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Taxonomy / Taxinomie

Mitochondrial DNA variability in *Giraffa camelopardalis*: consequences for taxonomy, phylogeography and conservation of giraffes in West and central Africa

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Abstract

The giraffe (*Giraffa camelopardalis*) still survives in four countries of West and central Africa. The populations of Niger and Cameroon are generally assigned to the subspecies *peralta*, but those of Chad and the Central African Republic are taxonomically problematic, as they are referred to as either *peralta*, or *antiquorum*, or *congoensis*. In this study, a mitochondrial fragment of 1765 nucleotide sites, covering the complete cytochrome *b* gene, three transfer RNAs and a large part of the control region, was sequenced to assess the relationships between several populations of giraffe. The phylogenetic analyses performed on the 12 identified haplotypes indicate that northern giraffes constitute a natural group, distinct from that of southern giraffes. Surprisingly, the giraffes of Niger are found to be more closely related to the giraffes of East Africa (subspecies *rothschildi* and *reticulata*) than to those of central Africa. We conclude therefore that the subspecies *peralta* contains only the Niger giraffes, whereas the subspecies *antiquorum* includes all populations living in Cameroon, Chad, the Central African Republic, and southwestern Sudan. We suggest that the ancestor of the Nigerian giraffe dispersed from East to North Africa during the Quaternary period and thereafter migrated to its current Sahelian distribution in West Africa, in response to the development of the Sahara desert. This hypothesis implies that Lake Mega-Chad acted as a strong geographical barrier during the Holocene, preventing any contact between the subspecies *peralta* and *antiquorum*. Our study has direct implications for conservation management, as we show that no subspecies *peralta* is represented in any European zoos, only in Niger, with a small population of less than 200 individuals. ***To cite this article: A. Hassanin et al., C. R. Biologies ••• (••••).***

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Résumé

Variabilité de l'ADN mitochondrial chez *Giraffa camelopardalis* : conséquences pour la taxinomie, la phylogéographie et la conservation des girafes en Afrique de l'Ouest et centrale. La girafe (*Giraffa camelopardalis*) subsiste encore à l'état sauvage dans quatre pays d'Afrique de l'Ouest et centrale. Alors que les populations du Niger et du Cameroun sont généralement classées dans la sous-espèce *peralta*, le statut taxinomique des girafes du Tchad et de la République centrafricaine est problématique, puisque ces populations peuvent être rattachées à trois sous-espèces différentes (*peralta, antiquorum*, ou *congoensis*). Lors de cette étude, un fragment mitochondrial de 1765 nucléotides, couvrant la totalité du gène du cytochrome b, trois ARN de transfert et une grande partie de la région de contrôle, a été séquencé afin d'évaluer les relations de parenté entre plusieurs populations de girafes. Les analyses phylogénétiques réalisées sur les 12 haplotypes identifiés indiquent que les girafes du Nord forment un groupe naturel, distinct de celui des girafes du Sud. De façon surprenante, la girafe du Niger apparaît plus proche des girafes d'Afrique de l'Est (sous-espèces *rothschildi* et *reticulata*) que de celles d'Afrique centrale. Du point de vue taxinomique, nos analyses montrent donc que la sous-espèce *peralta* correspond uniquement à la girafe du Niger, alors que la sous-espèce *antiquorum* regroupe toutes les populations vivant au Cameroun, au Tchad, en République centrafricaine et dans le Sud-Ouest du Soudan. Du point de vue phylogéographique, nos résultats suggèrent une dispersion de l'ancêtre de la girafe *peralta* à partir de l'Afrique de l'Est vers l'Afrique du Nord au cours de l'ère Quaternaire, suivie d'une migration vers le sud, pour finalement occuper sa distribution actuelle en Afrique de l'Ouest, et cela en réponse à l'extension du désert du Sahara. Cette hypothèse implique que le lac Méga-Tchad fut une importante barrière géographique durant l'Holocène, qui a empêché tout contact entre les sous-espèces *peralta* et *antiquorum*. Notre étude a des conséquences directes pour la conservation des girafes, puisque nous montrons que la sous-espèce *peralta* n'est malheureusement pas représentée dans les parcs zoologiques européens, et qu'elle est uniquement trouvée au Niger, où subsiste une petite population de moins de 200 individus. ***Pour citer cet article : A. Hassanin et al., C. R. Biologies ••• (••••).***

Keywords: Giraffe; *Giraffa camelopardalis*; mtDNA; Taxonomy; Phylogeography; Conservation

Mots-clés : Girafe ; *Giraffa camelopardalis* ; ADNmt ; Taxinomie ; Phylogéographie ; Conservation

1. Introduction

The giraffe, *Giraffa camelopardalis* (Linnaeus, 1758), belongs to the family Giraffidae, which also contains the okapi, *Okapia johnstoni*, confined to the Ituri rainforests in the Northeast of the Democratic Republic of Congo. Although different in appearance and behaviour, giraffe and okapi share a number of common features, including a long neck, a long and dark-coloured tongue, bilobed lower canines, and skin-covered horns, called ossicones. The giraffe formerly roamed the majority of savannahs and open woodlands in Africa, particularly in areas with an abundance of *Acacia*, *Commiphora*, and *Terminalia* trees [1]. Within historic times, the giraffe disappeared from most countries in northern Africa, due to increasing aridity and expanding human pressures, including hunting and farming [2]. However, the species *G. camelopardalis* is currently listed as Lower Risk by the IUCN [3], because wild populations remain common locally in eastern and southern Africa.

The male and female giraffe both have a typical coat pattern, consisting of large, irregularly shaped, chestnut-brown to black patches separated from one another by a network of white or yellowish–white bands. Nine subspecies of giraffe have been recognized on the basis of distinctive regional differences in colour and pattern of the coat [2] (Fig. 1B): *angolensis* (Angolan giraffe), *antiquorum* (Kordofan giraffe), *camelopardalis* (Nubian giraffe), *giraffa* (Southern/South African giraffe), *peralta* (Nigerian/West African giraffe), *reticulata* (reticulated giraffe), *rothschildi* (Baringo/Rothschild's/Uganda giraffe), *thornicrofti* (Thornicroft's giraffe), and *tippelskirchi* (Masai giraffe). The criteria used for defining the various subspecies are however suspect; firstly, because important coat variations have been observed between individuals of the same population, and secondly, because there are areas of range overlap, resulting in hybridization commonly occurring between described subspecies, e.g., between *rothschildi* and *reticulata* in Laikipia in central Kenya [4]. As considerable uncertainty surrounds the validity and geographical limits of most of the subspecies described in Dagg and Foster [2], only six groups of populations have been defined by the IUCN [5] (Fig. 1A). Whereas the three subspecies (1) *reticulata*, (2) *thornicrofti*, and (3) *tippelskirchi* were accepted for the IUCN classification, three new groups were considered: (4) the western group, which includes *antiquorum* and *peralta*, (5) the Nubian/Rothschild group, which contains *camelopardalis* and *rothschildi*, and (6) the southern group, which is composed of *angolensis* and *giraffa*.

Populations of the western group have drastically decreased over the last decades due to habitat destruction and poaching. In West Africa, the giraffe was formerly

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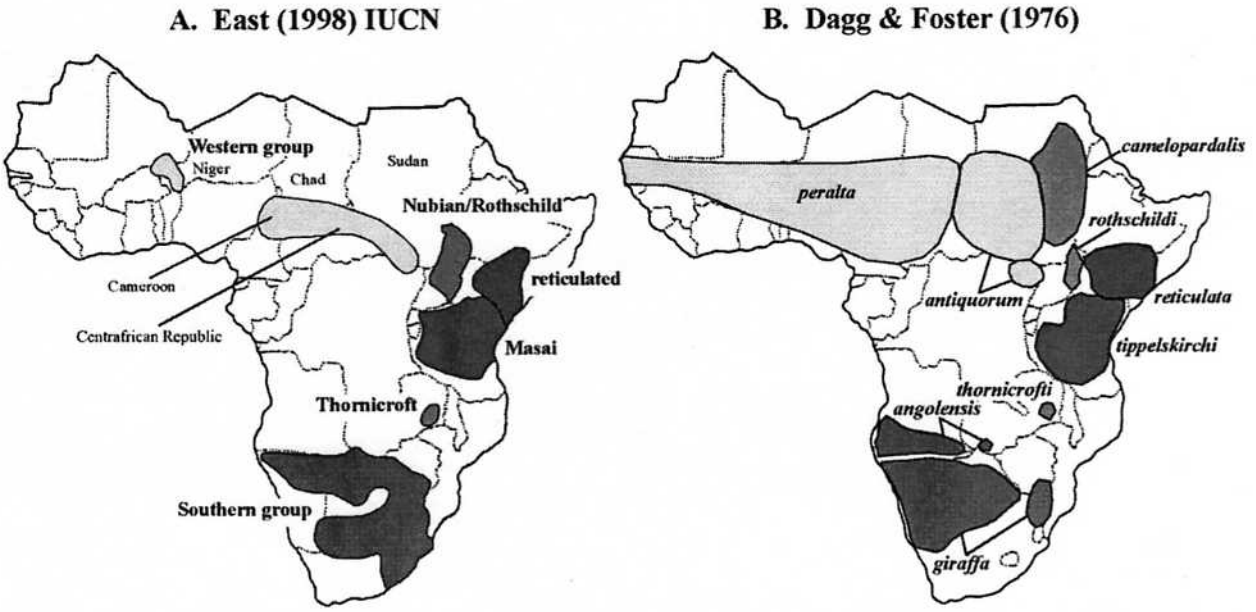

Fig. 1. Recent distribution of the giraffes at present (**A**, [5]) and within historic times (**B**, [2]). The colours indicate the six groups defined by the IUCN classification [5]. (For interpretation of the references to colour, the reader is referred to the web version of this article.)

found from Senegal to Lake Chad, but the only viable surviving population within this entire area is the small population of southwestern Niger (160–180 individuals, Omer Dovi, personal communication). The current attempt to protect Niger's remaining giraffes is a major international conservation priority [6]. The western giraffe survives in larger numbers in central Africa, but only the populations of northern Cameroon (Boubandjida, Benoue, and Waza National Parks) and Zakouma in Chad are reasonably secure [5]. Some urgent taxonomic research, based on a molecular approach, is therefore needed to clarify the status of these populations, because the absence of genetic information on the giraffe has led conservation agencies to employ management programmes based on poorly defined categories, which could reduce the long-term evolutionary potential of the species.

We report here the first molecular study dealing with the relationships among subspecies of *G. camelopardalis*. Our analyses are based on a mitochondrial DNA (mtDNA) fragment of 1765 nucleotides (nt), which covers the complete cytochrome *b* gene, three tRNA genes (Glu, Thr and Pro) and the 5′ region of the control region. The aims of this study were to (1) quantify the genetic variation between mitochondrial sequences of different populations of *G. camelopardalis*, (2) test the classifications proposed on the basis of coat variations, (3) determine the taxonomic status of the giraffes living in West and central Africa, and those held in captivity in European zoos, and (4) better understand the recent evolution of giraffes in Africa.

2. Material and methods

2.1. Samples used for DNA extraction

Our study includes 23 specimens of giraffe, representing all the nine subspecies of *G. camelopardalis* defined in Dagg and Foster [2], except the Thornicroft's giraffe (*thornicrofti*) from Zambia and the Nubian giraffe (*camelopardalis*) from eastern Sudan and western Eritrea (Table 1). The outgroup contains three species belonging to three different families of the suborder Ruminantia: *Bos grunniens* (Bovidae), *Muntiacus muntjak* (Cervidae), and *Okapia johnstoni* (Giraffidae), the closest living relative of *G. camelopardalis*.

Skin and blood samples were digested in CTAB (*hexade Cyl trimethylammonium bromide*) using the protocol detailed in Winnepenninckx et al. [7]. DNA was extracted from faecal samples using the method described in Porteous et al. [8]. DNA was purified in chloroform isoamyl alcohol and then precipitated with ethanol.

2.2. DNA amplification and sequencing

The mitochondrial DNA fragment selected for this study includes the 3′ end of the Glu-tRNA gene, the complete cytochrome *b* (*Cyb*) gene, the complete genes

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Table 1
Origin of the tissues used for DNA analyses

Classifications						
East (1998)	**Dagg and Foster (1976)**	**Name / N°**	**Sex**	**Origin** (*wild, °zoo)	**Collector**	**Tissue**
Western group	***G.c. peralta***	PER1	M	* Kouré Plateau, Niger	Bertrand Chardonnet	Faeces
		PER2	F	* Kouré Plateau, Niger	Bertrand Chardonnet	Faeces
		PER3	F	* Kouré Plateau, Niger	Bertrand Chardonnet	Faeces
		DOSSO	?	* Niamey-Dosso Road, Niger	Céline Houssin	Faeces
		BOUBA	?	* Boubandjida National Park, Cameroon	Bertrand Chardonnet	Faeces
		WAZA1	M	* Waza National Park, Cameroon	Bertrand Chardonnet	Skin
		WAZA2	?	* Waza National Park, Cameroon	Bertrand Chardonnet	Faeces
	G.c. peralta	Uma / ZA4124	F	° Vincennes Zoo, France	Alexis Lécu	Blood
		Rafiki / ZA4128	M	° Vincennes Zoo, France	Anne-Laure Gourmand	Faeces
	G.c. peralta x antiquorum	Valere / M9633B	M	° Vincennes Zoo, France	Anne-Laure Gourmand	Faeces
	G.c. antiquorum	ZAK1	M	* Zakouma National Park, Chad	Bertrand Chardonnet	Skin
		ZAK2	?	* Zakouma National Park, Chad	Bertrand Chardonnet	Skin
		Sarah / M9271	F	° Antwerp Zoo, Belgium	Francis Vercammen	Faeces
Reticulated	***G.c. reticulata***	Zénith / 4-3083	M	° Sigean African Reserve, France	Frédéric Tardy	Faeces
		Désirée	F	° Sigean African Reserve, France	Frédéric Tardy	Faeces
Nubian / Rotschild's	***G.c. rothschildi***	Eole / 1137	M	° La Palmyre Zoo, France	Thierry Petit	Faeces
		Robert / A4216	M	° Thoiry Zoological Park, France	Carine Alves	Faeces
Masai	***G.c. tippelskirchi***	Wari / 990681	F	° Basel Zoo, Switzerland	Friederike Von Holiwald	Faeces
		Saburi / 950677	M	° Basel Zoo, Switzerland	Friederike Von Holiwald	Faeces
Southern group	***G.c. angolensis***	Lisbon5A / 7382	M	° Lisbon Zoo, Portugal	Patricia Vilarinho	Faeces
		Lisbon6A / 6751	M	° Lisbon Zoo, Portugal	Patricia Vilarinho	Faeces
	G.c. giraffa	Tango / 97058	M	° Thoiry Zoological Park, France	Carine Alves	Faeces
		PHALA / GCG9	?	* Phalaborwa, Limpopo, South Africa	Hanneline Smit	Faeces
Okapi	***Okapia johnstoni***	Gunther / Z95145	M	° Vincennes Zoo, France	Geoffrey Fruleux	Faeces

for Thr- and Pro-tRNAs, and the 5′ part of the control region, also named D-loop in mammals. The sequences were obtained using several overlapping PCR amplifications. The exact matching between the overlapping portions of two different PCR fragments has been checked as a proof of authenticity of sequences. Most primers come from previous publications on the *Cyb* gene [9,10], but two new primers were specifically designed to amplify the D-loop region: 5′-CAT CGG ACA ACT AGC ATC TAT-3′ (direct, position 15222 in the sequence of *Muntiacus muntjak*, accession number NC_004563) and 5′-CCA GAT GTC TGA TAA AGT TCA-3′ (reverse, position 15882). Amplifications were done in 50 µl, using the following PCR standard conditions: buffer 10X with $MgCl_2$: 5 µl, dNTP: 5 µl (6.6 mM), Taq DNA polymerase (QBiogen, Illkirch, France): 0.3 µl (2.5 U), and primers: 2.5 µl at 10 µM. The standard PCR conditions used were: 94 °C for 4 min; 94 °C for 1 min, 50–58 °C for 1 min, 72 °C for 1 min (30 cycles). PCR products were sequenced by Genoscreen (Lille, France) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.3. Phylogenetic analyses

The DNA sequences were aligned by eye using SeAl v2.0a11 [11]. One ambiguous region, i.e., involving ambiguity for the position of the gaps, was excluded from the analyses to avoid erroneous hypotheses of primary homology: positions 15531–15603 in the sequence of *Muntiacus muntjak* (accession number NC_004563). The reduced alignment of mt sequences consists of 1689 nt. It is available upon request to AH.

Phylogenetic analyses were performed using Maximum Parsimony (MP), Maximum Likelihood (ML), Neighbour-Joining (NJ), and Bayesian methods. The MP analyses were conducted on PAUP 3.1.1 [12] with differential weighting of the character-state transformations using the product CI_{ex} S (CI_{ex}: consistency index excluding uninformative characters, S: slope of saturation) [9,13]. Bootstrap percentages (BP_{MP}) were computed after 1000 replicates using the closest stepwise addition option. ML and NJ analyses were carried out under PAUP 4.0b10 [14], and Bootstrap proportions (BP_{ML} and BP_{NJ}) were obtained after 1000 replicates. MrModeltest 2.2 [15] was used for choosing the model of DNA substitution that best fits the data. The selected likelihood model was the Hasegawa–Kishino–Yano model [16] with among-site substitution rate heterogeneity described by a gamma distribution (HKY + G). Bayesian posterior probabilities (PP) were calculated on MrBayes 3.1.2 [17] using five independent Markov chains run for 2 000 000 Metropolis-coupled MCMC generations, with tree sampling every 100 generations and a burn-in of 2000 trees.

3. Results

3.1. Mitochondrial DNA variation in Giraffa camelopardalis

A total of 12 mitochondrial haplotypes (mitotypes) was identified among the 23 samples. Sequences of these haplotypes have been deposited in the EMBL/GenBank/DDBJ database (accession numbers EF442263-EF442274). In Fig. 2, haplotypes are coded by geographical locality or zoological park with individual identification numbers. In general, giraffes of the same zoological park share the same haplotype (Basel, Sigean, and Vincennes). However, two mitotypes were found in the Lisbon zoo (Nos. 7382 and 6751), which differ by only one nucleotide (Fig. 2; transition C-T in the D-loop, position 1566 of the alignment).

Two haplotypes were found for the subspecies *antiquorum*: the first one diagnoses two wild giraffes of Zakouma (Chad, ZAK 1 and 2), and the second one characterizes the maternal lineage of the Antwerp zoo (specimens named Sarah and Valère; the latter is a hybrid produced from crossing a female *antiquorum* from the Antwerp zoo with a male *peralta* from the Vincennes zoo).

Three haplotypes were found for the subspecies *peralta*: the first one diagnoses all the wild giraffes of Niger (PER1-3, and DOSSO); the second one defines the three wild giraffes of northern Cameroon (BOUBA, WAZA1, and 2); the third one characterizes the giraffes of the Vincennes zoo (Uma and Rafiki).

The 12 haplotypes can be classified into three distinct lineages, i.e. northern giraffe, Angolan giraffe, and the southeastern group, composed of the subspecies *giraffa* and *tippelskirchi*, as their sequence divergences range from 3.1 to 4.4% for the total mitochondrial fragment (1765 nt), from 2.5 to 3.8% for *Cyb* (1140 nt), and from 3.9 to 6.9% for the 5′ region of the D-loop (464 nt). The northern group includes the populations previously included in the subspecies *peralta*, *antiquorum*, *rothschildi*, and *reticulata*.

3.2. Phylogenetic analyses

Out of 1689 total unambiguous characters kept for the phylogenetic analyses, 1239 were constant, 207 were variable but parsimony-uninformative and 243

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