does not assign individuals to a population as STRUCTURE does but instead identifies the major axes of variability in the data based on allele frequencies, and each individual can be plotted in a coordinate system (Hahn, 2019). Thus, the population structure and the ideal number of populations are primarily identified by eye or by confidential intervals around predefined populations.

POTENTIAL BARRIERS TO GENE FLOW

Given the limited scientific literature about giraffe biology in general, little is known about potential barriers to gene flow among giraffe populations. Since probably a century, giraffe populations are often separated by distance due to a loss of suitable habitats caused mainly by growing human populations and consequential land conversions. These recent barriers caused by an expanding human population and land use, however, affected giraffe only since the last 100 years, or approximately ten generations. Before that, natural geographic or biological barriers must have dominated because giraffe species and populations have been in closer contact than today.

Still, there are geographic regions where different giraffe taxa have adjacent ranges. In East Africa, three of the four giraffe species (northern giraffe, reticulated giraffe, Masai giraffe, Fig. 4) occur in close proximity without known geographic barriers that would prevent migration, and some of their ranges potentially even overlap in some areas. Nevertheless, the findings reported here show that gene flow is limited among species without notable geographic barriers, keeping them genetically distinct. In many cases, they are even phenotypically distinguishable (see Fig. 2). This is unexpected because it is known that most giraffe taxa can hybridize in zoos (Gray, 1972; Lackey, 2011; Lönnig, 2011). There are only a few anecdotal sightings of potential hybrids, identified by their phenotypes. Further support for genetic barriers comes from the fact that in this study, which involved 137 individuals, only a single potential hybrid individual from the wild was identified (Winter et al., 2018b).

Due to the known ability to hybridize and form fertile hybrid offspring in captivity, postzygotic reproduction barriers, such as reduced zygote viability or low hybrid viability, alone are an unlikely explanation for the limitation of gene flow among giraffe.

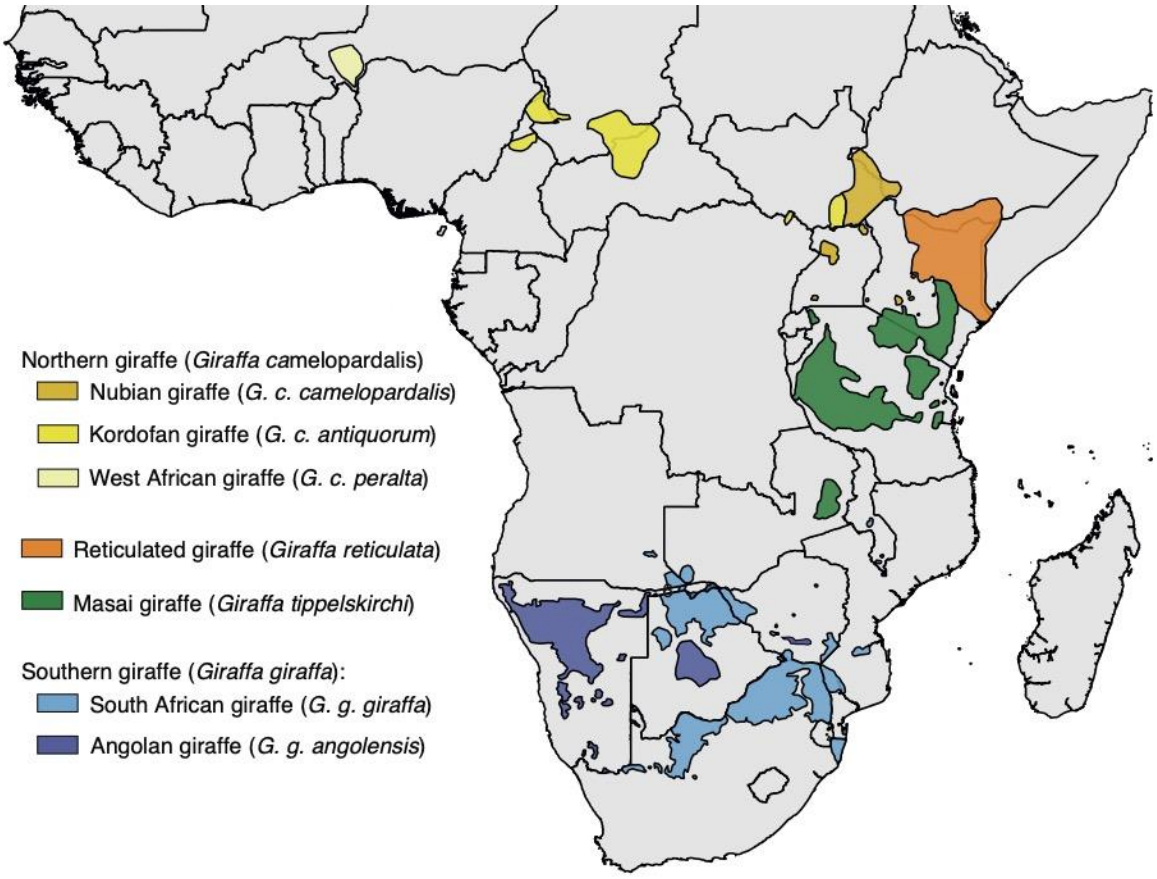

Figure 4. Distribution of giraffe species and subspecies according to Winter et al. (2019).

This leaves prezygotic reproduction barriers a likely scenario to keep giraffe populations separate. Many studies have shown that prezygotic or premating isolation is often a more effective barrier to gene flow and the most crucial factor in maintaining species differences when there is a lack of geographic barriers (Coyne and Orr, 2004; Futuyma, 2009; Jiggins et al., 2001; Kirkpatrick and Ravigné, 2002). Prezygotic reproduction barriers often develop when postzygotic reproduction barriers are already in place but still incomplete (Coyne and Orr, 2004). This process is called reinforcement and might be of high importance for the completion of speciation.

There are no present geographic barriers that hinder the migration of the three species into each other's ranges in Kenya. In fact, the current distribution areas of northern, reticulated, and Masai giraffe have common borders (Fig. 4). Therefore, it is likely that they are isolated mainly by their behavior. To date, only one possible reproductive isolation mechanism has been described based on abiotic discontinuity. Thomassen et al. (2013) have observed that giraffe reproduction seems to be synchronized to seasonal rainfall cycles

varying between geographic regions. Thus, resulting in different mating seasons and birth times, potentially contributing to reproductive isolation between the three in East Africa occurring giraffe taxa (Thomassen et al., 2013).

Another mechanism for prezygotic, even premating isolation, could be partner recognition. It has been suggested, though only as a speculation, that giraffe calves memorize their mothers' pelage patterns and potentially develop mating preferences for similar-looking coat patterns, thus acting as a reproduction barrier between the different giraffe taxa (Dagg, 2014).

POPULATION STRUCTURE AND DISTRIBUTION

The distribution of the different giraffe populations has been based on the historical records of their distribution. The assignment of populations to a subspecies was done by morphology, in particular, the characteristics of ossicones and pelage patterns (Dagg, 2014).

Genetic studies of some of the African populations have shown that several populations were identified as the wrong taxon based on these morphological characteristics (Bock et al., 2014).

My studies have genetically identified the most eastern distribution of the Angolan giraffe to date. Giraffe from the Bubye Valley Conservancy in southern Zimbabwe were expected to be South African giraffe (Dagg and Foster, 1976; Fennessy et al., 2016; Muller et al., 2018). Ten individuals have been sampled, and the analyses for their mitochondrial haplotypes demonstrated they are closely related to giraffe from the Central Kalahari Game Reserve in Botswana. Therefore, this giraffe population now represents the most easterly known natural population of the Angolan giraffe. (Winter et al., 2018a, see Publications, Publication 1). This demonstrates our limited knowledge of the geographic range of the different giraffe taxa.

The taxonomic affiliation of many populations, especially in southern Africa, is still unclear because of hitherto inadequate and unsuitable marker systems that have been used to identify them. Also, the assignment of populations to a subspecies has been complicated by translocations across countries and national parks for which there are only poor or no records (Deacon and Tutchings, 2018).

The Malawi government has planned to augment its small giraffe population with individuals from outside the country. Given the limited knowledge about the true nature of

the subspecies status of giraffe populations in Southern Africa, it was crucial to study Malawi's giraffe before supplementing their population with giraffe from outside the country. Malawi's giraffe were suspected of having originated from a population in eastern Zimbabwe for which the taxonomic affiliation was unclear. This has been of particular concern after identifying the presence of Angolan giraffe in southern Zimbabwe (Giraffe Conservation Foundation, 2015; Winter et al., 2018a). There have also been reports of unverified translocations and migrations of Masai giraffe from Zambia into Malawi that could have resulted in a hybrid population of southern and Masai giraffe, which would complicate the augmentation programs (Briggs, 2013; Giraffe Conservation Foundation, 2015).

MtDNA, as expected, identified Malawi's giraffe as South African giraffe. However, mtDNA is only maternally inherited and, therefore, is not able to reliably identify hybridization. Thus, nuclear intron sequences with known fixed variants between southern and Masai giraffe were used to rule out hybridization (Winter et al., 2018b). Only two individuals share a single allele with Masai giraffe. The shared allele alone is, however, not a sign of recent hybridization but rather of either ancestral hybridization or trans-species polymorphism (Klein et al., 1998).

This is supported by the mtDNA phylogeny showing South African giraffe being more closely related to Masai giraffe despite grouping together with their sister subspecies Angolan giraffe in the nuclear tree (Winter et al., 2019). This may be evidence for ancient mitochondrial capture, which is the replacement of the mitochondrial genome by that of another taxon, enabled by at least one hybridization event (Perea et al., 2016). In this case South African giraffe likely captured an ancient mitochondrial haplotype from the Masai lineage, which then spread in the population and replaced their original haplotype.

The genetic population structure of Angolan giraffe populations within Namibia is varied and particular for different populations. An important case is the desert-dwelling giraffe of the Hoanib to the Nadas (south to north) ephemeral river systems. These giraffe represent a population of only about 250 individuals that live in extreme desert conditions. They have a lighter pelage (Fennessy, 2004), and it was unknown if they are in contact with giraffe from the Etosha National Park (ENP).

The analyses have shown that matrilines of the desert-dwelling giraffe in the Namib desert in the northwest of Namibia and the giraffe from Etosha National Park (ENP) are genetically different despite only being separated by 200 km (Flanagan et al., 2016). These

genetic differences are unexpected, because there are no known geographic barriers, to the contrary, both areas are linked by ephemeral rivers, making the distance easy to traverse for a vagile animal like the giraffe (Flanagan et al., 2016).

The distinctness of the matrilines of these two nearby populations suggests that female giraffe are likely philopatric, i.e., females stay in the area where they were born, allowing them to differentiate into their own matrilines (Bock et al., 2014). This way, adaptation to desert conditions in the desert-dwelling giraffe population would be possible over a longer time to differentiate from the ENP giraffe. Calculations of divergence times of about 40,000 years for the separation of these two populations in a stable environment, the Namib desert is 35 million years old, support this idea.

Although the two populations, in general, exhibiting distinct matrilines, two of the desert-dwelling giraffe individuals group together with giraffe from ENP in the phylogenetic tree and the haplotype network. These are not natural migrants but can be traced back to a translocation of 22 giraffe from ENP into the desert population of 80 individuals in 1991, which was supposed to augment a human-induced declining population by civil war (Fennessy, 2004). Therefore, we would expect to find ENP haplotypes in approximately 20% of individuals, due to the ratio of translocated vs. resident individuals (n=80) in 1991. However, only two individuals out of 23 exhibits an ENP haplotype (~10%). This suggests that the translocated individuals might not have flourished in the desert condition despite more than 25 years or nearly three generations since introduction (Muller et al., 2018). The population has increased from 100 to 250 individuals in these ~25 years, mainly due to increased conservation efforts, which resulted in a decrease in poaching.

Interestingly, giraffe from ENP itself form two distinct clades (see Winter et al., 2018a, Fig. 2a). One clade is more closely related to the more eastern populations of Angolan giraffe (Botswana, Zimbabwe). Even though the branches in the phylogeny are not significantly supported, this pattern could be explained by an “out of Etosha” radiation of Angolan giraffe. Thus, ENP was possibly the origin of an Angolan giraffe dispersal to the east, ensued by a back-migration into ENP.

IMPLICATIONS FOR CONSERVATION

The species is the basic unit in biological systematics, evolution, and conservation biology. Therefore, it is crucial to delineate species accurately and know how many species of

a certain taxon exist, because threat categories on the IUCN Red List of Threatened Species are usually accessed on the species level rather than subspecies or population level (IUCN, 2019).

Presently, giraffe are still assessed by the IUCN as a single species with nine subspecies and listed as “Vulnerable” due to an about 40% population decline over the past 30 years (Muller et al., 2018). Yet, eight of the nine traditionally recognized giraffe subspecies have been recently assessed separately (Bercovitch et al., 2018; Bolger et al., 2019; J. Fennessy et al., 2018; Fennessy and Marais, 2018; S. Fennessy et al., 2018; Marais et al., 2018; Muneza et al., 2018; Wube et al., 2018). However, the recognition of four distinct giraffe species calls for a reevaluation of their assigned threat category and the development of independent conservation strategies for each species. Three of the four giraffe species are facing declining populations.

During the last three decades, northern giraffe numbers have declined by about 95%, and currently, there are little more than 5.000 individuals left in the wild. This is comparable in numbers to the black rhinoceros, which is listed as “Critically Endangered” (Emslie, 2012). Thus, the northern giraffe would likely also be listed by the IUCN as “Critically Endangered”, highlighting the need for increasing conservation measures.

The reticulated giraffe was recently assessed, still as a subspecies of a single giraffe species, and listed as “Endangered”. The reason for this is a decline of about 56% over the last three 30 years, which represents three generations (Muneza et al., 2018). Even though the reticulated giraffe has already been assessed individually as subspecies, elevation to species rank would highlight the importance of a specific conservation strategy for this species.

A population decline of 49-51%, likely due to habitat loss and poaching, over the last three generations, justifies categorizing the Masai giraffe as “Endangered” (Bolger et al., 2019). Recognizing the proposed four species systematic by (Fennessy et al., 2016) that elevates Masai giraffe into species rank and subsumes Thornicroft’s giraffe into Masai giraffe, which was further supported by this thesis and the underlying publication (Winter et al., 2018b), would likely not change the assessment for Masai giraffe. However, it would, similar to the reticulated giraffe, highlight the necessity for a species-specific conservation strategy. Furthermore, it would result in placing Thornicroft’s giraffe, which is currently listed as “Vulnerable”, into a higher threat category now being a population of Masai giraffe. Yet, the current genetic analyses show two distinct clusters within Masai giraffe, meaning that there

are potentially two subspecies. These clusters, however, do not necessarily correspond to the previously recognized subspecies boundaries between Masai and Thornicroft's giraffe (Winter et al., 2018b), calling for further genetic evaluations.

So far, only the Angolan giraffe, one of the subspecies within southern giraffe, has been assessed. Due to increased conservation efforts over the last three decades, population numbers for Angolan giraffe have been increasing. Therefore, Angolan giraffe has been listed as "Least Concern" (Marais et al., 2018). Preliminary assessment results suggest that the South African giraffe population has increased by 150% during the last 30 years (Giraffe Conservation Foundation, 2019a). Therefore, it is expected that South African giraffe, the second subspecies of southern giraffe, will also be categorized as "Least Concern". Thus, it is very likely that southern giraffe will be classified as "Least Concern" if recognized as distinct species. This shows that targeted conservation measures, which explicitly includes regulated trophy hunting, can successfully reverse population trends.

Yet, this thesis also highlights that too little is known about the distribution of the southern giraffe subspecies. This makes the previous assessments for each subspecies incomplete. Furthermore, it shows that pre- and post-translocation genetic assessments are still rarely conducted despite being of special importance to evaluate the success of such a translocation. Unsurprisingly, a high level of inbreeding was identified in Malawi's giraffe population of approximately 30 individuals, which goes back to a founder population of only five individuals (Winter et al., 2019).

Overall, adequately assessed threat categories for the four distinct giraffe species, updated range maps due to genetic taxonomic assignments of populations, and conservation genetic assessments would facilitate the development of well-adapted conservation strategies and could increase the support granted by the respective governments.

CONCLUSION AND OUTLOOK

The increased dataset of 21 nuclear intron sequences for 137 giraffe used to study giraffe systematics in this thesis, allowed to make the first comprehensive gene flow analyses among giraffe. The analyses identified limited gene flow of less than one effective migrant per generation among all of the four giraffe species. Thus, in combination with population genetic analyses, these analyses have further supported that the genus *Giraffa* consists of four extant species. These findings call for the recognition of the four distinct

species by the IUCN. As a consequence, a reassessment of their conservation status and the development of species-specific conservation strategies are necessary and currently debated.

The analyses also highlight the urgent need in general biology for a clear definition of gene flow under the Biological Species Concept and further development of modern species delineation methods, which include detailed gene flow analyses. This is necessary because genome-scale data will allow more detailed analyses of this process. It will yield a better understanding of the genetic basis of reproductive isolation barriers and enable to study the phenomenon of reverse speciation by simulating the amount of gene flow essential for the fusion of two species into one.

Even though the increased dataset of this thesis clearly supports four distinct species of giraffe, Petzold and Hassanin (2020) concluded, mainly based on an implied phylogenetic species concept (see above), that three species are more likely. However, this thesis and ongoing population genomic studies based on whole-genome sequences from all giraffe taxa (unpublished) further support the four giraffe species hypothesis. Sampling and sequencing are continuing to make whole-genome analyses with a focus on East Africa, where the ranges of three giraffe species are in close geographic proximity. It will be possible to identify natural giraffe hybrid individuals unambiguously, if existing, define potential hybrid zones, and enable to estimate the recent frequency of introgressive hybridization among giraffe species.

Finally, an ongoing morphology-based revision of giraffe taxonomy by modern digital morphometrics of museum and newly collected giraffe skulls will provide new and numerous data and, in combination with genomics, possibly reach a consensus on giraffe systematic within the scientific community.

The combination of genetic and morphometric studies will allow the development of species-specific conservation strategies that will help to save giraffe across Africa.

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PUBLICATIONS

PUBLICATION 1: MATRILINEAL POPULATION STRUCTURE AND DISTRIBUTION OF THE ANGOLAN GIRAFFE IN THE NAMIB DESERT AND BEYOND

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What are the contributions of the doctoral candidate and his co-authors?

(1) Concept and design

SW:	50%	
AJ:	30%	
JF:	20%	

(2) Conducting tests and experiments

SW:	90%	DNA isolation, PCRs, Sanger Sequencing
JF, SF:	10%	Sampling (remote skin biopsies of giraffe)

(3) Compilation of data sets and figures

SW:	100%	Compilation of all data sets and all figures (incl. sequence editing and alignments)

(4) Analysis and interpretation of data

SW:	80%	Phylogenetic tree, haplotype network, PCA
SW, AJ:	20%	Summary interpretation

(5) Drafting of manuscript

SW:	70%
AJ:	20%
JF, SF:	10%

I hereby certify that the information above is correct.

Date and place — Signature doctoral candidate

Date and place — Signature supervisor

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Matrilineal population structure and distribution of the Angolan giraffe in the Namib desert and beyond

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Desert

ABSTRACT

The distribution maps of giraffe (*Giraffa*) subspecies in southern Africa are based on historical assumptions, yet some populations have likely been misidentified, hindering effective conservation efforts. Particularly, the populations in Zimbabwe are poorly studied, and translocations, such as the 1991 movement of giraffe from Namibia's Etosha National Park to mitigate the human-induced decline of Namibia's desert-dwelling giraffe in the lower Hoanib and Hoarusib Rivers, was concluded without consideration of genotype. Mitochondrial DNA sequence analyses from cytochrome *b* and control region revealed that instead of the South African giraffe subspecies (*G. giraffa giraffa*), the Angolan giraffe (*G. g. angolensis*) occurs in southern Zimbabwe's Bubye Valley Conservancy. Furthermore, Namibia's desert-dwelling giraffe and those from Etosha National Park form distinct matrilineal lineages within the Angolan giraffe subspecies clade. Thus, despite the translocation and proximity of the Etosha National Park, the Etosha giraffe genotype has not spread amongst the desert-dwelling giraffe. Use of mutation rate estimates indicates that there has been no matrilineal exchange between Etosha and the desert-dwelling giraffe for ca. 40,000 years. While philopatry could produce distinct mitochondrial lineages, the short geographical distance and long time involved, makes it more likely that the Etosha giraffe cannot successfully compete with a putatively better adapted desert-dwelling giraffe. The analyses also show that the distribution of the Angolan giraffe extends further eastwards than expected. These findings provide important implications for giraffe conservation, and translocation in particular, which may not always be an effective means of improving genetic diversity.

1. Introduction

Giraffe (*Giraffa*) as a single species have recently been classified as "Vulnerable" on the IUCN RedList, due to a decline of approximately 40% over the past three decades [1]. However, the classification did not consider that genetic taxonomy suggests four distinct species of giraffe, which in turn has important implications for giraffe conservation [2]. To date, the distribution of the currently recognized nine subspecies [2] is not well understood, because most distribution maps are based on historical assumptions and anecdotal evidence.

In southern Africa, the southern giraffe (*G. giraffa*) is divided into two subspecies: the South African giraffe (*G. g. giraffa*) mainly occurring in Botswana, South Africa, Zambia and Zimbabwe, and the Angolan giraffe (*G. g. angolensis*) prevalent primarily throughout Botswana and Namibia (Fig. 1). In southern Africa, the distinction between the southern giraffe subspecies is historically based on pelage pattern, morphometrics and geography [3]. However, the pelage pattern of the two subspecies is very similar and, therefore, the geographic distribution of the subspecies in southern Africa is uncertain [1]. Genetic analyses of most populations are missing, and only few giraffe studies have been undertaken in Zimbabwe [4–6]. These studies led to varying classifications over the years as either Angolan or South African giraffe subspecies [5,7,8].

In Namibia, the Angolan giraffe occurs naturally across the central and northern parts of the country with major populations in Etosha National Park (ENP), communal northwestern Namib Desert and private land throughout the country. The pelage of the desert-dwelling giraffe is often paler in comparison to Angolan giraffe from ENP [9] and a status as separate subspecies has been suggested [10]. However, genetic studies so far did not find differences to justify a separate subspecies or population [9,11,12]. In 1991, 22 giraffe individuals were translocated from western ENP to the lower Hoarusib River to augment the desert-dwelling giraffe population, which at the time comprised approximately 100 individuals [9]. However, failure to conduct pre-

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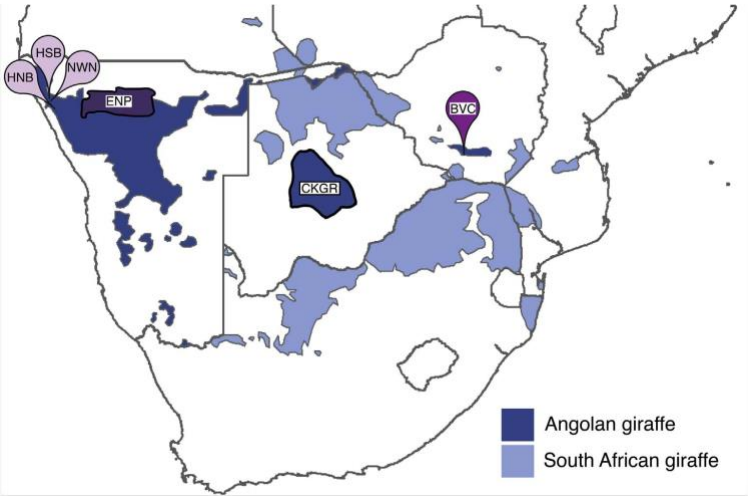


Fig. 1. The distribution of the southern giraffe subspecies. Map of southern Africa highlighting the assumed distribution of southern giraffe subspecies, as estimated by the Giraffe Conservation Foundation, 2017. Labeled pins represent the main sampling locations for this study: Hoanib River Catchment (HNB), Hoarusib River Catchment (HSB), northwestern Namibia (NWN), and Bubye Valley Conservancy (BVC). Areas with a black frame indicate the location of two additional major Angolan giraffe populations, the Etosha Nationalpark (ENP) in Namibia, and the Central Kalahari Game Reserve (CKGR) in Botswana. According to the new findings, the giraffe population of the BVC in Zimbabwe is shown as the Angolan giraffe and not as expected South African giraffe subspecies. It illustrates the dispersed distribution of the Angolan giraffe to the east in southern Africa.

and post-translocation surveys impeded assessment of translocation success [9].

Genetic analysis of mitochondrial DNA is a powerful tool to aid in distinguishing giraffe subspecies [2,12,13]. Here, cytochrome *b* and the control region from 33 southern giraffe, including Namibia's desert-dwelling and Bubye Valley Conservancy (BVC) giraffe from Zimbabwe (Fig. 1), were analyzed, to determine the biogeography of southern giraffe and to investigate whether the translocation of giraffe from a nearby population increased the genetic diversity of the desert-dwelling giraffe in Namibia.

2. Material and methods

Tissue samples from southern giraffe individuals were obtained by remote biopsy darting in southern Zimbabwe's BVC (n = 10), as well as the desert-dwelling giraffe of the lower Hoanib (HNB) (n = 13) and Hoarusib (HSB) (n = 3) River catchments, and other regions of northwestern Namibia (NWN) (n = 7) (Fig. 1). Similarly, reticulated giraffe (*G. reticulata*) samples were collected at Ishaqbini Conservancy (n = 4) in Kenya, to add wild reticulated giraffe to the dataset of all giraffe species and subspecies. All samples were collected by the Giraffe Conservation Foundation (GCF) and partners with permits from the respective governments. Biopsy samples were preserved in 99% ethanol. DNA was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel). Cytochrome *b* and control region were PCR amplified using the VWR Taq DNA Polymerase 2X Master Mix with 2.0 mM $MgCl_2$. PCR amplification and sequencing followed previously published protocols [13].

Cycle sequencing was performed using the BigDye terminator sequencing kit 3.1 (Applied Biosystems) and analyzed on an ABI 3730 DNA Analyzer, manually edited in Geneious version 6.1.8 [14] and aligned using MAFFT version 7 [15]. All data generated during this study are available from GenBank under the accession numbers KY865101 - KY865174. Accession numbers for additional data analyzed in the current study can be found in the references (see Supplementary material, Table S1).

Published sequences from other giraffe (sub)species were aligned to Okapi (*Okapia johnstoni*) orthologs, which served as an outgroup in the full sample analysis (see Supplementary material, Table S1). A reduced dataset limited to southern giraffe where six northern giraffe (*G. camelopardalis*) individuals served as an outgroup, allowed for higher resolution depiction of topology.

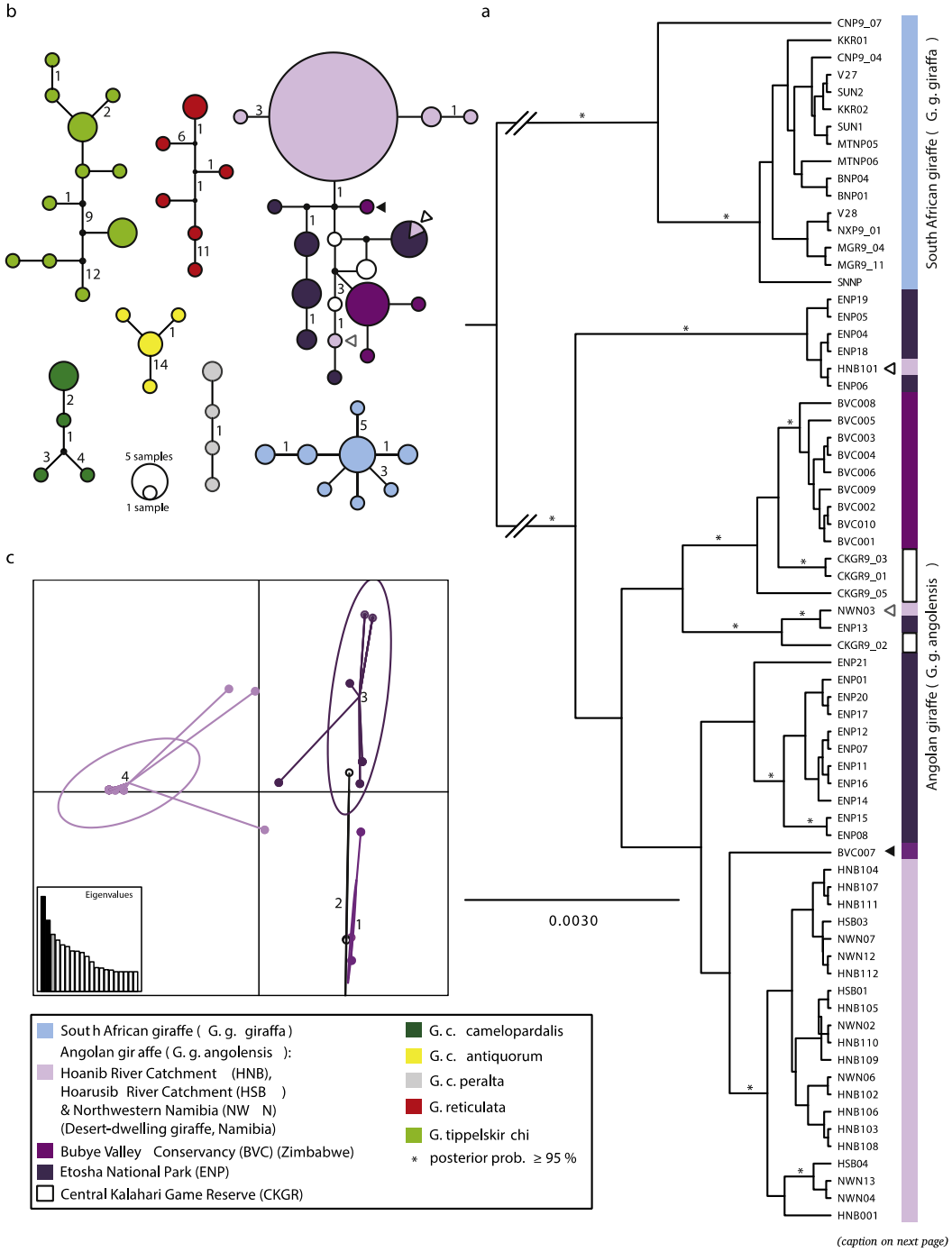
BEAST version 1.8.3 [16] using an uncorrelated lognormal relaxed clock [17] and 20 million MCMC chains produced Bayesian phylogenies. The HKY + 4G + I substitution model [18] was suggested by jModelTest2 [19]. Convergence of the BEAST run was evaluated in Tracer version 1.6.0 [20]. A maximum clade credibility tree was generated using TreeAnnotator version 1.8.3 [21] with 10% burnin. Additionally, we used MEGA version 7.0.16 [22] to calculate a Maximum-Likelihood tree under HKY + 4G + I and Geneious version 6.1.8 [14] for Neighbor-Joining tree analysis under HKY. Both analyses were performed with 100 bootstrap replicates.

Statistical parsimony networks of both mtDNA loci were calculated using TCS version 1.21 [23] with a connection limit of 95%. Haplotypes for Angolan giraffe individuals were coded with DnaSP version 5.10.01 [24] for both loci separately. Principal Component Analysis (PCA) was performed using adegenet [25] for R version 3.3.2.

3. Results

Aligning 1140 nt of the cytochrome *b* gene and 418 nt of control region sequences yielded a 1558 bp long dataset for each of the 116 giraffe individuals. Fig. 2a shows a Bayesian tree for the South African and Angolan giraffe only. Tree analyses using Maximum Likelihood or Neighbor-Joining, and a Bayesian tree that includes all (sub)species, are shown in Supplementary material (Figs. S1–3) and generally agree with the tree shown in Fig. 2a. Surprisingly, nine giraffe from the BVC, southern Zimbabwe, which were expected to be South African giraffe, form a distinct clade and group within the Angolan giraffe lineage close to a cluster of giraffe from the Central Kalahari Game Reserve (CKGR) in Botswana (Fig. 2a). Individual BVC007 groups outside the BVC cluster, being closely related to the desert-dwelling giraffe. Except for two giraffe individuals (NWN03 and HNB101), the desert-dwelling giraffe (HNB, HSB, and NWN) form a distinct cluster with significant support (posterior probability ≥0.95), within the Angolan giraffe clade. In the tree analysis and haplotype networks giraffe from ENP form two distant clades. One splits at the basis of Angolan giraffe, and one is reconstructed as sistergroup to the desert-dwelling giraffe (Fig. 2a). While these branches are not significantly supported, the two distinct clades within the ENP population could be explained by an "out of Etosha" radiation with the ENP as origin of an Angolan giraffe dispersal to the east, followed by a back-migration into ENP.

Assuming a human mitochondrial mutation rate 0.118/sites/Myr



(caption on next page)

Fig. 2. Phylogeny and population structure among southern giraffe. a. MtDNA BEAST tree of southern giraffe, rooted by northern giraffe (not shown). Except for two individuals, the investigated desert-dwelling giraffe form a distinct cluster. Bubye Valley Conservancy (BVC) giraffe are nested within Angolan giraffe. Open arrowheads indicate desert-dwelling giraffe grouping with Etosha National Park (ENP); filled arrowhead indicates the BVC individual grouping outside the BVC cluster. b. Statistical parsimony networks of mtDNA sequences. Numbers indicate the number of potential but not sampled haplotypes. Circles are scaled to the number of individuals with the same haplotype (see nested circles for scale). c. PCA axes 1–2 for the four different populations of Angolan giraffe [1: Bubye Valley Conservancy (BVC), 2: Central Kalahari Game Reserve (CKGR), 3: Etosha National Park (ENP), 4: Hoanib River Catchment (HNB) + Hoarusib River Catchment (HSB) + northwestern Namibia (NWN)]. The oval outlines represent the 95% confidential intervals.

[26] for giraffe, some 184 mutations per million years would be expected for the 1558 bp long concatenated mtDNA sequences. The average of seven observed substitutions between the desert-dwelling and ENP giraffe suggests that these populations separated approximately 38,000 years ago.

Statistical parsimony analysis of the concatenated dataset (Fig. 2b) conforms with the phylogenetic tree analyses. The giraffe subspecies do not connect with each other to the network at a 95% connection limit, indicating considerable genetic distance, which is expected for mtDNA sequences of giraffe subspecies. Except for one individual (BVC007, black triangle Fig. 2b), the BVC giraffe form a distinct cluster and are clearly placed within the Angolan giraffe haplotype network. Within the Angolan subspecies, the desert-dwelling giraffe form a separate cluster that is distinct from the ENP and other giraffe (Fig. 2b), except for individuals (NWN03 and HNB101, open triangles Fig. 2b) that fall close to or within the ENP giraffe cluster. The PCA for the Angolan giraffe clade (Fig. 2c) conforms with the phylogenetic and clustering analyses, showing a significant differentiation between the desert-dwelling giraffe (number 4) and the other populations.

4. Discussion

This study provides the first population genetic analyses of giraffe in southern Zimbabwe and investigates the success of translocation to augment the desert-dwelling giraffe population in Namibia.

Zimbabwe's BVC giraffe were historically expected to represent the most northeastern distributional range of the South African giraffe subspecies [1,2,27]. However, the mtDNA analyses unambiguously identified all sampled individuals of the Zimbabwe's BVC as Angolan giraffe, and most are closely related to the geographically nearest known Angolan giraffe population in the CKGR, which is expected. However, individual BVC007 groups together with the geographically most distant desert-dwelling giraffe, but the current dataset does not allow any explanation for that. It is important to note that no translocation event has been reported that could explain these unexpected results. The BVC giraffe have distinct mtDNA haplotypes that are close to the Angolan giraffe subspecies, but not to the South African giraffe. Thus, the Angolan giraffe subspecies has a more easterly distribution in southern Africa than anticipated and comprises of a number of distinct haplotypes that are separate but related to the Angolan giraffe. The isolated eastern population of Angolan giraffe at BVC (Fig. 1) is noteworthy because it exemplifies our limited knowledge of giraffe distribution. Ongoing studies in southern Africa will determine if there are additional Angolan giraffe mtDNA haplotypes in Zimbabwe, and will reveal a more accurate distribution of Angolan giraffe, possibly extending further to the East and North.

In Namibia, the matrilines of the desert-dwelling giraffe are genetically different from the nearest Angolan giraffe population in the ENP, suggesting that there has been very limited, if any, natural exchange of matrilines between these populations. This is unexpected, as the populations are only separated by 200 km [28]. In addition, the areas where they occur are linked by ephemeral rivers making the distance easy to traverse [28]. The strict separation of these populations suggests that female giraffe are likely philopatric, i.e. females stay in the area where they were born, and differentiated into own matrilines [13]. While we are aware of the limitations of the rough divergence time estimation, an approximate separation time of some 40,000 years (or even an order of magnitude less) for the ENP and desert-dwelling giraffe is unexpectedly old for a vagile animal like giraffe. Therefore, the exchange of matrilines might be hindered instead by adaptation to desert conditions of the local desert-dwelling giraffe population. Given the old age of the Namib Desert with stable climatic conditions for million of years [29], it is conceivable that giraffe and other local animals have had ample time to adapt to the arid conditions.

Two desert-dwelling giraffe individuals, HNB101 and NWN03, did not fall into the expected clade, but group with haplotypes of ENP giraffe. This is explained by translocation of 22 giraffe from ENP into the lower Hoarusib River in 1991 to augment a human-induced declining population of the desert-dwelling giraffe [9]. Hence, these haplotypes are likely remnants of this translocation. Since then, the desert-dwelling giraffe population has increased from 100 to approximately 250 individuals today. Thus, after blending in 20% individuals from ENP into the desert dwelling giraffe population, a similar ratio of the respective genotypes of ENP giraffe would be expected. With only two of the 23 desert-dwelling giraffe (10%) having haplotypes corresponding to those from ENP, it appears that the translocated individuals did not flourish within the desert population despite more than 25 years, and approximately three generations lapsing since introduction [1]. Importantly, the increase in population size is manly due to a decrease in poaching and community-based conservation measures.

5. Conclusion

This study shows that the Angolan giraffe is spread further east than expected, and the genetically distinct desert-dwelling giraffe suggests that this iconic population is on a separate evolutionary trajectory. While, morphological, physiological and behavioral differences relative to non-desert populations could not be identified [30], they are possible and discussed elsewhere [9]. The genetically separate desert-dwelling giraffe, exemplifies once more that conservation efforts must take population genetics into consideration when establishing new and/or augmenting current giraffe ranges, and that translocation might not always be an effective tool for increasing genetic diversity.

Conflicts of interest

None.

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Authors contribution

AJ and JF conceived and financed the project. JF and SF collected samples. SW generated and analyzed the data. SW and AJ wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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Environment & Tourism, Namibia.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.egg.2018.03.003.

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Supplementary material

Matrilineal population structure and distribution of the Angolan giraffe in the Namib desert and beyond

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Table S1. Location, abbreviation, number of individuals (n), (sub)species and source of analyzed giraffe and Okapi sequences.
Individual sample ID´s see Figure S1.

Location	Abbreviation	n	(Sub)species	Source
Badingilo National Park, South Sudan	BaNP	2	*G. c. camelopardalis*	Fennessy et al., 2016[1]
Bubye Valley Conservancy, Zimbabwe	BVC	10	*G. g. angolensis*	**Present study**
Bwabwata National Park, Namibia	BNP	2	*G. g. giraffa*	Bock et al., 2014[2]
Chobe National Park, Botswana	CNP	2	*G. g. giraffa*	Bock et al., 2014[2]
Central Kalahari Game Reserve, Botswana	CKGR	4	*G. g. angolensis*	Bock et al., 2014[2]
Etosha National Park, Namibia	ENP	17	*G. g. angolensis*	Bock et al., 2014[2]
Gambella National Park, Ethiopia	ETH	1	*G. c. camelopardalis*	Fennessy et al., 2016[1]
Garamba National Park, DR Congo	GNP	3	*G. c. antiquorum*	Bock et al., 2014[2]
Hoanib River Catchment, Namibia	HNB	13	*G. g. angolensis*	**Present study**
Hoarusib River Catchment, Namibia	HSB	3	*G. g. angolensis*	**Present study**
Ishqbini Conservancy, Kenya	ISC	4	*G. reticulata*	**Present study**
Khamab Kalahari Reserve, South Africa	KKR	2	*G. g. giraffa*	Bock et al., 2014[2]
Koure, Niger	WA	5	*G. c. peralta*	Bock et al., 2014[2]
Luangwa Valley National Park, Zambia	LVNP	5	*G. tippelskirchi*	Fennessy et al., 2013[3]
Moremi Game Reserve, Botswana	MGR	2	*G. g. giraffa*	Fennessy et al., 2013[3]
Mosi-oa-Tunya National Park, Zambia	MTNP	2	*G. g. giraffa*	Fennessy et al., 2016[1]
Murchison Falls National Park, Uganda	MF	5	*G. c. camelopardalis*	Bock et al., 2014[2]
Northwestern Namibia (Namib desert), Namibia	NWN	7	*G. g. angolensis*	**Present study**
Nxai Pans, Botswana	NXP	1	*G. g. giraffa*	Fennessy et al., 2013[3]
Nürnberg Zoo, Germany	RET	4	*G. reticulata*	Fennessy et al., 2016[1]
Selous Game Reserve, Tanzania	SGR	5	*G. tippelskirchi*	Bock et al., 2014
Shambe National Park, South Sudan	SNR	2	*G. c. camelopardalis*	Fennessy et al., 2016[1]
Sioma Ngwezi National Park, Zambia	SNNP	1	*G. g. giraffa*	Fennessy et al., 2016[1]
Sun hotel, Livingstone, Zambia	SUN	2	*G. g. giraffa*	Fennessy et al., 2016[1]
Vumbura Concession, Botswana	V	2	*G. g. giraffa*	Bock et al., 2014[2]
Zakouma National Park, Chad	ZNP	1	*G. c. antiquorum*	Bock et al., 2014[2]
Basel Zoo, Switzerland	Okapi	1	*O. johnstoni*	Fennessy et al., 2013[3]
Additional sequences from NCBI				
Location	**Accession No.**		**(Sub)species**	**Source**
Basel Zoo, Switzerland	EF442269		*G. tippelskirchi*	Hassanin et al., 2007[4]
unknown	EU088330		*G. c. camelopardalis**	Brown et al., 2007[5]
unknown	EU088332		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088335		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088336		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088339		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088340		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088341		*G. tippelskirchi*	Brown et al., 2007[5]
unknown	EU088342		*G. tippelskirchi*	Brown et al., 2007[5]

* questionable species assignment

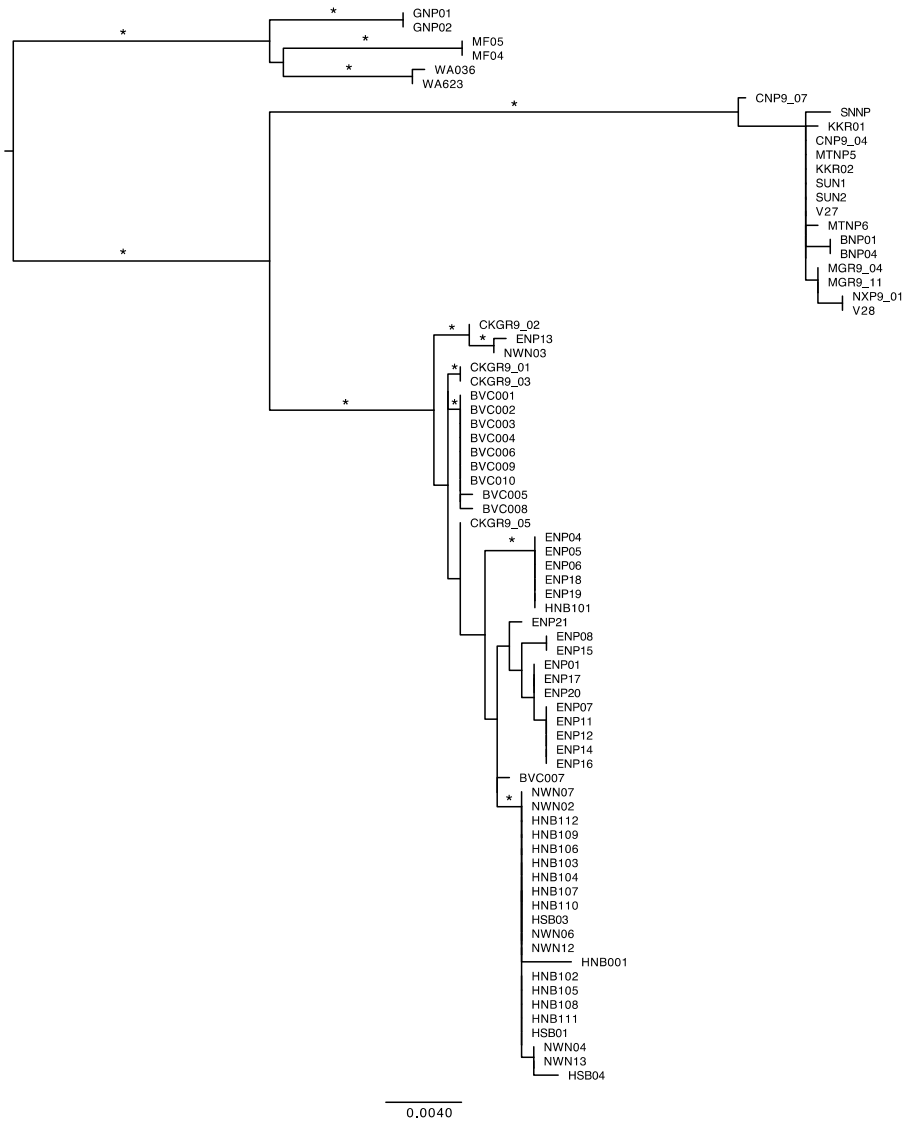

Figure S1. Maximum Likelihood tree analysis of the southern giraffe
Analysis was generated with MEGA 7[6] under HKY + 4G +I substitution model with 100 Bootstrap replicates, and rooted with six Northern giraffe individuals (GNP, WA, MF). Asterisks indicate a Bootstrap support ≥ 80 %.

Figure S2. Neighbor-Joining tree analysis of the southern giraffe. Analysis was generated with Geneious version 6.1.8[7] under HKY substitution model and 100 Bootstrap replicates, and rooted with six Northern giraffe individuals (GNP, WA, MF). Asterisks indicate a Bootstrap support ≥ 80 %

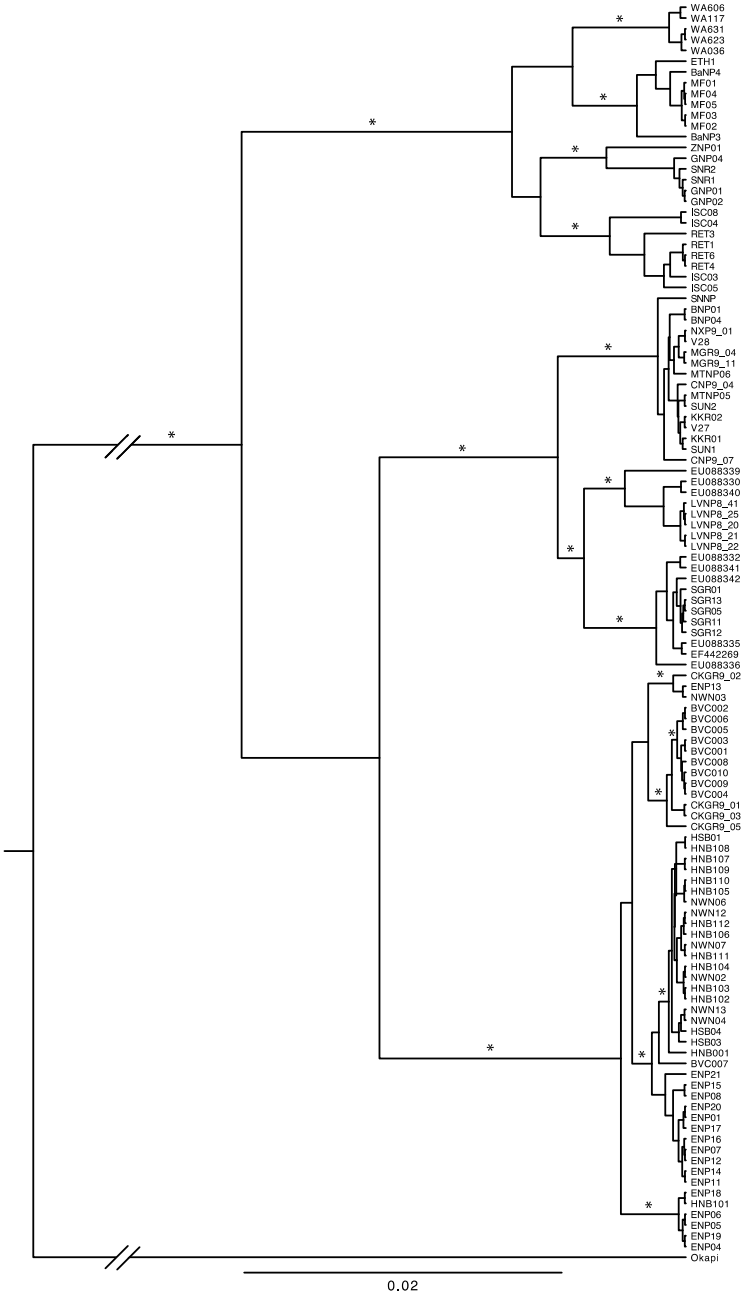

Figure S3. Bayesian analysis of 116 giraffe individuals including sequences of all species and subspecies. Analysis was rooted with Okapi. The asterisks indicate a posterior probability ≥ 95 %. All subspecies are well separated and form significantly supported monophyletic groups. Within the Angolan giraffe clade Zimbabwe's Bubye Valley Conservancy and Namibia's desert-dwelling giraffe form distinct clusters with few exceptions. Note – the okapi branch is not in scale.

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PUBLICATION 2: LIMITED INTROGRESSION SUPPORTS DIVISION OF GIRAFFE INTO FOUR SPECIES

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(1) Concept and design

SW:	60%	
AJ:	30%	
JF:	10%	

(2) Conducting tests and experiments		
SW:	90%	DNA isolation, Primer design, Marker development, PCRs, Sanger Sequencing
JF:	10%	Sampling (remote skin biopsies of giraffe) and sampling coordination

(3) Compilation of data sets and figures		
SW:	100%	Compilation of all data sets and all figures

(4) Analysis and interpretation of data		
SW:	80%	Phylogenetic trees, Structure analyses, PCA, gene flow analyses
SW, AJ:	20%	Summary interpretation

(5) Drafting of manuscript	
SW:	80%
JF, AJ:	20%

I hereby certify that the information above is correct.

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ORIGINAL RESEARCH

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Limited introgression supports division of giraffe into four species

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Abstract

All giraffe (*Giraffa*) were previously assigned to a single species (*G. camelopardalis*) and nine subspecies. However, multi-locus analyses of all subspecies have shown that there are four genetically distinct clades and suggest four giraffe species. This conclusion might not be fully accepted due to limited data and lack of explicit gene flow analyses. Here, we present an extended study based on 21 independent nuclear loci from 137 individuals. Explicit gene flow analyses identify less than one migrant per generation, including between the closely related northern and reticulated giraffe. Thus, gene flow analyses and population genetics of the extended dataset confirm four genetically distinct giraffe clades and support four independent giraffe species. The new findings support a revision of the IUCN classification of giraffe taxonomy. Three of the four species are threatened with extinction, and mostly occurring in politically unstable regions, and as such, require the highest conservation support possible.

KEYWORDS

conservation, gene flow, giraffe, hybridization, speciation

1 | INTRODUCTION

Traditionally, giraffe, with their long necks and striking silhouettes (Figure 1), were classified as a single species (*Giraffa camelopardalis*) with up to eleven subspecies proposed (Lydekker, 1904). However, until recently, the classification into nine subspecies was generally the most accepted one (Dagg & Foster, 1976). It has been shown that in captivity some giraffe subspecies hybridize (Gray, 1972; Lackey, 2011; Lönnig, 2011), which supported the traditional single species concept for giraffe. However, multi-locus analyses of wild giraffe nuclear loci identified four monophyletic, distinct and evolutionary old groups of giraffe that should be recognized as four distinct species (Fennessy et al., 2016). This finding conflicts with former classifications and has been questioned based on the limited interpretation of traditional data for example, pelage pattern, number of ossicones and geographic distribution (Bercovitch et al., 2017). The initial findings of four giraffe species (Fennessy et al., 2016) could, however, be criticized because it did not involve explicit gene flow analyses. Gene flow analyses are imperative to understanding speciation from a genetic perspective, especially since reproductive isolation is the keystone of the biological species concept (BSC), which is one of the most widely applied (Coyne & Orr, 2004; Mayr, 1942). The BSC implies that there is no or only very limited gene flow between species, but currently, it lacks a clear definition how to deal with gene flow. It has been proposed that one or a limited number of effective migrants (up to 10) per generation (N_em) avoids genetic differentiation of populations and escapes a substantial loss of genetic diversity for neutral traits (Lacy, 1987; Mills & Allendorf, 1996; Vucetich & Waite, 2000; Wright, 1969). Thus, it may be a conservative estimate that limited gene flow of <1 migrant per generation ($N_em < 1$) can lead to speciation, despite the occurrence of hybridization. It should be noted, that the number of effective migrants is not the same as

the actual number of migrating individuals, but an abstract value correlated to the effective population size N_e. The N_e is typically much smaller (~10%) than the census population size and is equivalent to the size of an idealized population that loses genetic diversity at the same rate as the real population (Frankham, Ballou, & Briscoe, 2010).

The BSC definition might need to be amended as some species naturally hybridize in the wild and produce fertile offspring for example, bears (Arnold, 2016; Kelly, Whiteley, & Tallmon, 2010; Kumar et al., 2017) and whales (Bérubé & Aguilar, 1998; Spilliaert et al., 1991), and divergence can occur under genetic exchange (Arnold, 2016). While the distinction of four giraffe species is consistent with population genetic analyses (Fennessy et al., 2016), gene flow among giraffe species has not yet been sufficiently analyzed. Here, we revisit the hypothesis of four giraffe species using population genetic methods that explicitly involve gene flow analyses with an increased dataset of 21 nuclear loci and 137 giraffe individuals from 21 locations across Africa (Figure 2; Supporting information Table S1).

2 | MATERIALS AND METHODS

2.1 | Sampling and DNA Extraction

Tissue samples from all giraffe species and five subspecies were collected by the Giraffe Conservation Foundation (GCF) and partners using remote biopsy darts with country-specific research permits between 2009 and 2016 in accordance with ethical guidelines and regulations of the respective governments and institutions. All samples were stored either in RNAlater (Invitrogen) or in >95% ethanol. Additional sequences of giraffe individuals were added to the mtDNA dataset of Fennessy et al. (2016) resulting in a total number of 217 giraffe. The geographical origins and individual IDs are shown in Supporting information Table S1. Sample locations and geographical distributions are shown in Figure 2. Additional southern giraffe individuals were included only if they were from a hitherto unrepresented region. DNA was extracted using either a Macherey-Nagel NucleoSpin Tissue Kit or a standard phenol-chloroform extraction method. All experimental protocols are in compliance with the guidelines for the best ethical and experimental practices of the Senckenberg Society, as well as with national guidelines of the respective countries.

2.2 | Amplification and sequencing

We PCR amplified and sequenced the seven intron sequences previously published (Fennessy et al., 2016) for 32 new individuals and developed 14 additional intron sequences as described (Fennessy et al., 2016). The 14 new intron sequences were amplified and sequenced for a total number of 137 individual giraffe and the okapi (*Okapia johnstoni*). The putatively independent and neutral 21 nuclear gene loci are on different chromosomes or are widely separated from each other in the bovine genome, a close relative with available chromosome level genome data (Supporting information Table S2). PCRs were performed with 10 ng genomic DNA and giraffe and okapi specific primers. Supporting information Table S2 details primer sequences and PCR conditions. We also amplified and sequenced the mitochondrial cytochrome b and control region for all new individuals as described previously (Bock et al., 2014). Each PCR was examined using agarose gel electrophoresis on a 1% agarose gel with ethidium bromide.

Sanger sequencing was performed for the forward and reverse strand using the BigDye terminator sequencing kit 3.1 (Applied Biosystems) with 5 ng of PCR product for each reaction and analyzed on an ABI 3730 DNA Analyzer.

The sequences were manually edited and aligned in Geneious v.6.1.8 (Kearse et al., 2012). Heterozygous insertions/deletions of nuclear sequences were resolved manually or using Indelligent v.1.2 (Dmitriev & Rakitov, 2008) and verified by allele-specific primers, as necessary. PHASE implemented in DnaSP v.5.10.01 (Librado & Rozas, 2009) was used to derive the allele haplotypes of the nuclear sequences using a threshold of 0.6 and allowing for recombination. All analyses, except of a mtDNA tree analysis, were performed using phased nuclear allele sequences.

2.3 | Tree analyses

The mitochondrial cytochrome b and control region sequences of 217 giraffe, including new and published sequences (Bock et al., 2014; Brown et al., 2007; Fennessy et al., 2016; 2013; Hassanin et al.,

FIGURE 1 Reticulated giraffe (*Giraffa reticulata*) in the Samburu National Reserve, Kenya (©GCF)

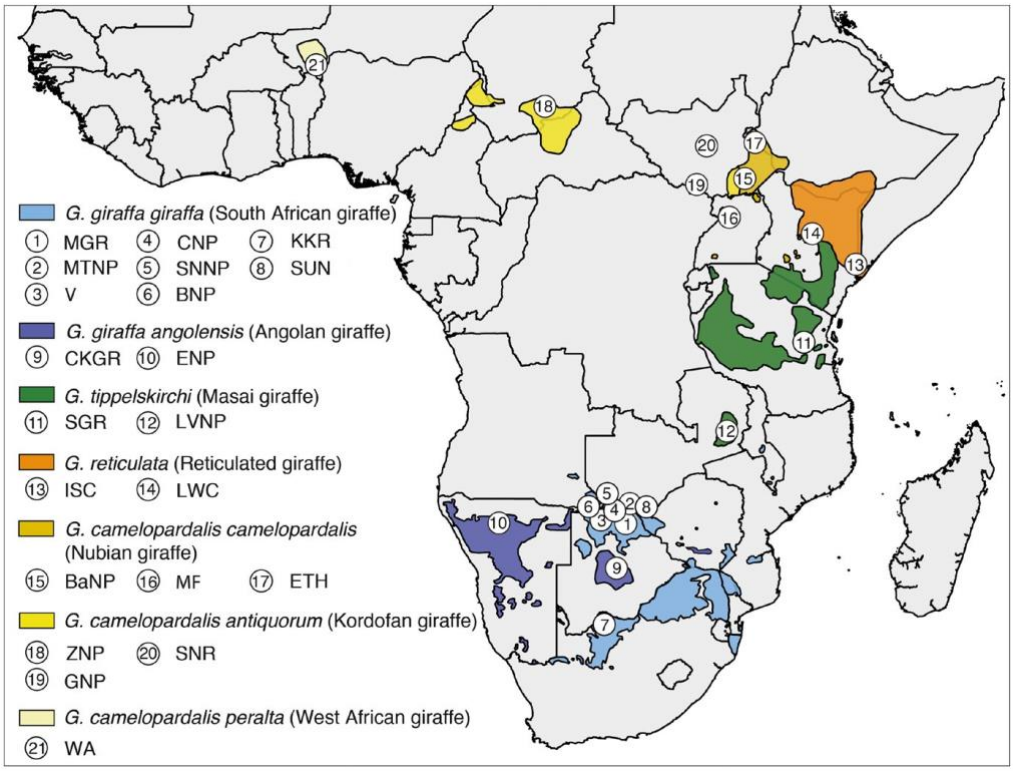

FIGURE 2 Map of Sub-Saharan Africa with giraffe (sub)species distributions and sampling locations. Geographic ranges (colored shadings) of giraffe as identified by the Giraffe Conservation Foundation (2017) were plotted on a map of Sub-Saharan Africa. Numbered circles represent sampling locations (for details see Supporting information Table S1). Species and common names as per Fennessy et al. (2016)

2012; 2007; Winter, Fennessy, Fennessy, & Janke, 2018; Supporting information Table S1) were aligned and concatenated, and a Bayesian analysis was conducted in BEAST v.2.4.5. (Bouckaert et al., 2014). We used the HKY model of sequence evolution (Hasegawa, Kishino, & Yano, 1985), as suggested by jModelTest v.2.1.1 (Darriba, Taboada, Doallo, & Posada, 2012), a log-normal relaxed clock with 10^9 generations and sampled every 20,000th iteration. Sequences of two okapis were used as an outgroup.

A multi-locus Bayesian phylogenetic tree of the 21 intron sequences for 137 individuals and the okapi as outgroup was generated with the StarBEAST2 (Ogilvie, Bouckaert, & Drummond, 2017) package in BEAST v.2.4.5. (Bouckaert et al., 2014, p. 2) under the JC model of nucleotide evolution suggested as best fitting model by jModelTest v.2.1.1 (Darriba et al., 2012). A log-normal relaxed clock was used with 10^9 generations and sampling every 20,000th iteration. Convergence of the MCMC runs was analyzed with Tracer v.1.6.0 (Rambaut, Suchard, Xie, & Drummond, 2014), and TreeAnnotator v.2.4.5 (Rambaut & Drummond, 2016) was used to construct a maximum clade credibility tree with 30% burn-in for both the mtDNA and the nuclear sequences.

2.4 | Population genetic analyses

Haplotype information for each locus deduced by DnaSP (Librado & Rozas, 2009) was used to code each individual. The haplotype matrix was then used to infer admixture using the Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4. (Pritchard, Stephens, & Donnelly, 2000). For the maximum number of populations (K) between 1 and 10, we sampled 250,000 steps following a 100,000-step burn-in, with 40 replicates each. The CLUMPAK webserver (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015) was used to average the results and to infer the most likely K based on the posterior probability of K (Pritchard et al., 2000) and ΔK (Evanno, Regnaut, & Goudet, 2005). Additionally, the most likely K was deduced by eye based on the plot of estimated Ln probability of data (Ln Pr($X|K$)) for K between 1 and 10 as described in Pritchard,

Wen, and Falush (2010), generated using Structure Harvester (Earl & vonHoldt, 2012). Principal Component Analyses (PCAs) were performed with the R package adegenet (Jombart, 2008) in R v.3.2.3 (R Core Team, 2015) to assess the degree of similarity between defined population scenarios. Pairwise fixation index (F_{ST}) values were calculated in Arlequin v.3.5.2.1 (Excoffier & Lischer, 2010) based on the nuclear haplotypes.

2.5 | Gene flow analyses

Long-term average gene flow among and within the giraffe species was calculated in the coalescent genealogy sampler MIGRATE-N v.3.6.11 (Beerli, 2006; Beerli & Felsenstein, 2001) by estimating the mutation-scaled population sizes (Θ) for each population and migration rates (*M*) for each direction between a pair of populations. We used the Brownian motion mutation model and the Bayesian inference analysis strategy, as some parameter combinations are better estimated using the Bayesian approach compared to the Maximum-likelihood approach (Beerli, 2012). The transition/transversion ratio was set to 2.31 as estimated in MEGA v.7.0.16 (Kumar, Stecher, & Tamura, 2016) based on a concatenated alignment of all 21 loci. Variable mutation rates were considered among loci. We used the default settings for the Θ uniform priors and adjusted the *M* uniform priors (0; 5,000; 10,000; 1,000) because the upper prior boundary appeared to be too small in initial analyses. Several short runs were performed to check for convergence of the runs. Three long-chain runs were performed for six million Markov Chain Monte Carlo (MCMC) iterations (60,000 recorded steps) and a burn-in of 600,000 iterations. An adaptive heating scheme was used with four chains and temperatures set by default with a swapping interval of one. Convergence of the runs was evaluated by the posterior distributions, Effective Sample Size (ESS) with a threshold of ESS ≥ 5,000, and consistency of results between runs. In addition, we estimated short-term gene flow, as well as the probability of recent hybridization for each individual in BayesAss v.3.0.4 (Wilson & Rannala, 2003) using 100 million MCMC iterations, a burn-in of 10 million and a sampling interval of 1,000 iterations. Mixing of the chain was improved by adjusting the acceptance rates for proposed changes to the parameters (allele frequencies and inbreeding coefficient) by adapting the mixing parameters for allele frequencies (Δ*A*) and inbreeding coefficients (Δ*F*) to 0.30. Convergence was checked in Tracer v.1.6.0 (Rambaut et al., 2014) and by consistency of results of several runs with different initial seeds. Results for short-term gene flow were visualized in circos plots using the Circos Table Viewer v.0.63-9 (Krzywinski et al., 2009).

2.6 | Calculation of gene flow rate

We calculated the N_em using the coalescent-based estimates for the mutation-scaled population size Θ and the mutation-scaled immigration rate *M* derived from MIGRATE-N. For autosomal markers, Equation (1) expresses the relationship between Θ_j (population size of the population receiving migrants) and M_{ij} (corresponding migration rate; Marko & Hart, 2011):

$$N_em_{i>j} = \frac{M_{i>j} \times \Theta_j}{4}. \quad (1)$$

3 | RESULTS

A Bayesian multi-locus tree analysis of the 21 nuclear loci (total of 16,969 nucleotides) for 137 giraffe, including all traditionally recognized giraffe subspecies (Figure 3a), implies a clear separation into four giraffe clades: (a) a northern giraffe cluster including West African (*G. c. peralta*), Kordofan (*G. c. antiquorum*), and Nubian giraffe (*G. c. camelopardalis*) which includes the former Rothschild's giraffe (*G. c. rothschildi*), (b) the reticulated giraffe (*G. reticulata*), (c) the Masai giraffe (*G. tippelskirchi*) including former Thornicroft's giraffe (*G. c. thornicrofti*), and (d) a southern giraffe cluster (*G. giraffa*) including Angolan giraffe (*G. g. angolensis*) and South African (*G. g. giraffa*). The monophyly of each of these four clades is supported by a posterior probability of $p \geq 0.95$. However, in the analyses, the exact relationships of southern and Masai giraffe relative to northern and reticulated giraffe could not be determined with significant probability ($p \approx 0.81$) (not shown).

A mtDNA Bayesian tree (Supporting information Figure S1) confirms the reciprocal monophyly of six distinct subspecies clusters with posterior probability ≥0.95 (Bock et al., 2014; Brown et al., 2007; Fennessy et al., 2016). MtDNA does not support two subspecies of Masai giraffe, even though they appear paraphyletic in the tree, because individuals, which are designated Masai giraffe individuals disrupt a possible reciprocal monophyly and the branch dividing the Masai giraffe into two clusters is not significantly supported. For reticulated giraffe, three individuals do not group as expected but rather fall within the northern giraffe, indicating possible hybridization. However, two of these individuals are from zoos, where hybridization can occur, and have an unknown breeding history. The third individual (LWC01) is a wild giraffe from a geographic range adjacent to the northern giraffe and is a possible natural hybrid. During tissue sampling, this individual was identified phenotypically as reticulated giraffe (J. Fennessy pers. obs.).

Multi-locus population STRUCTURE analyses (Pritchard et al., 2000) of 21 nuclear loci (Figure 3b) proposes the best clustering into four distinct populations (optimal $K = 4$) based on the graphical display. At $K = 3$ the analyses merge the reticulated and the northern giraffe and at $K \geq 5$ the analyses do not produce further clustering. Three different statistical methods to interpret the STRUCTURE results (Evanno et al., 2005; Pritchard et al., 2000, 2010) confirm $K = 4$ being the best fitting number of populations (Supporting information Figure S2). These four clusters conform to the four giraffe clades identified by tree analyses. Intriguingly, STRUCTURE also identifies three potential hybrids between the northern and reticulated giraffe within the reticulated giraffe clade (Figure 2a,b). The distinctness of four unique giraffe clades is in addition supported by Principal Component Analyses (PCAs; Figure 3c) with significant nonoverlapping 95% confidence

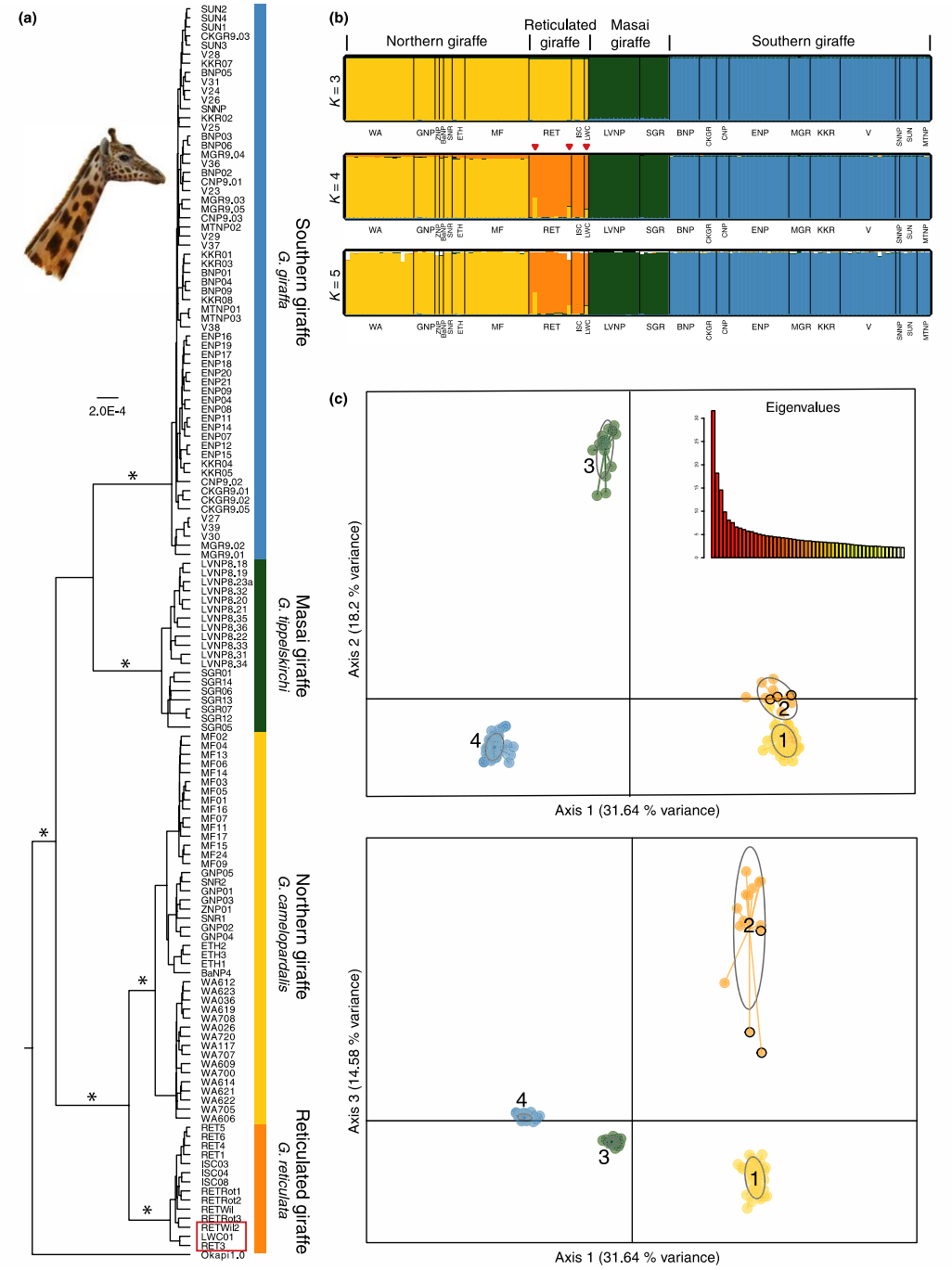
(a)
2.0E-4
Southern giraffe
G. giraffa
Masai giraffe
G. tippelskirchi
Northern giraffe
G. camelopardalis
Reticulated giraffe
G. reticulata
(b)
Northern giraffe
Reticulated giraffe
Masai giraffe
Southern giraffe
K = 3
K = 4
K = 5
WA
GNP
MF
RET
LVNP
SGR
BNP
CKGR
CNP
ENP
MGR
KKR
V
SNNP
SUN
MTNP
(c)
Eigenvalues
Axis 2 (18.2 % variance)
Axis 1 (31.64 % variance)
Axis 3 (14.58 % variance)
Axis 1 (31.64 % variance)

FIGURE 3 Nuclear phylogeny and population structuring of giraffe. (a) Bayesian multi-locus tree from 21 nuclear loci and 137 giraffe individuals reconstruct four significant supported ($p \geq 0.95$) giraffe clades, corresponding to the four giraffe species (Fennessy et al., 2016). The okapi is used as the outgroup. The asterisks indicate branches with statistical significant support ($p \geq 0.95$). The red frame indicates the potential hybrids. (b) STRUCTURE analysis of the dataset, excluding the okapi. The colors indicate the membership in a cluster for each sampling location and individual. $K = 4$ shows four well-resolved groups and is supported as best fitting number of clusters by several statistical methods (see Supporting information Figure S2). The grouping into four clusters is consistent with the Bayesian multi-locus analysis: yellow: northern giraffe, orange: reticulated giraffe, green: Masai giraffe, and blue: southern giraffe. Three individuals within the reticulated giraffe cluster (red arrowheads) indicate potential hybridization with admixture from the northern giraffe. $K = 3$ merges northern and reticulated giraffe, and at $K \geq 5$ no further clustering is evident. (c) PCA axes 1-2 and axes 1-3 for four distinct giraffe clusters (1: northern; 2: reticulated; 3: Masai; 4: southern). Colors as in Figure 3b. The 95% confidence intervals are shown as oval outlines. Note that the nonoverlapping confidential intervals in the PCA axes 1-2, as well as, axes 1-3 indicate significantly different clusters. Potential hybrids are indicated by black circles. *Note*. The drawing by Jon B. Hlidberg shows a Nubian giraffe

intervals. PCAs using groups of the seven mtDNA clades do not find more than four distinct clusters (Supporting information Figure S3). Finally, pairwise fixation indices (F_{ST}) of ≥0.237 (statistically significant at $p < 0.001$) are consistent with the four distinct clusters of giraffe in the tree analyses (Supporting information Table S3).

Separate PCAs and STRUCTURE analyses for each species (Supporting information Figures S4–S7) indicate population substructure within northern giraffe, and potentially in the Masai giraffe, but no further population substructure in southern and reticulated giraffe. Within northern giraffe, STRUCTURE analyses and PCAs up to four clusters can be identified. However, the sample sizes of some populations (three for Ethiopia) are arguably insufficient to draw definitive conclusions if there are further clusters. Within the Masai giraffe, STRUCTURE and PCAs identify potentially two separate clusters, indicating a possible separation of the two geographically most distant populations that have been analyzed for nuclear intron sequences to date. Consistent with the STRUCTURE and PCA analyses, pairwise F_{ST} analyses within each giraffe species find a high level of population differentiation within northern and possibly Masai giraffe, and little differentiation within southern and reticulated giraffe (Supporting information Table S4).

We estimated long-term gene flow between all four giraffe clades, as well as among subspecies within each of the giraffe species, which show population substructure in STRUCTURE and PCAs using MIGRATE-N (Beerli, 2006; Beerli & Felsenstein, 2001). All

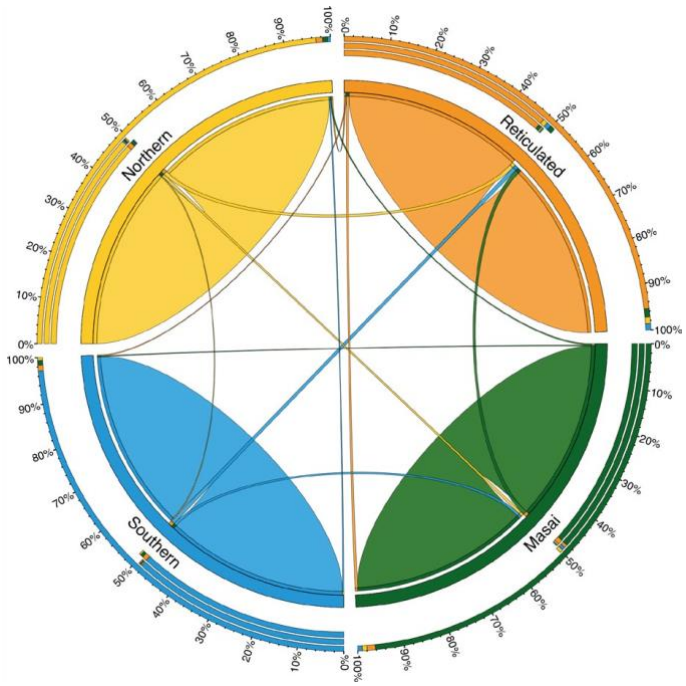

FIGURE 4 Circular migration plot of recent migration rates among four giraffe clades. Recent directional migration rates (m) as estimated by BayesAss and indicated by ribbons connecting one species to another. The color coding of the four species is according to the STRUCTURE clusters (Figure 3b). Peripheral concentric stack bars show relative migration rates in percent. Whereas the inner stack bar shows the outgoing ribbon sizes, the middle stack bar the incoming ribbon sizes and the outer stack bar the combination of both

parameters had an ESS > 5,000. Assuming similar mutation rates among all giraffe species, the mutation-scaled population size theta (Θ) estimates for the four species suggest that the effective population size (N_e) is smaller in southern giraffe and Masai giraffe than in northern and reticulated giraffe (Supporting information Table S5a). Thus, the population size in the northern and reticulated giraffe had been larger in the past considering their current numbers (Giraffe Conservation Foundation, 2017). The calculated effective numbers of migrants per generation or gene flow rate (N_em) based on Θ and the mutation-scaled migration rate (M) (Supporting information Table S5b) indicate generally very low level of gene flow among most of the four giraffe clades with a maximum of one migrant per five generations ($N_em \leq 0.179$), with one exception. A higher N_em occurs between the northern and reticulated giraffe with nearly one migrant per generation in the direction of the reticulated giraffe ($N_em = 0.945$), but much less migration is observed in the opposite direction to northern giraffe ($N_em = 0.179$). There is little (ca. one in ten) directional gene flow from Masai to reticulated giraffe ($N_em = 0.107$) and from southern to reticulated giraffe ($N_em = 0.104$) with nearly zero gene flow in the opposite direction. The gene flow rates for all other species pairs are very low ($N_em < 0.065$). Within species long-term gene flow rates are on average higher ($N_em > 1$) (Supporting information Table S6b). However, between some subspecies, gene flow is also limited, in particular, the geographically isolated West African giraffe (WA).

Finally, short-term migration rates (m) estimated with BayesAss (Wilson & Rannala, 2003; Figure 4; Supporting information Table S5b) confirm low levels of gene flow among the four giraffe species for the past three generations. The highest migration rates occur from northern, Masai and southern giraffe in the direction of reticulated giraffe, which is expected due to adjacent ranges. The data suggest that approximately 2% ($m = 0.021$) of the reticulated giraffe population are derived from each of these neighboring species. In comparison with the other gene flow analyses, BayesAss identifies somewhat higher recent migration rates (m) among subspecies within species (Supporting information Table S6b). This is consistent with the lack of genetic differentiation identified by PCA and F_{ST} analyses. The recent migration rates estimated by BayesAss analyses suggest directional gene flow between West African and Kordofan giraffe ($m = 0.064$), and find gene flow between South African and Angolan giraffe ($m = 0.052$). Most importantly, however, is that BayesAss does not find any first or second generation hybrids.

4 | DISCUSSION

Morphology, ecology, and genetic analyses suggest that there are more than one giraffe species (Brown et al., 2007; Fennessy et al., 2016; Groves & Grubb, 2011; Thomassen, Freedman, Brown, Buermann, & Jacobs, 2013). Here we expand our previous dataset three-fold and improve the sampling of northern and reticulated giraffe to further study if there is indeed more than one species. The new data allow for the first-time detailed gene flow and migration analyses with an appropriate amount of data. Among the four giraffe species, gene flow and migration are very limited. As such, the new analyses of the extended nuclear data corroborate the identification of four genetically distinct giraffe species (Fennessy et al., 2016).

Several attempts have been made to define a species, but an unequivocal consensus has not yet been reached (Coyne & Orr, 2004; De Queiroz, 2007). The most commonly applied model is the BSC, which suggests that reproductive isolation is essential to delineate species (Dobzhansky, 1970; Mayr, 1942). By contrast, subspecies or evolutionary significant units are often debated distinctions within a species. Reproductive isolation is also a cornerstone of other species concepts that define species as distinct evolutionary units with limited gene flow to other such units (Avise & Ball, 1990). Therefore, analyzing gene flow among species is a central analysis to delineate species, especially if, like in giraffe, they possibly hybridize in nature. It has been suggested that gene flow among species must be limited to allow genetic differentiation. A value below one migrant per generation ($<1\ N_em$) may be a conservative estimate (Wright, 1969), even if other studies are more liberal and suggest that gene flow rates of $<5\ N_em$ (Lacy, 1987) or even $<10\ N_em$ (Mills & Allendorf, 1996; Vucetich & Waite, 2000) can allow genetic differentiation and consequently speciation.

The initial finding of four giraffe species was unexpected (Fennessy et al., 2016), as some giraffe interbreed in captivity (Gray, 1972; Lackey, 2011; Lönnig, 2011), and they are highly mobile in the wild (Flanagan, Brown, Fennessy, & Bolger, 2016), both processes which would facilitate admixture. However, we do not observe this in the four giraffe species. If giraffe was in fact one species, mathematical models suggest that long-term effective gene flow rates in excess of 1–10 migrants per generation would be required to avoid differentiation between populations (Lacy, 1987; Mills & Allendorf, 1996; Vucetich & Waite, 2000; Wright, 1969).

Yet, even among the closely related and neighboring northern and reticulated giraffe lower long-term effective gene flow rates, $<1\ N_em$, and substantial genetic differentiation are observed, which is consistent with being genetically differentiated species. In addition, among all 137 individuals from a wide geographic distribution, only one natural hybrid was genetically identified. The rare occurrence of hybrids in the wild hints to prezygotic reproduction barriers, because successful hybridization in captivity excludes postzygotic barriers. This further supports the existence of four giraffe species. So far only one possible mechanism for reproductive isolation has been published. Reproduction seems to be synchronized to geographically distinct seasonal rainfall cycles and may contribute to reproductive isolation between the three giraffe species in East Africa (Thomassen et al., 2013).

Population genetic analyses, such as STRUCTURE and PCA of the data set, support the results from the gene flow analyses. The new results are inconsistent with past suggestions of one or possibly six or seven distinct giraffe species (Brown et al., 2007). These previous results were based on nonstringent conclusions from STRUCTURE analyses with 11 separate genetic clusters at $K = 13$

based on 14 microsatellites, and results of six to seven giraffe clusters based on mtDNA phylogeny (Brown et al., 2007): "11 of the 18 sampling localities resolved as distinct genetic clusters at $K = 13$," however, the authors concluded that only "the seven lineages that are reciprocally monophyletic in the mtDNA tree need to be considered evolutionary significant units if not species." Other findings of up to eight giraffe species were proposed based on a combination of limited genetic analyses (Brown et al., 2007; Hassanin, Ropiquet, Gourmand, Chardonnet, & Rigoulet, 2007) and morphological characteristics (Groves & Grubb, 2011); however, the sample locations of some samples were inaccurate.

Both, Fennessy et al. (2016) and Bock et al. (2014) suggested to subsume Rothschild's giraffe (MF) into the Nubian giraffe, as well as Thornicroft's giraffe (LVNP) into the Masai giraffe because they lack differentiation at mtDNA sequences. Evolutionary differentiation of populations is often first evident in mtDNA because theory suggests that this locus, due to its maternal inheritance and nonrecombining nature, reaches fixation 4-times more rapidly than nuclear loci (Zink & Barrowclough, 2008). Such population differentiation processes have been reported in natural population of bears (Hailer et al., 2012), humpback whales (Palumbi & Baker, 1994) and macaques (Melnick & Hoelzer, 1992).

While the current mtDNA analyses support previous findings (Fennessy et al., 2016) of Thornicroft's giraffe being subsumed into the Masai giraffe, new and extended nuclear gene datasets identify subtle substructure among them. We emphasize, however, that the nuclear loci have only been sampled from across a limited distribution of the Masai giraffe (Fennessy et al., 2016). Additional sampling of intermediate Masai giraffe populations and additional nuclear gene loci will be necessary to yield more definite results. The first detailed mtDNA analyses on Thornicroft's giraffe (Fennessy, Bock, Tutchings, Brenneman, & Janke, 2013) proposed that while they are not reciprocal monophyletic, the geographic location in Zambia's Luangwa Valley is unique and should, for conservation efforts, tentatively maintain its subspecies status as Thornicroft's giraffe within Masai giraffe (*Giraffa tippelskirchi thornicrofti*).

Within the northern giraffe, some substructure is evident in PCAs and STRUCTURE analyses for nuclear sequences (Supporting information Figure S6). However, the West African giraffe is a geographically very isolated and small population of ~600 individuals. The geographic distinction between the former Nubian and Kordofan giraffe is unclear and current data suggest that they are not genetically isolated (Fennessy et al., 2016).

With little more than 5,000 northern giraffe, < 15,000 reticulated giraffe and ~34,000 Masai giraffe remaining in the wild (Giraffe Conservation Foundation, 2017), recognizing these—and the southern giraffe—as separate species has an impact on giraffe conservation. Their decline in numbers over the last thirty years (three generations)—northern giraffe (~95%), reticulated giraffe (~60%) and Masai giraffe (~52%)—highlight that these species are threatened with extinction (IUCN, 2017). Giraffe, as a single species, and not four, were recently listed as "Vulnerable" on the IUCN Red List (Muller et al., 2016). The mounting evidence of four giraffe species that now also includes gene flow analyses requires a re-evaluation of the current IUCN giraffe taxonomy. A higher threat category may be granted to increase conservation management actions.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

AJ, JF, and SW designed and conceived the study. AJ and JF funded the project, and JF collected the samples and provided biological data. SW developed markers, generated and analyzed the data. SW and AJ wrote the manuscript with input from JF.

DATA ACCESSIBILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the article and its Supporting Information files. Sequences generated during the study are available at GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) under the Accession Numbers MG257948–MG262301.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplemental material

Limited Introgression Supports Division of Giraffe into Four Species

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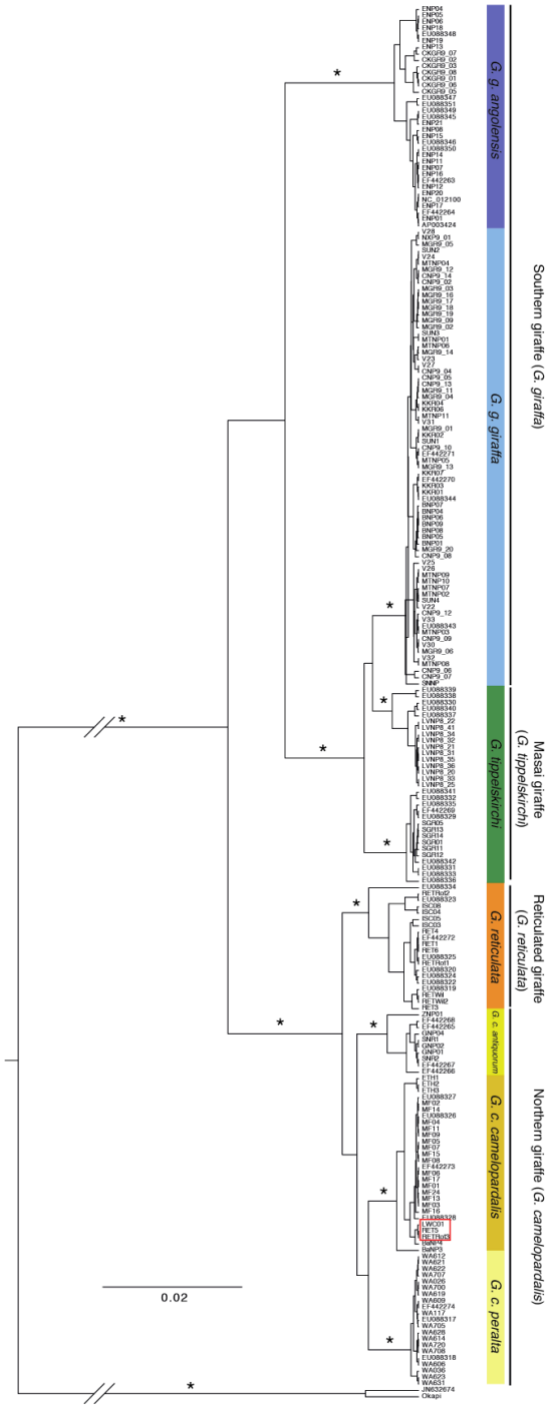
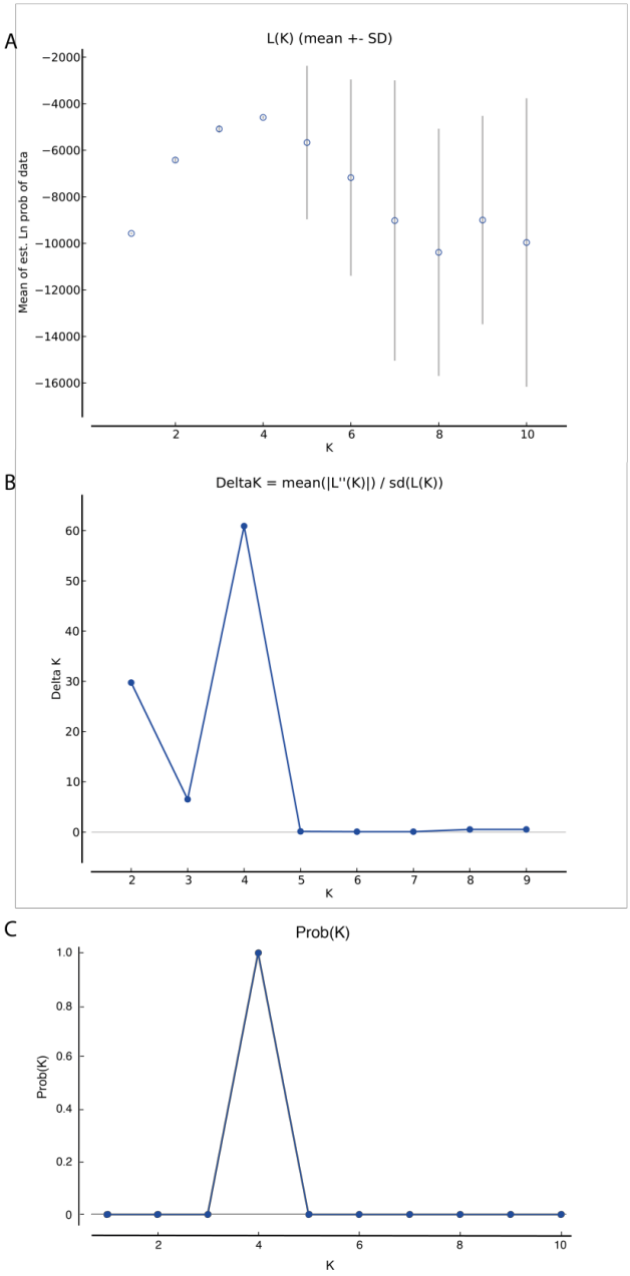


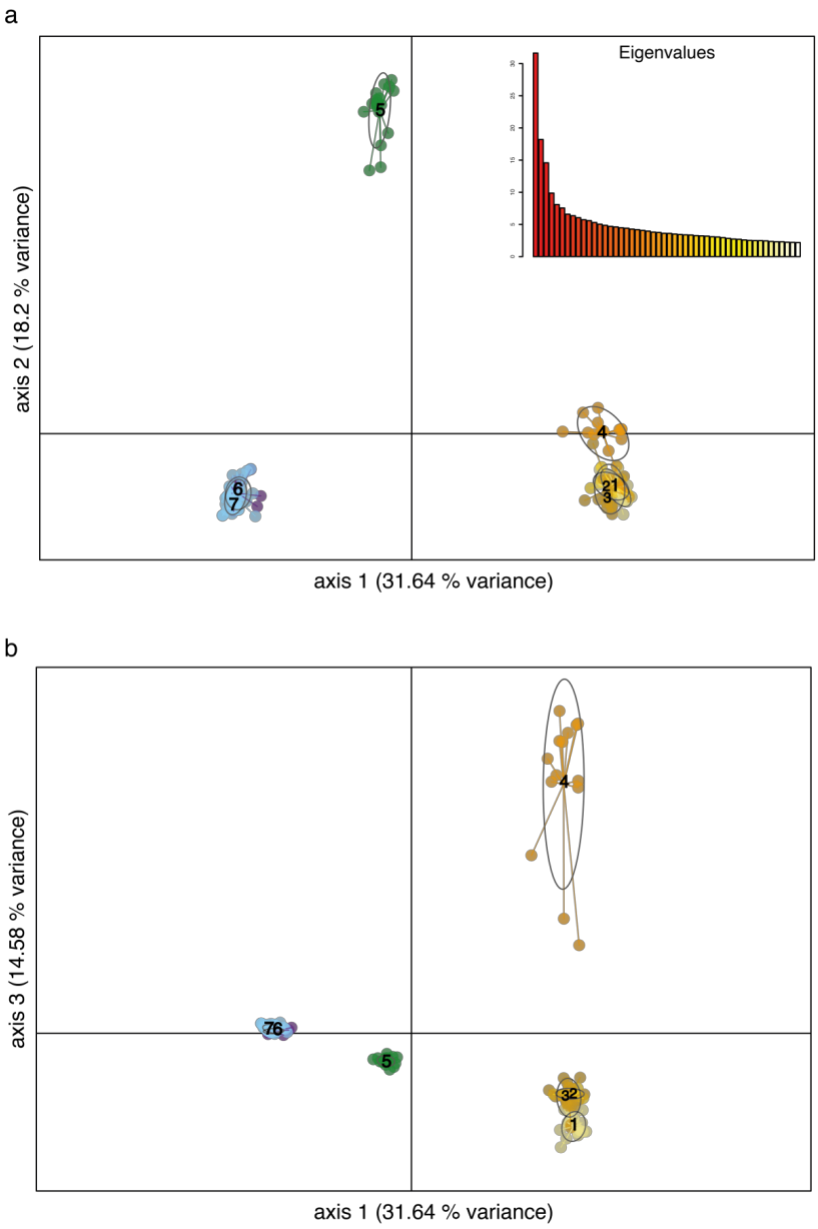
Supplementary Fig. 1. MtDNA Bayesian tree of 217 giraffe individuals
Asterisks show major branches with a posterior probability ≥ 0.95 and the red frame shows potential reticulated giraffe / Nubian giraffe hybrids. Two different okapi individuals were used as an outgroup. Note, the root is not to scale.

1



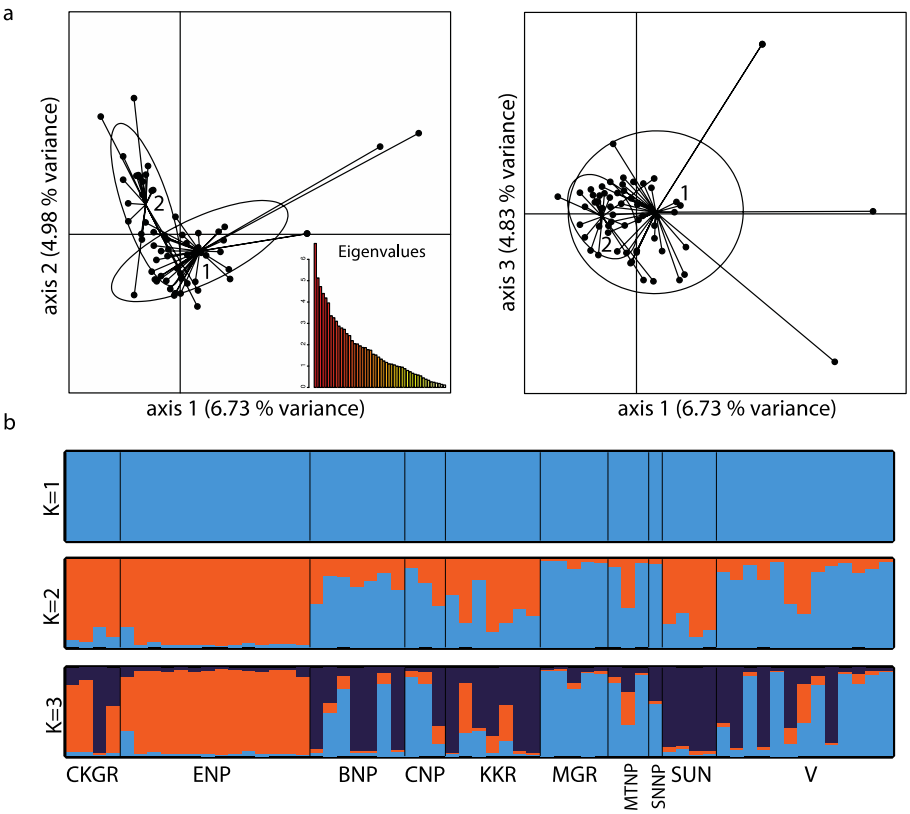
Supplementary Fig. 2. Statistical calculations to evaluate the best fitting K in STRUCTURE
(A) Mean likelihood (L(K)) and variance per K value from STRUCTURE according to (Pritchard, Wen, & Falush, 2010). (B) Delta K plot as per Evanno, Regnaut, & Goudet (2005) to find the best fitting number of populations (K) for the data. K = 4 has the highest Delta K. (C) Probability by K plot according to Pritchard, Stephens, & Donnelly (2000). K = 4 shows the highest probability.

2



Supplementary Fig. 3. Additional PCAs based on mtDNA clades.
PCA axes 1-2 and axes 1-3 for seven giraffe mtDNA clades (1: West African; 2: Kordofan; 3: Nubian; 4: reticulated; 5: Masai; 6: Angolan; 7: South African). Colored as in Supplementary Fig. 1. The 95% confidential intervals are shown as grey colored oval outlines. Note that the confidence intervals in the PCA axes 1-2, as well as axes 1-3, indicate the same four significantly different clusters as seen in Fig. 3c.

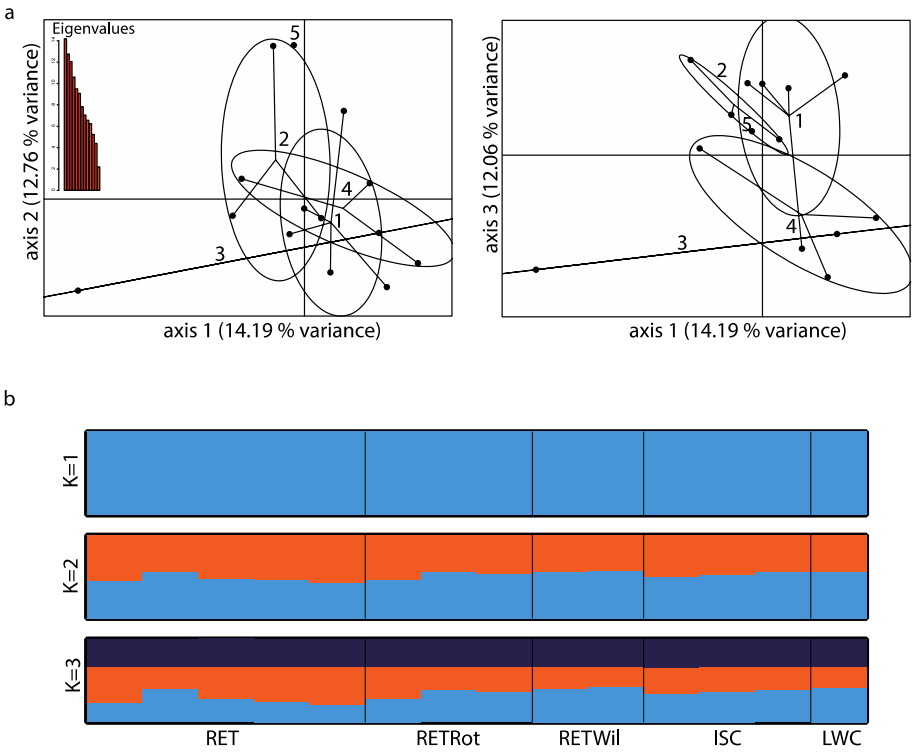
3



Supplementary Fig. 4. Additional PCAs and STRUCTURE analyses for southern giraffe

(a) PCA axes 1-2 and axes 1-3 for the southern giraffe populations (1: South African giraffe; 2: Angolan giraffe). The 95% confidential intervals are shown as oval outlines. Note, the confidence intervals in the PCA axes 1-2, as well as axes 1-3, indicate no substructure within the southern giraffe.

(b) STRUCTURE analysis for the southern giraffe populations (CKGR and ENP are Angolan giraffe, the remaining populations are South African giraffe) does not show additional clustering but a high level of admixture, which contradicts the clear separation of subspecies by mtDNA. Note, detailed information about the populations are listed in Supplementary Table 1.



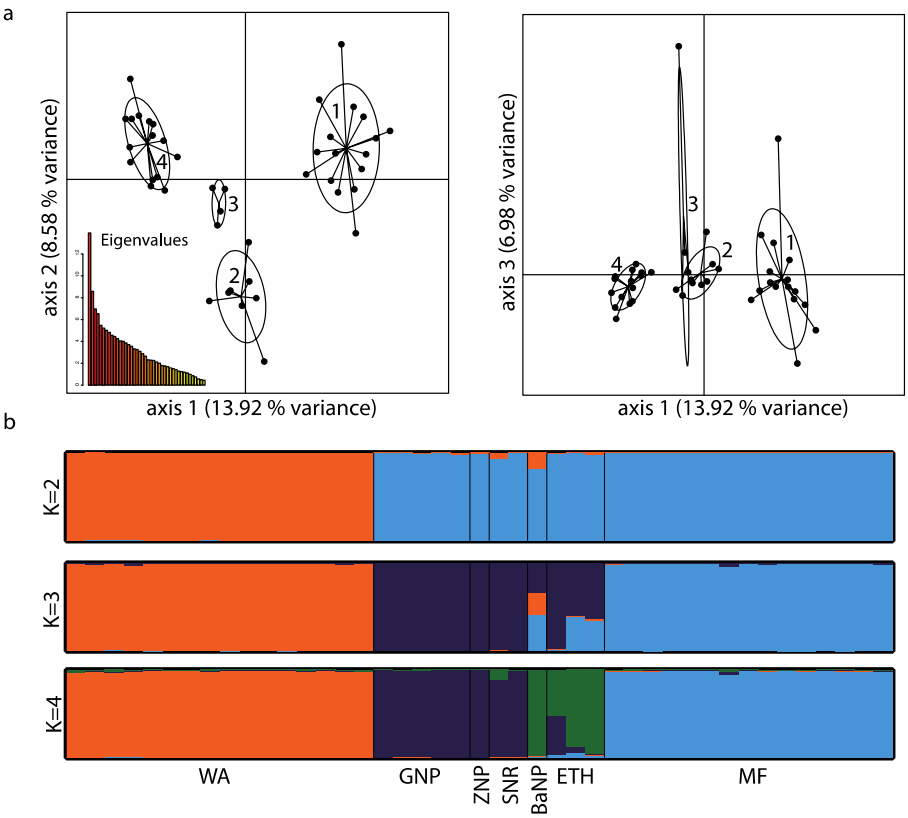
Supplementary Fig. 5. Additional PCAs and STRUCTURE analyses for reticulated giraffe

(a) PCA axes 1-2 and axes 1-3 for the sampled reticulated giraffe populations (1: RET; 2: RETRot; 3: RETWil; 4: ISC; 5: LWC). The 95% confidence intervals are shown as oval outlines. Note that the confidence intervals in the PCA axes 1-2, as well as axes 1-3, indicate no substructure within the reticulated giraffe.

(b) Separate STRUCTURE analysis for reticulated giraffe populations (RET, RETRot and RETWil: captive animals; ISC and LWC: wild populations) shows no additional clustering.

Note, detailed information about the populations are listed in Supplementary Table 1.

5

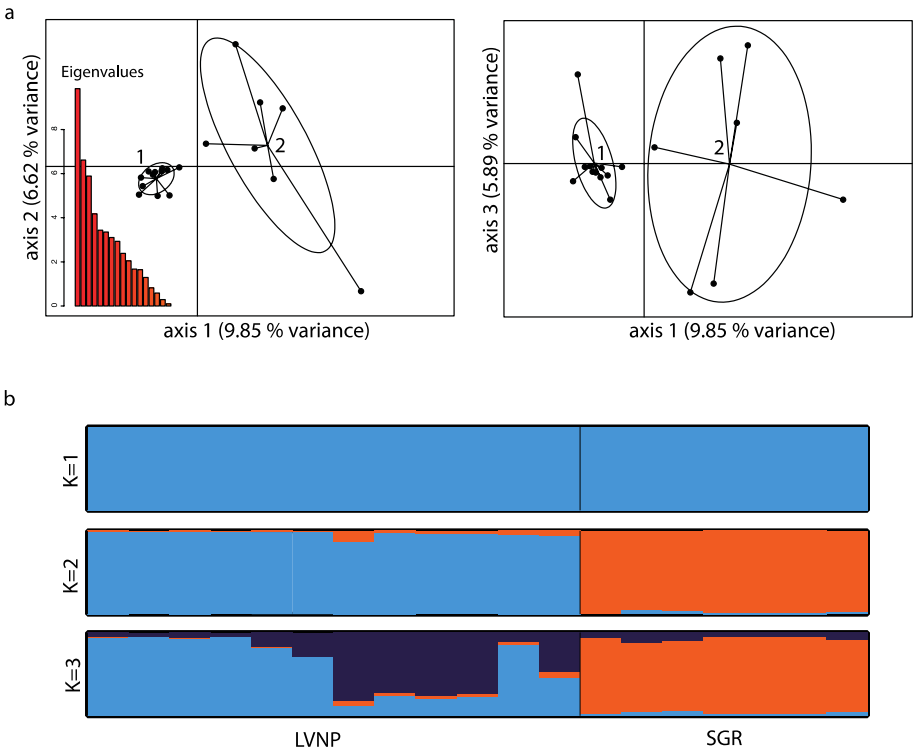


Supplementary Fig. 6. Additional PCAs and STRUCTURE analyses for northern giraffe

(a) PCA axes 1-2 and axes 1-3 for the sampled northern giraffe populations (1: West African giraffe; 2: Kordofan giraffe; 3: Nubian giraffe; 4: former Rothschild's giraffe). The 95% confidence intervals are shown as oval outlines. Note, the confidence intervals in the PCA axes 1-2, as well as axes 1-3, indicate some substructure within the northern giraffe. However, there is no clear distinction between Kordofan and Nubian giraffe.

(b) Separate STRUCTURE analysis for the northern giraffe populations (WA: West African giraffe; GNP, ZNP, and SNR: Kordofan giraffe; BaNP and ETH: Nubian giraffe; MF: former Rothschild's giraffe) shows additional substructure for up to four clusters, but also highlights admixture at K=3.

Note, detailed information about the populations are listed in Supplementary Table 1.



Supplementary Fig. 7. Additional PCAs and STRUCTURE analyses for Masai giraffe
(a) PCA axes 1-2 and axes 1-3 for the two sampled Masai giraffe populations (1: LVNP; 2: SGR). The 95% confidence intervals are shown as oval outlines. Note that the confidence intervals in the PCA axes 1-2, as well as axes 1-3, indicate some substructure within the Masai giraffe.
(b) Separate STRUCTURE analysis for the Masai giraffe populations shows additional clustering for two populations.
Note, detailed information about the populations are listed in Supplementary Table 1.

Supplementary Table 1. Location, abbreviation, number of individuals (n), (sub)species and source of analyzed giraffe and okapi sequences for mtDNA and nuclear analyses.

Individual sample ID´s see Figure 2 and Figure S1. Bold numbers indicate populations newly added in this study and underlined numbers indicate populations with increased sample size.

Location	Abbreviation	n (mtDNA)	n (nuclear DNA)	(Sub)species
Badingilo National Park, South Sudan	BaNP	2	1	*G. c. camelopardalis*
Basel Zoo, Switzerland	Okapi	1	1	*O. johnstoni*
Bwabwata National Park, Namibia	BNP	7	7	*G. g. giraffa*
Central Kalahari Game Reserve, Botswana	CKGR	7	4	*G. g. angolensis*
Chobe National Park, Botswana	CNP	11	3	*G. g. giraffa*
Etosha National Park, Namibia	ENP	17	14	*G. g. angolensis*
Gambella National Park, Ethiopia	ETH	3	3	*G. c. camelopardalis*
Garamba National Park, DR Congo	GNP	5	5	*G. c. antiquorum*
Ishqbini Conservancy, Kenya	ISC	4	**3**	*G. reticulata*
Khamab Kalahari Reserve, South Africa	KKR	6	7	*G. g. giraffa*
Koure, Niger	WA	18	16	*G. c. peralta*
Loisaba Wildlife Conservancy, Kenya	LWC	1	**1**	*G. reticulata*
Luangwa Valley National Park, Zambia	LVNP	11	12	*G. tippelskirchi*
Moremi Game Reserve, Botswana	MGR	16	5	*G. g. giraffa*
Mosi-oa-Tunya National Park, Zambia	MTNP	11	3	*G. g. giraffa*
Murchison Falls National Park, Uganda	MF	16	15	*G. c. camelopardalis*
Nürnberg Zoo, Germany	RET	5	5	*G. reticulata*
Nxai Pans, Botswana	NXP	1	–	*G. g. giraffa*
Rotterdam Zoo, Netherlands	RETRot	**3**	**3**	*G. reticulata*
Selous Game Reserve, Tanzania	SGR	6	7	*G. tippelskirchi*
Shambe National Park, South Sudan	SNR	2	2	*G. c. antiquorum*
Sioma Ngwezi National Park, Zambia	SNNP	1	1	*G. g. giraffa*
Sun hotel, Livingstone, Zambia	SUN	4	4	*G. g. giraffa*
Vumbura Concession, Botswana	V	11	13	*G. g. giraffa*
Wilhelma Stuttgart, Germany	RETWil	2	2	*G. reticulata*
Zakouma National Park, Chad	ZNP	1	1	*G. c. antiquorum*

Additional mtDNA sequences from GenBank

Accession No.	(Sub)species	Source
EF442263 - EF442274	*Giraffa spp.*	Hassanin et al., 2007
EU088317 - EU088320	*Giraffa spp.*	Brown et al., 2007
EU088322 - EU088351	*Giraffa spp.*	Brown et al., 2007
AP003424	*G. g. angolensis*	Yasue et al. unpublished
JN632674	*O. johnstoni*	Hassanin et al., 2012
NC_012100	*G. g. angolensis*	Yasue et al. unpublished

Supplementary Table 2. Giraffe and okapi specific primer sequences and PCR conditions for 14 new nuclear intron loci.

Primer sequences and PCR conditions of the remaining seven intron loci and the mtDNA loci can be found in Bock et al. (2014) and Fennessy et al. (2016).

Name & locus	Primer sequence 5'-3'	PCR conditions
Intron SW05 (RFC5, intron 9, chromosome 17)	for: GATCACTCTGGAACCTGCTCA rev: CATACCTGTGGTTCTGCGGT	TD-PCR (T_a = 70-60 °C; 10 cycles), standard PCR (T_a = 60 °C; 30 cycles)
Intron SW07 (USP33, intron 18, chromosome 3)	for: TGACGACCAGAGTGTCACTG rev: TCTTTTTGTGCTTCTTCACTGCT	TD-PCR (T_a = 67-57 °C; 10 cycles), standard PCR (T_a = 57 °C; 30 cycles)
Intron SW21 (MACF1, intron 46, chromosome 3)	for: GCTCACGACCTCATGGAAAT rev: GTTGGAAATGGCTGAGGATG	TD-PCR (T_a = 66-59 °C; 14 cycles), standard PCR (T_a = 59 °C; 26 cycles)
Intron SW40 (IGF2B1, intron 11, chromosome 19)	for: GGCAGCACATCAAACAGCTC rev: GGGGTCCAGTGATGATGACC	See Intron SW05
Intron SW43 (COL5A2, intron 32, chromosome 2)	for: AATGGCTGGAGGACATGGTC rev: GCCGGAAGTTCCTGCAATTC	See Intron SW05
Intron SW44 (CTAGE5, intron 8, chromosome 21)	for: CCCTCAAATCACAAGTAGCTGA rev: TCTGGCTTTCCTGAAGTTGAGA	See Intron SW05
Intron SW51 (NOTCH2, intron 33, chromosome 3)	for: AAAAATGGGGCCAACCGAGA rev: GGGCAGCAAGAAACAGAGGT	See Intron SW05
Intron SW68 (CCT2, intron 14, chromosome 5)	for: TGAAGGCAAAACAACCGCTG rev: CGCTTCACTTGGAAACTTTCTGT	See Intron SW05
Intron SW84 (DHX36, intron 4, chromosome 1)	for: CATCCTTCCCTGACCTCTCAG rev: TCCACAACCAGTTTCACCACT	See Intron SW05
Intron SW108 (C1orf74, intron 1, chromosome 16)	for: TCCAGTGTTGTTGCTGCTGA rev: TCTGGGAGGACCTCGTTTCT	See Intron SW05
Intron SW111 (SAP130, intron 5, chromosome 2)	for: CCCCCTCTTCACATTGGAGC rev: AACTGGACATCACTGCAGCA	See Intron SW05
Intron SW113 (PLCE1, intron 20, chromosome 26)	for: ACTCTGCTTGCAACAAAGGA rev: TCTGGTGCAATCTGTCTGCT	See Intron SW05
Intron SW117 (DDX1, intron 15, chromosome 11)	for: TTCCTGGTCCTGGATGAAGC rev: AACCTTGAGAAAGAAGCCCAT	See Intron SW05
Intron SW123 (USP54, intron 9, chromosome 28)	for: CCCCCAAGTTGAGTTCCAGT rev: CCGTTGAGGAATCGGTTCGA	See Intron SW05

Note – for: forward primer. rev: reverse primer. TD-PCR: touchdown PCR. T_a: primer annealing temperature. The locus is the gene name of the human orthologs, the respective intron, and the chromosome of *Bos taurus* it is located on.

Supplementary Table 3. List of pairwise F_{st} values for 21 nuclear loci of four giraffe species.

	Northern	Reticulated	Masai	Southern
Northern	–			
Reticulated	0.23725**	–		
Masai	0.57289**	0.57813**	–	
Southern	0.62072**	0.63408**	0.68790**	–

Note – ** indicates significance of F_{st} values at $p < 0.001$.

Supplementary Table 4. Pairwise F_{st} values for 21 nuclear loci between subspecies / populations within four giraffe species.

a. Pairwise F_{st} values among southern giraffe subspecies

	South African giraffe	Angolan giraffe
South African giraffe	–	
Angolan giraffe	0.10199**	–

b. Pairwise F_{st} values among reticulated giraffe populations

	RET	ISC	LWC
RET	–		
ISC	0.04000	–	
LWC	0.09065	0.10268	–

c. Pairwise F_{st} values among northern giraffe subspecies (incl. former Rothschild's giraffe)

	West African giraffe	Kordofan giraffe	Nubian giraffe	"Rothschild's giraffe"
West African giraffe	–			
Kordofan giraffe	0.21326**	–		
Nubian giraffe	0.15513**	0.12841**	–	
"Rothschild's giraffe"	0.26777**	0.27702**	0.15245**	–

d. Pairwise F_{st} values among Masai giraffe populations

	SGR	LVNP
SGR	–	
LVNP	0.39517**	–

Note – ** indicates significance of F_{st} values at $p < 0.001$. Detailed information about the populations are listed in Supplementary Table 1.

Supplementary Table 5. Long- and short-term gene flow estimates among four giraffe species.

a. Mutation-scaled effective population size derived from MIGRATE-N of the four giraffe species

Species	Estimated Θ (95 % conf. int.)
Northern giraffe	0.00137 (0.0 – 0.00287)
Reticulated giraffe	0.00110 (0.0 – 0.00267)
Masai giraffe	0.00003 (0.0 – 0.00153)
Southern giraffe	0.00003 (0.0 – 0.00193)

b. Estimates of gene flow derived from MIGRATE-N and BayesAss among the four giraffe species

Migration route	M (95 % conf. int.) [MIGRATE-N]	N_em	m (+/- sdev.) [BayesAss]
Reticulated → Northern	523.3 (253.3 – 793.3)	0.17923	0.0071 (0.0070)
Masai → Northern	190.0 (0.0 – 373.3)	0.06508	0.0071 (0.0069)
Southern → Northern	123.3 (0.0 – 306.7)	0.04223	0.0071 (0.0069)
Northern → Reticulated	3436.7 (3140.0 – 4246.7)	0.94509	0.0209 (0.0196)
Masai → Reticulated	390.0 (120.0 – 693.3)	0.10725	0.0208 (0.0195)
Southern → Reticulated	376.7 (0.0 – 660.0)	0.10359	0.0208 (0.0196)
Northern → Masai	276.7 (20.0 – 553.3)	0.00208	0.0144 (0.0138)
Reticulated → Masai	796.7 (366.7 – 1313.3)	0.00598	0.0145 (0.0139)
Southern → Masai	503.3 (186.7 – 786.7)	0.00377	0.0144 (0.0138)
Northern → Southern	176.7 (0.0 – 360.0)	0.00133	0.0052 (0.0051)
Reticulated → Southern	176.7 (0.0 – 366.7)	0.00133	0.0051 (0.0051)
Masai → Southern	243.3 (6.7 – 493.3)	0.00182	0.0051 (0.0050)

Supplementary Table 6. Long-term and short-term gene flow estimates between giraffe subspecies (populations) within species.

a. Mutation-scaled effective population size derived from MIGRATE-N for subspecies (populations) within giraffe species

Species	Estimated Q (95 % conf. int.)
West African giraffe	0.00130 (0.0 – 0.00287)
Kordofan giraffe	0.00150 (0.0 – 0.00307)
Nubian giraffe	0.00110 (0.0 – 0.00273)
South African	0.00137 (0.0 – 0.00313)
Angolan	0.00050 (0.0 – 0.00213)
SGR	0.00143 (0.0 – 0.00300)
LVNP	0.00097 (0.0 – 0.00260)

b. Estimates of gene flow derived from MIGRATE-N and BayesAss among subspecies (populations) within species

Migration route	M (95 % conf. int.) [MIGRATE-N]	N_em	m (+/- sdev.) [BayesAss]
Kordofan → West African	1611.7 (873.3 – 2563.3)	0.52380	0.0211 (0.0168)
Nubian → West African	2631.7 (1756.7 – 3520.0)	0.85530	0.0150 (0.0141)
West African → Kordofan	3285.0 (2413.3 – 4186.7)	1.23188	0.0636 (0.0364)
Nubian → Kordofan	4215.0 (3523.3 – 4990.0)	1.58063	0.0386 (0.0344)
West African → Nubian	2301.7 (1516.7 – 3193.3)	0.63298	0.0168 (0.0158)
Kordofan → Nubian	1438.3 (863.3 – 2276.7)	0.39553	0.0347 (0.0228)
South African → Angolan	8130.0 (6773.3 – 9420.0)	1.01625	0.0521 (0.0328)
Angolan → South African	8456.7 (7913.3 – 9446.7)	2.89642	0.0223 (0.0154)
Masai			
SGR → LVNP	1610.0 (940.0 – 2793.3)	0.39043	0.0239 (0.0221)
LVNP → SGR	8250.0 (6853.3 – 9746.7)	2.94938	0.0405 (0.0362)

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PUBLICATION 3: SPECIES ASSIGNMENT AND CONSERVATION GENETICS OF GIRAFFE IN THE REPUBLIC OF MALAWI

Sven Winter, Raphael T. F. Coimbra, Anna Bronec, Craig Hay, Amanda L. Salb, Julian Fennessy, Axel Janke

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Sven Winter (SW), Raphael T. F. Coimbra (RC), Anna Bronec (AB), Craig Hay (CH), Amanda L. Salb (AS), Julian Fennessy (JF), Axel Janke (AJ)

What are the contributions of the doctoral candidate and his co-authors?

(1) Concept and design

SW:	70%
AJ:	20%
JF, CH:	10%

(2) Conducting tests and experiments		
SW:	70%	DNA isolation, PCRs, Sanger Sequencing, Fragment analyses (STRs)
AB:	10%	DNA isolation, PCRs, Sanger Sequencing (mtDNA), Fragment analyses (STRs)
RC:	10%	PCR, Sanger Sequencing of nuclear introns
CH, AS, JF:	10%	Sampling (remote skin biopsies of giraffe)

(3) Compilation of data sets and figures		
SW:	80%	Compilation of mtDNA and microsatellite data sets and figures
RC:	20%	Compilation of nuclear intron data sets and Structure plots

(4) Analysis and interpretation of data		
SW:	80%	Phylogenetic trees, genetic diversity analyses, interpretation of all data
RB:	20%	Structure analyses, PCA

(5) Drafting of manuscript

SW:	80%
RC, AB, CH, AS, JF, AJ:	20%

I hereby certify that the information above is correct.

Date and place

Signature doctoral candidate

Date and place

Signature supervisor

Conservation Genetics
https://doi.org/10.1007/s10592-018-01142-4

SHORT COMMUNICATION

Species assignment and conservation genetics of giraffe in the Republic of Malawi

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Abstract

Historically, giraffe have been translocated across Africa to supplement extant populations, reintroduce extinct populations or to establish new populations, often for conservation and tourism. Such faunal relocations were often carried out disregarding taxonomic affiliation. Today, the small giraffe populations in the Republic of Malawi are assumed to consist of South African giraffe (*Giraffa giraffa giraffa*), which have likely descended from five individuals translocated from Imire Game Park (Zimbabwe) to Nyala Game Park (Malawi) in 1993. However, during the last 25 years, unknown additional translocations, migrations or unrecognized local populations of potential Masai giraffe (*Giraffa tippelskirchi*) in Malawi may have resulted in introgressive hybridization. Thus, the current taxonomic affiliation for Malawi's giraffe is uncertain, calling for a genetic assessment to implement further management. We analyzed mitochondrial sequences and nuclear introns for 14 individuals, representing approximately half of the known Malawian population, to genetically determine the (sub)species of giraffe that occur in the Republic of Malawi by comparison with a comprehensive *Giraffa* dataset. Additionally, we genotyped individuals at ten microsatellite loci to determine the level of inbreeding and potential introgression. All data identify individuals unambiguously as South African giraffe, although two individuals shared a single nuclear allele with Masai giraffe. The low microsatellite genetic variability suggests high inbreeding in the current population. Thus, supplementing Malawi's giraffe populations with *G. g. giraffa* will prevent further loss of their genetic diversity and avoid inbreeding depression.

Keywords Hybridization · Inbreeding · Population genetics · Southern giraffe · Translocation · Wildlife management

Introduction

During the last three giraffe generations, or three decades, Africa's giraffe population has declined by 40% to less than 100,000 individuals. Consequently, in 2016 the IUCN classified giraffe as a single species as being "Vulnerable" (Muller et al. 2018). However, recent genetic studies suggest that there are four distinct giraffe species, of which three species would be highly threatened (Online Resource 1, Fig. S1) (Fennessy et al. 2016; Winter et al. 2018b). This would call for a higher conservation status for some of these species. Consequently, the recent assessment by the IUCN classified several (sub)species as "Critically Endangered" or "Endangered"; only the Masai giraffe (*Giraffa tippelskirchi*) and the South African giraffe (*Giraffa giraffa giraffa*), a subspecies of the southern giraffe (*G. giraffa*), are not yet assessed.

The Republic of Malawi (henceforth Malawi) has only 28 giraffe individuals that occur in three parks and their taxonomic history is confounded by numerous translocations.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10592-018-01142-4) contains supplementary material, which is available to authorized users.

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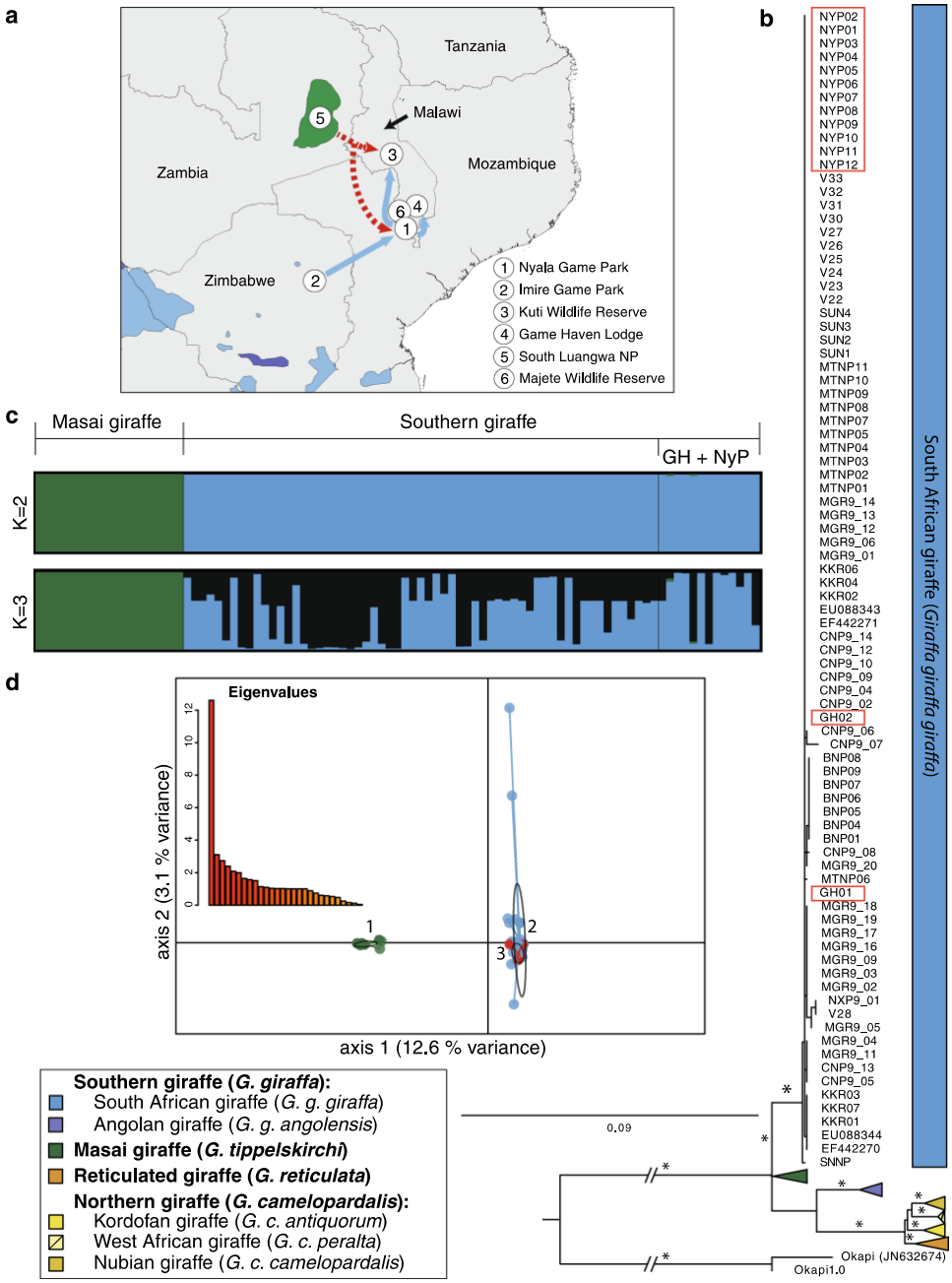
a
Tanzania
Malawi
Zambia
Mozambique
Zimbabwe
1 Nyala Game Park
2 Imire Game Park
3 Kuti Wildlife Reserve
4 Game Haven Lodge
5 South Luangwa NP
6 Majete Wildlife Reserve
b
NYP02
NYP01
NYP03
NYP04
NYP05
NYP06
NYP07
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MGR9_04
MGR9_11
CNP9_13
CNP9_05
KKR03
KKR07
KKR01
EU088344
EF442270
SNNP
South African giraffe (Giraffa giraffa giraffa)
0.09
Okapi (JN632674)
Okapi1.0
c
Masai giraffe
Southern giraffe
GH + NyP
K=2
K=3
d
Eigenvalues
axis 2 (3.1 % variance)
axis 1 (12.6 % variance)
Southern giraffe (G. giraffa):
South African giraffe (G. g. giraffa)
Angolan giraffe (G. g. angolensis)
Masai giraffe (G. tippelskirchi)
Reticulated giraffe (G. reticulata)
Northern giraffe (G. camelopardalis):
Kordofan giraffe (G. c. antiquorum)
West African giraffe (G. c. peralta)
Nubian giraffe (G. c. camelopardalis)

◂**Fig. 1** Map of Malawi with neighboring states and population genetic analyses of Malawi's giraffe. **a** Map of Malawi and adjacent states. Colored shadings show giraffe distribution in the area as identified by the Giraffe Conservation Foundation (2017) and amended after Winter et al. (2018a). Light blue indicates the distribution of *Giraffa giraffa*, while green shows that of *G. tippelskirchi*. The blue arrows indicate recorded translocations from Imire Game Park, Zimbabwe (2) to Nyala Game Park, Malawi (1) (in 1993) and, later on, from Nyala Game Park to Kuti Wildlife Reserve (3) and Game Haven Lodge (4). The dotted red arrows indicate possible translocations of Masai giraffe from the South Luangwa National Park, Zambia (5) into Malawi. **b** ML concatenated Cytb + CR tree of Giraffa (all taxa, n = 231). Except for the South African giraffe, in which all individuals from Malawi are grouped, all other clades are collapsed for better resolution of the tree. The newly studied individuals are framed in red boxes: Nyala Game Park (NyP) and Game Haven Lodge (GH); the original tree is shown in Online Resource 1, Fig. S2. The asterisks indicate branches with bootstrap support ≥ 80%. Note that the root is not to scale. **c** structure bar plot of Malawi's giraffe with southern and Masai giraffe controls. **d** PCA axes 1–2 show two distinct clusters. Cluster A: Masai giraffe (1) and cluster B: southern giraffe (2) and Malawi giraffe (3). The 95% confidence intervals are shown as oval outlines. Note that the blue color in **c** and **d** represents the southern giraffe species instead of the subspecies South African giraffe

The current largest giraffe population lives in the Nyala Game Park (Fig. 1a). Its individuals are expected to be the descendants of five presumptive South African giraffe animals that were introduced from Imire Game Park (Zimbabwe) (Fig. 1a) in 1993 (Giraffe Conservation Foundation 2015). The other two small populations occur in the Kuti Wildlife Reserve (Fig. 1a) and the Game Haven Lodge (Fig. 1a). In 2007, two giraffe individuals that originated from Nyala Game Park were reintroduced to Game Haven Lodge (Fig. 1a). To add to the confusion, it is possible that in the early 2000's Masai giraffe from the South Luangwa National Park in Zambia (Fig. 1a) were translocated to Nyala Game Park and Kuti Wildlife Reserve in Malawi (Fig. 1a) (Briggs 2013).

The traditional taxonomy of giraffe is largely based on morphological traits, such as pelage patterns and distributional data, however, these characteristics have been proven to be inaccurate in some cases (Dagg 2014). This is exemplified by giraffe in an area of Zimbabwe where South African giraffe were expected, but genetic analyses have identified them as Angolan giraffe (*G. g. angolensis*), making them the eastern-most distribution of that subspecies (Winter et al. 2018a). This opens the possibility that the giraffe that were translocated 25 years ago may not be South African giraffe. This renders the taxonomic status of Malawi's giraffe population even more uncertain because, over time, a mix of southern and Masai giraffe may have developed.

Malawi has ideal habitat conditions for a much larger giraffe population (Leslie 2017). The vernacular name for giraffe, *kadyamnsonga* in Chichewa (one of the official languages in Malawi), indicates that giraffe were once widespread and common in the country. Although there is no documentation, the extinction of giraffe in Malawi occurred probably more than a century ago.

Before augmenting Malawi's small giraffe population, it is important to clarify the taxonomic status of its major populations. This will make it possible to identify the best-suited source population for the planned reintroduction of giraffe into the Majete Wildlife Reserve (Malawi) (Fig. 1a).

Materials and methods

Skin biopsy samples from 14 wild giraffe in Malawi were collected by the African Parks Network and the Lilongwe Wildlife Trust in 2017 using remote biopsy darting (DanInject) at the Nyala Game Park (NyP, Fig. 1a) (n = 12) and the Game Haven Lodge (GH, Fig. 1a) (n = 2), with approval from the National Commission for Science and Technology (Malawi), reference number NCST/RTT/1/20. Biopsy samples were preserved in 80% ethanol and DNA was extracted using the Macherey–Nagel NucleoSpin® Tissue kit. We PCR amplified the mitochondrial cytochrome b (cytb) and control region (CR), as well as seven nuclear introns (for details see Online Resource 1, Table S1), following previously established protocols (Bock et al. 2014; Fennessy et al. 2016; Winter et al. 2018b). Sequences were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems) and manually edited in GENEIOUS v.11.1.3 (Kearse et al. 2012). We aligned the mtDNA sequences to a reference dataset (Winter et al. 2018b) containing all species and subspecies of giraffe and the okapi (*Okapia johnstoni*) as an out-group using MAFFT v 7 (Katoh and Standley 2013). The identities of the samples are detailed in Online Resource 1, Table S2. The nuclear sequences were aligned to a reference dataset containing sequences of both giraffe species potentially occurring in Malawi, the southern and Masai giraffe species (Winter et al. 2018b) (Online Resource 1, Table S2). All sequences generated for this study are available from GenBank under the accession numbers MH782194-MH782221 (mtDNA) and MH806895-MH807076 (nuclear DNA).

MEGA v.10.0.4 (Kumar et al. 2018) was used to construct a maximum-likelihood (ML) tree for the concatenated alignment of cytochrome b and the control region. We used the HKY + G + I substitution model, as suggested, to be the best suited by JMODELTEST v.2.1.10 (Darriba et al. 2012) with 1,000 bootstrap replicates.

Allele haplotypes for the nuclear sequences were derived using PHASE implemented in DNASP v.5.10.0143 (Librado and Rozas 2009), with a threshold of 0.6 and allowing for recombination after resolving heterozygous insertions/deletions manually, or with the help of INDELLIGENT v.1.2 (Dmitriev and Rakitov 2008). The Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Pritchard et al. 2000) was used to infer potential hybridization and admixture within the Malawi giraffe

population. We ran 500,000 iterations following a 250,000-step burn-in for each possible number of populations (K), assumed to range from one to five. Each value of K was replicated ten times. The results were averaged and plotted using the CLUMPAK webserver (Kopelman et al. 2015). Additionally, Principal Component Analyses (PCA) were conducted in (R Core Team 2015) using the ADEGENET package (Jombart 2008).

We genotyped all 14 giraffe individuals at ten microsatellites using six previously used loci [Gca_01, Gca_16, Gca_21, Gca_25, EM2 [same as Gca_24 but using a different set of primers, see Online Resource 1, Table S3, Carter et al. (2012)] and Gica9976, Crowhurst et al. (2013)], two newly tested microsatellite loci (EM22 and EM44) from a list of additional microsatellite loci (Carter et al. 2012) and two microsatellites (Girµ25 and Girµ26) from a list of putatively informative microsatellite loci, identified as described in (Fennessy et al. 2016). These loci were chosen because of their variability within different populations of giraffe (unpublished data). Details of the microsatellite loci are shown in Online Resource 1, Table S3. PCR amplifications of the microsatellites were performed in a 15 µl reaction volume using the VWR Taq Polymerase 2x Master Mix with 2.0 mM $MgCl_2$ and approximately 10 ng genomic DNA. PCR amplification conditions described in Carter et al. (2012) were applied using the annealing temperatures (T_A) listed in Table 1. Forward primers were fluorescently labeled using the Applied Biosystems Standard Dye Set DS-33 G5. DNA Fragment Analyses were performed on an ABI 3730 DNA Analyzer (Applied Biosystems) as per the manufacturer's recommendations using the GeneScan-600 LIZ® size standard. Fragment length were determined using the Microsatellite Plugin v.1.4.4 in GENEIOUS v.11.1.3 (Kearse et al. 2012). We conducted three independent PCR amplifications for each locus and sample to assess PCR errors and to obtain consensus genotypes.

Calculations of the observed (H_o) and expected heterozygosity (H_e), number of alleles (N_a) for each locus and tests for deviations from the Hardy–Weinberg equilibrium (HWE) with implemented Bonferroni correction were performed in CERVUS v.3.0.7 (Kalinowski et al. 2007). GENEPOP v.4.7.0 (Rousset 2008) was used to calculate the inbreeding coefficients (F_{is}). Tests for linkage disequilibrium (LD) were performed in ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010) and manually corrected using the Bonferroni correction (Rice 1989). As all samples were considered as a single population, we did not identify private alleles. We used the one-tailed Wilcoxon sign rank test (100,000 iterations) in BOTTLENECK v.1.2.02 (Piry et al. 1999) to detect heterozygote excess, assuming that all microsatellite loci fit the two-phase mutation model (TPM) (proportion of stepwise mutation model (SMM) = 30%, variance = 30.00).

Results

The mtDNA alignment resulted in a 1,560nt long concatenated sequence (cytb − 1,140nt, CR − 420nt). All of the 14 individuals have identical cytb and CR sequences and cluster together with the South African giraffe in the ML tree (Fig. 1b).

Not all of the seven nuclear loci could be sequenced for the individual NyP05, which was, therefore, removed from downstream analyses of the nuclear data. STRUCTURE analyses of the seven nuclear intron loci show two separate clusters at K = 2 and no further clustering at K = 3, or above (Fig. 1c). One cluster consists, unambiguously of assigned Masai giraffe,

Table 1 Microsatellite genetic diversity of Malawi's giraffe (n = 14)

Locus	T_A (°C)	N_a	H_o	H_e	Probability of HWE	F_{is}	References
Gca_01	62.5	1	0.000	0.000	–	–	a
Gca_16	62.5	2	0.286	0.254	0.551	−0.130	a
Gca_21	62.5	2	0.000	0.138	0.009	1.000	a
Gca_25	62.5	2	0.500	0.389	0.575	−0.300	a
EM2 (Gca_24)	56.0	2	0.000	0.476	0.001*	1.000	a
EM22	62.5	2	0.429	0.349	0.774	−0.238	a
EM44	62.5	3	0.571	0.442	0.590	−0.308	a
Gica9976	62.5	4	0.357	0.743	0.001*	0.529	b
Girµ25	56.0	2	0.143	0.349	0.090	0.600	c
Girµ26	56.0	1	0.000	0.000	–	–	c
Mean		2.100	0.229	0.314		0.28	

T_A annealing temperature, N_a number of alleles, H_o and H_e observed and expected heterozygosity, F_{is} inbreeding coefficient

References (a) Carter et al. (2012); (b) Crowhurst et al. (2013); (c) Fennessy et al. (2016). The * indicates significant deviation from the Hardy–Weinberg equilibrium after Bonferroni correction. The level of significance was set to α = 0.05 and α = 0.0036 after Bonferroni correction

while the other is formed by known southern giraffe individuals and the 13 giraffe from Malawi. Two of these (GH02 and NyP03) share one allele with Masai giraffe at a single locus (I21). PCA of the same dataset also identifies a single cluster, including all Malawi giraffe and southern giraffe, with no admixture of the individuals from Malawi (Fig. 1d).

All DNA isolates were amplified three times for each of the ten microsatellite loci. There was no evidence for allelic dropout or false alleles and a complete dataset without missing data was obtained. The number of alleles amplified in the ten loci ranged from 1 to 4 (Table 1). Two loci (Gca_1 and Girµ26) were monomorphic. Linkage disequilibrium was detected at four pairwise loci comparisons, but all comparisons were non-significant following Bonferroni correction. Significant deviations from HWE were detected in Gca_01 ($P = 0.001$), Gca_21 ($P = 0.009$) and Gica9976 ($P = 0.001$) (Table 1), however, following Bonferroni correction, the deviation from HWE in Gca_21 was found to be non-significant (Table 1). H_e values ranged from 0 to 0.743 in Gica9976, resulting in a mean H_e of 0.314 (Table 1), whilst the mean H_o was even lower (0.229) (Table 1). Four loci displayed a higher level of heterozygosity than expected by the number of alleles, indicated by negative F_{is} values. However, the mean inbreeding coefficient (mean F_{is}) of 0.28 indicates a high level of inbreeding (Table 1). Finally, the Wilcoxon sign rank test in BOTTLENECK detected significant heterozygote excess ($P = 0.037$) in Malawi's giraffe population.

Discussion

The first conservation population genetic study of giraffe in Malawi identified these, unambiguously, as South African giraffe (*G. g. giraffa*) with no detectable signal of introgression from other taxa. To date, the taxonomic affiliation of Malawi's giraffe was unclear because of a possible uncertain subspecies assignment of giraffe from the Zimbabwean source population of the 1993 translocation (Giraffe Conservation Foundation 2015). According to recent studies, South African or Angolan giraffe may be present in Zimbabwe (Winter et al. 2018a). An unverified report suggests that translocations of Masai giraffe from the South Luangwa National Park (Zambia) into Malawi in the early 2000's (Briggs 2013) may have resulted in a mixed population of southern and Masai giraffe. The single allele that two individuals of Malawi's giraffe share with the Masai giraffe is likely to be a signal of an ancient hybridization event, or a trans-species polymorphism (Klein et al. 1998).

The invariant mitochondrial sequences and diversity statistics of the nuclear DNA suggest a single, maternal origin and inbreeding among Malawi's giraffe. This is consistent with the documented history of translocating only five South African giraffe individuals into Malawi; these were likely to be the single founders of the current population. The microsatellite diversity of Malawi's giraffe is the lowest so far observed in the genus *Giraffa* (Brown et al. 2007; Brenneman et al. 2009; Carter et al. 2012; Crowhurst et al. 2013; Austin et al. 2018) and further supports the historic records. However, these results should be interpreted cautiously as previous studies used different sets of microsatellites. Nevertheless, the analysis of the microsatellite data shows a high level of inbreeding which even exceeds that expected for full-siblings mating (Frankham et al. 2010). In addition, the significant heterozygote excess may indicate a recent founder effect (Tarr et al. 1998).

Inbreeding depression results in the reduction of fertility and survival of inbred individuals (Hedrick and Kalinowski 2000; Amos and Balmford 2001) and can consequently drive small populations to extinction. The lower fitness of inbred individuals could possibly account for the frequent observations of giraffe with mange and ticks in Nyala Game Park (C. Hay, pers. obs.). Inbreeding is notoriously associated with higher susceptibility to infections and higher disease severity for wildlife, often resulting in a high mortality rate (Coltman et al. 1999; Fuchs 2014). Thus, the giraffe populations in Malawi would benefit from genetic rescue through additional translocations of new individuals to prevent further inbreeding depression and to cope with future environmental changes.

It is unclear if the historically natural populations of Malawi consisted of *Giraffa giraffa* or *G. tippelskirchi*. According to their current distribution, both giraffe species may have been present historically in the country, with Masai giraffe being geographically closer to Malawi. The reintroduction of the South African giraffe in 1993 defined the current composition of giraffe populations in Malawi, an area that was devoid of these animals at that time. Therefore, based on our findings, the source population for any future translocation should be the South African giraffe. Furthermore, the population at Nyala Game Park is currently overstocked given its small area (A. Salb pers. obs.). Therefore, moving a proportion of the population into the Majete Wildlife Reserve and augmenting both populations is necessary to prevent inbreeding depression and, consequently, to reduce the risk of extinction of giraffe in Malawi.

While recent ICUN Red List assignment has shown a positive trend for populations of the South African giraffe over recent years, creating new, (re-)introduced populations of South African giraffe into Malawi will play an important role in providing safety nets for giraffe in general. The (re-) introduction of giraffe also holds considerable conservation tourism value for Malawi, generating general awareness for giraffe conservation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Electronic Supplementary Material

Title:

Species assignment and conservation genetics of giraffe in the Republic of Malawi

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Table S1. Locus information and PCR conditions for the two mitochondrial and the seven nuclear intron loci.

Name (locus)	Primer sequences 5'-3'	PCR conditions	Source
Cytochrome b (mtDNA)	F: GAAAAACCATCGTTGTCG R: TGGGAGTATATTAATAGC	Standard PCR (Ta= 50 °C; 30 cycles)	Fennessy et al., 2016
Control region (mtDNA)	F: TACACTGGTCTTGTAAGC R: TCGCTTTGGTGTTTAAGC	Standard PCR (Ta= 50 °C; 30 cycles)	Fennessy et al., 2016
SW05 (RFC5)	F: GATCACTCTGGAACCTGCTCA R: CATACCTGTGGTTCTGCGGT	TD-PCR (Ta = 70-60 °C; 10 cycles), standard PCR (Ta = 60 °C; 30 cycles)	Winter et al., 2018
SW07 (USP33)	F: TGACGACCAGAGTGTCACTG R: TCTTTTTGTGCTTCTTCACTGCT	TD-PCR (Ta = 67-57 °C; 10 cycles), standard PCR (Ta = 57 °C; 30 cycles)	Winter et al., 2018
SW40 (IGBF2B1)	F: GGCAGCACATCAAACAGCTC R: GGGGTCCAGTGATGATGACC	See Intron SW05	Winter et al., 2018
SW43 (COL5A2)	F: AATGGCTGGAGGACATGGTC R: GCCGGAAGTTCCTGCAATTC	See Intron SW05	Winter et al., 2018
SW44 (CTAGE5)	F: CCCTCAAATCACAAGTAGCTGA R: TCTGGCTTTCCTGAAGTTGAGA	See Intron SW05	Winter et al., 2018
SW108 (C1orf74)	F: TCCAGTGTTGTTGCTGCTGA R: TCTGGGAGGACCTCGTTTCT	See Intron SW05	Winter et al., 2018
I21 (RASSF4)	F: CAGTGTCCATCACACAAC R: GCACCGGCATTTCAAACTTA	TD-PCR (Ta =65-55°C; 10 cycles), standard PCR (Ta =55°C; 30 cycles)	Fennessy et al., 2016

Note – F: forward primer. R: reverse primer. TD-PCR: touchdown PCR. Ta: primer annealing temperature. The locus is the gene name of the human ortholog.

Table S2. Location, abbreviation, number of individuals (n), (sub)species and source of analyzed giraffe and okapi sequences for mtDNA and nuclear analyses.
Individual sample ID´s see Fig. S1.

Location	Abbreviation	n (mtDNA)	n (nuclear DNA)	(Sub)species
Badingilo National Park, South Sudan	BaNP	2	–	*G. c. camelopardalis*
Basel Zoo, Switzerland	Okapi	1	–	*O. johnstoni*
Bwabwata National Park, Namibia	BNP	7	7	*G. g. giraffa*
Central Kalahari Game Reserve, Botswana	CKGR	7	4	*G. g. angolensis*
Chobe National Park, Botswana	CNP	11	3	*G. g. giraffa*
Etosha National Park, Namibia	ENP	17	14	*G. g. angolensis*
Gambella National Park, Ethiopia	ETH	3	–	*G. c. camelopardalis*
Game Heaven Lodge, Malawi	GH	2	2	*G. g. giraffa*
Garamba National Park, DR Congo	GNP	5	–	*G. c. antiquorum*
Ishqbini Conservancy, Kenya	ISC	4	–	*G. reticulata*
Khamab Kalahari Reserve, South Africa	KKR	6	7	*G. g. giraffa*
Koure, Niger	WA	18	–	*G. c. peralta*
Loisaba Wildlife Conservancy, Kenya	LWC	1	–	*G. reticulata*
Luangwa Valley National Park, Zambia	LVNP	11	12	*G. tippelskirchi*
Moremi Game Reserve, Botswana	MGR	16	5	*G. g. giraffa*
Mosi-oa-Tunya National Park, Zambia	MTNP	11	3	*G. g. giraffa*
Murchison Falls National Park, Uganda	MF	16	–	*G. c. camelopardalis*
Nürnberg Zoo, Germany	RET	5	–	*G. reticulata*
Nxai Pans, Botswana	NXP	1	–	*G. g. giraffa*
Nyala Game Park, Malawi	NyP	12	11	*G. g. giraffa*
Rotterdam Zoo, Netherlands	RETRot	3	–	*G. reticulata*
Selous Game Reserve, Tanzania	SGR	6	7	*G. tippelskirchi*
Shambe National Park, South Sudan	SNR	2	–	*G. c. antiquorum*
Sioma Ngwezi National Park, Zambia	SNNP	1	1	*G. g. giraffa*
Sun hotel, Livingstone, Zambia	SUN	4	4	*G. g. giraffa*
Vumbura Concession, Botswana	V	11	13	*G. g. giraffa*
Wilhelma Stuttgart, Germany	RETWil	2	–	*G. reticulata*
Zakouma National Park, Chad	ZNP	1	–	*G. c. antiquorum*

Additional mtDNA sequences from GenBank

Accession No.	(Sub)species	Source
EF442263 - EF442274	*Giraffa spp.*	Hassanin, Ropiquet, Gourmand, Chardonnet, & Rigoulet, 2007
EU088317 - EU088320	*Giraffa spp.*	Brown et al., 2007
EU088322 - EU088351	*Giraffa spp.*	Brown et al., 2007
AP003424	*G. g. angolensis*	Yasue et al. unpublished
JN632674	*O. johnstoni*	Hassanin et al., 2012
NC_012100	*G. g. angolensis*	Yasue et al. unpublished

Table S3. Locus information and PCR conditions for ten microsatellite loci.

Locus	Primer sequences 5'-3'	Repeat motif	PCR conditions	Accession No.	Source
Gca_01	F: GCATGCTACCAACACCTCTG R: ACCAATCGAAGGACTGTTGC	$(AC)_{13}$	Standard PCR (Ta= 62.5 °C; 35 cycles)	JQ973777	Carter et al., 2012
Gca_16	F: GCAACCTTCCCAGTTTCCAG R: AAGACCCTGAGAGTGAGCAC	$(AC)_{8}$	Standard PCR (Ta= 62.5 °C; 35 cycles)	JQ973781	Carter et al., 2012
Gca_21	F: GAGACACAGAACCAACAGGC R: TCATACTTTGAGCATCCCAGC	$(AGAT)_{11}$	Standard PCR (Ta= 62.5 °C; 35 cycles)	JQ973782	Carter et al., 2012
Gca_25	F: TGAAGTTGCCAGGGAGATCC R: AGAGTCCACTGAAGCTGGTG	$(AAT)_{10}$	Standard PCR (Ta= 62.5 °C; 35 cycles)	JQ973785	Carter et al., 2012
EM2 *	F: GCTTTGGGCCACTACCTCTT R: GAGGGCGATCTAAGCCAGTA	$(AC)_{17}$	Standard PCR (Ta= 56 °C; 35 cycles)		Carter et al., 2012 Supplementary material (GSIYPAC01D8O73)
EM22	F: CTGCATGAGCCTGAGGAGAT R: GCAAGCTGCTTCTTTGTTCTG	$(GT)_{10}$	Standard PCR (Ta= 62.5 °C; 35 cycles)		Carter et al., 2012 Supplementary material (cons_gr111_2)
EM44	F: AGGCATATCAACACTTTGGG R: AACCAACACAGACATGTGCAA	$(AT)_{11}$	Standard PCR (Ta= 62.5 °C; 35 cycles)		Carter et al., 2012 Supplementary material (GSIYPAC01EF5KV)
Gica9976	F: GGGAGGAGACTGGATTGTCA R: AGT GGCTCTCCAAAGCACAT	$(GT)_{18}$	Standard PCR (Ta= 62.5 °C; 35 cycles)	JX424293	Crowhurst et al., 2013
Girµ25	F: CTTCCAGCAGTGTAATAGTG R: CAAGTGACTTAATGATAAATGC	$(AAT)_{45}$	Standard PCR (Ta= 56 °C; 35 cycles)		Fennessy et al., 2016
Girµ26	F: GTCCACTTAGGGAAGATTG R: GAAGTCTCCTTTCTGTTTCTC	$(AAT)_{41}$	Standard PCR (Ta= 56 °C; 35 cycles)		Fennessy et al., 2016

Note – F: forward primer. R: reverse primer. Ta: primer annealing temperature. The locus is the gene name of the human ortholog. * same locus as Gca_24, but a different pair of primers.

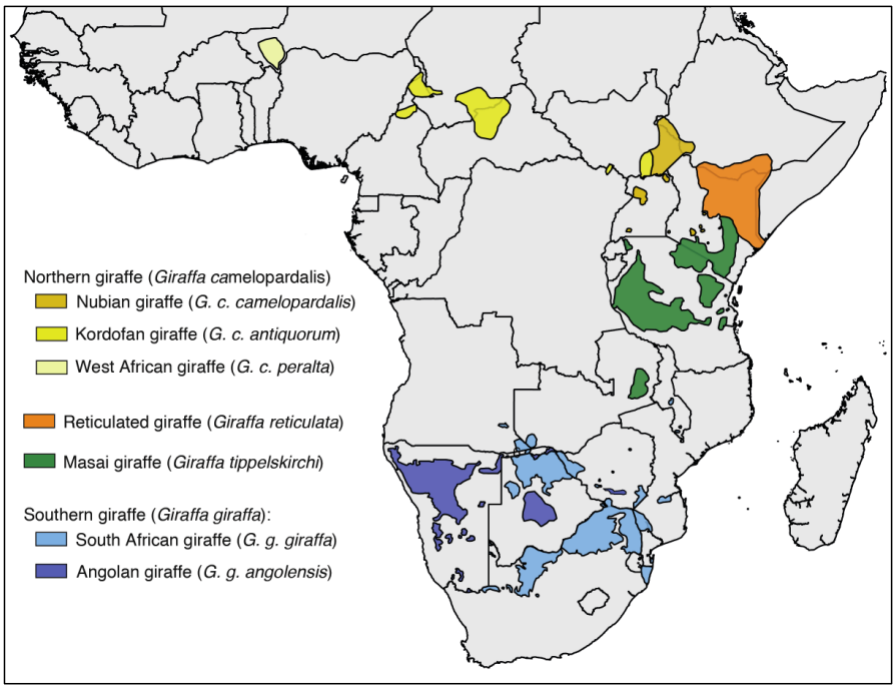

Fig. S1. Giraffe distributions
Geographic ranges (colored shadings) of giraffe plotted on a map of Sub-Saharan Africa (Amended from Winter et al. (2018)).


0.09
Okapi
South African giraffe (G. giraffa giraffe)
Masai giraffe (G. tippelskirchi)
Nubian giraffe (G. camelopardalis)
West African giraffe (G. c. peralta)
Kordofan giraffe (G. c. antiquorum)
Reticulated giraffe (G. reticulata)
Angolan giraffe (G. giraffa angolensis)

Fig. S2. Mitochondrial maximum-likelihood tree of 231 giraffe
Asterisks show major branches with a bootstrap support of ≥80%. Two different okapi individuals were used as an outgroup.

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APPENDIX

RESPONSE TO "HOW MANY SPECIES OF GIRAFFE ARE THERE?"

Julian Fennessy, **Sven Winter**, Friederike Reuss, Vikas Kumar, Maria A. Nilsson, Melita Vamberger, Uwe Fritz, Axel Janke

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separate species and is based upon a sample size of five individuals [1] out of a population estimated at 500–600 [2,5]. In summary, Fennessy *et al*. [1] present a new perspective on giraffe taxonomy, but the conclusions should not be accepted unconditionally.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found online at http://dx.doi.org/10.1016/j.cub.2016.12.039.

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Response to "How many species of giraffe are there?"

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It is not unexpected that a proposal, such as ours [1], of four new mammalian species stirs up controversy, as evident in the correspondence by Bercovitch *et al*. [2]. We appreciate that their concerns are unrelated to the quality of the genetic data, the methodological approach or analyses, but are focused on the interpretation. Thus, we provided an analysis of giraffe speciation based on genomic sequence data, and not just "another viewpoint on giraffe taxonomy" [2]. We maintain our perspective that there is not only one but four species of giraffe (Figure 1).

Bercovitch *et al*.'s [2] concerns focus on the concordance of results, interpretation of data in relation to previous findings, morphological data, and conservation issues. Implicit in their correspondence [2] is an unspecific critique about species delineation and genetics, the latter being an increasingly valuable and objective tool to study speciation. In our analyses, we randomly chose neutrally evolving autosomal loci for sequence variability. The analysis of neutral loci is a prerequisite for coalescent-based methods and allows a conservative approach for species delineation by other methods, because neutral loci require more time to become fixed than loci under selection. Compared to microsatellite data, DNA sequences allow estimating divergence times and, finally, autosomal loci are preferred over uniparental inherited loci (i.e. mitochondrial DNA, mtDNA) for species delineation [3]. The reason is that in species with non-dispersing females (philopatry), mtDNA can show local or regional subdivisions and ancient maternal lineages that may not be consistent with the nuclear gene pool [4]. The fact that mtDNA and multi-locus analyses do not agree in every topological aspect with the nuclear gene tree is therefore not unexpected. Still, there is concordance for at least four distinct groups of giraffe [1] fulfilling the concordance criterion for species delineation in integrative taxonomy. Notably, mtDNA analysis is also consistent with Thornicroft's giraffe nested within Masai giraffe [5]. Disagreement about the exact grouping can be attributed to limited resolution, limited sampling, misidentified individuals [5] or mitochondrial capture.

We agree with the hypothesis of Bercovitch *et al*. [2] that sexual selection on pelage pattern may contribute to giraffe speciation and add to species delineation. However, this is not fully supported by data, and pelage pattern is a poor estimator of species delineation. In giraffe, pelage pattern and ossicones were described as unreliable taxonomic characters, because of sexual differences and variations within populations [6]. Thus, other morphological measurements e.g. from skulls were suggested for taxonomic purposes [6] and further research will assess these morphological traits among and between the four species. Interestingly, three giraffe species reproduce at different times according to regional differences in rainfall [7], a factor that could act as a mechanism to isolate the giraffe species in that area.

Bercovitch *et al*. [2] imply that we suggest the findings of Brown *et al*. [8] were "based on faulty statistics". Yet, the previous Structure analyses [8] did not calculate additional statistics such as a ΔK, a measure for the fit of the data to the number of assumed clusters. This method was available then to avoid speculating on the number of clusters. The absence of admixture in lower cluster numbers [8], however, complements our findings [1]. Also, three other species delineating methods agreed with four giraffe species: PCA, BPP and Bayesian multi-locus analyses [1].

Furthermore, the claim of Bercovitch *et al*. [2] that we ignore absence of admixture and hybridization in captivity for taxonomy is based on an out-of-context quote. The corresponding paragraph describes that levels of admixture among the four giraffe clusters are very limited despite the ability to interbreed in captivity. There is strong differentiation between the four groups of giraffe into distinct units despite the lack of a reproductive barrier and being highly vagile animals, which

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Figure 1. The differences in coat pattern are consistent with four giraffe species.
From left to right: northern giraffe (*Giraffa camelopardalis*), reticulated giraffe (*G. reticulata*), Masai giraffe (*G. tippelskirchi*), southern giraffe (*G. giraffa*). Images: Masai giraffe: B. Dodson. Other three: J. Fennessy Giraffe Conservation Foundation.

strengthens our conclusions for four species [1]. Numerous species that are unambiguously regarded as distinct, such as polar and brown bears or tiger and lion, interbreed in captivity and even in the wild.

Conservation is predominantly undertaken at the species level and sadly often used as a political tool, e.g., the species debate over forest versus savanna elephants [9]. Concerning the species status of the Thornicroft's giraffe, Bercovitch *et al*. [2] ignore that there is little or no genetic difference between them and Masai giraffe [1]. Implying that sampling 1% of Thornicroft's giraffe population (5/500 individuals) is a limited approach [2], overlooks that Thornicroft's giraffe is genetically depauperate, and thus more individuals would not increase the resolution. It is interesting that Bercovitch *et al*. [2] quote [8] in favour of six giraffe species when [8] vaguely suggested a minimum of "six potential giraffe species". Suggesting that [10] had evidence for eight giraffe species Bercovitch *et al*. [2] refer to a non-reviewed book chapter that provides limited analyses and data partially obtained from unknown locations. Some of the proposed species [10] are at odds with the lack of genetic differentiation [1].

Many of the latest numbers of giraffe for the IUCN assessment are based on the data from GCF Country Profiles, which is thus a valid reference. A change in giraffe taxonomy will in the short term not change their conservation status, but the conservation efforts of the endangered species will benefit in the future. Recently, giraffe as a single species was uplisted to 'Vulnerable' on the IUCN Red List. Division into four giraffe species would likely propose to classify three of these species in higher categories of threat. Yet, with the population increase over the last three decades of both Rothschild's and West African giraffe, it is possible that these currently listed 'Endangered' taxa could be downlisted. However, whether or not four giraffe species are suitable management units is independent from their species status.

Our multi-locus analyses objectively demonstrated the presence of four distinct giraffe species with limited gene flow among them [1]. Four giraffe species also appear to be consistent with giraffe coat patterns (Figure 1) and other genetic analyses [8]. Our recent and subsequent studies will hopefully garner African and international interest to implement increased conservation measures for preserving these species for future generations. The first multi-locus analyses of giraffe [1] have brought the threat of giraffe to the attention of the public and create a basis for future taxonomy discussions and conservation efforts.

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