Introduction

The phylogeographic pattern of most of the savanna mammals distributed across Africa can be partitioned into two to four main lineages. These lineages are typically associated with a West-Central, Eastern, Southern and/or South-East African distribution. For example, the hartebeest (Alcelaphus buselaphus) and the common warthog (Phacochoerus africanus) comprise three lineages associated with West-Central, Eastern and Southern Africa [1,2]. The wildebeest (Connochaetes taurinus), the topi (Damaliscus lunatus), the greater kudu (Tragelaphus strepsiceros), and the wild dog (Lycaon pictus) comprise at least two lineages associated with Eastern and Southern Africa [1,3–5]. A partitioning into two lineages between West-Central and East-Southern Africa is observed for the kob (Kobus kob), the African lion (Panthera leo), the roan antelope (Hippotragus equinus), the waterbuck (Kobus ellipsiprymnus), the savanna elephant (Loxodonta africana) and the bushbuck (Tragelaphus scriptus) [6–12]. Species that have a largely East to Southern (including South-West) African distribution often divide into two or three lineages, such as impala (Aepyceros melampus) which has a distinct South-West African lineage [13], or sable antelope (Hippotragus niger) which has two East African lineages, a South-West lineage and a Southern lineage [14–16]. The congruency of phylogeographic patterns among taxonomic groups and trophic levels is generally attributed to the existence of similar forces shaping the evolutionary history of species, with common African refugia [1,7,9,17] and speciation events being climatically medi-
Paleoclimatic reconstructions show that the African continent was marked by pronounced monsoonal changes during the Pleistocene (i.e., climatic oscillations), paced by earth’s orbital variations [18]. The vegetation changes associated with these climatic changes were probably the main driver of population expansions in savanna species during cool and dry phases, and population contraction during wet and warm phases [2]. The location of these refugia were purportedly in West, East, South and South-Western Africa [1,9,10,19,20].

Phylogeographic studies that accurately reflect the pattern of spatial genetic variation require access to large sample collections distributed across the entire range of the target species. Perhaps because of the difficulty in obtaining samples over such a large geographic area, as well as the inaccessibility of remote areas in Africa, it is rare to find studies involving more than 200 samples or which cover the entire distribution of the model species. To improve our understanding of African biogeography, we studied the spatial genetic structure of the African buffalo (*Syncerus caffer*) at a continental scale. The African buffalo is a good model because, together with a few other large taxa such as e.g., the elephant and the giraffe, it is distributed throughout most of sub-Saharan Africa. The species is primarily found in savannas, but also occurs in other habitats, including clearings in the rainforest belt [21–23]. The African buffalo exhibits extreme morphological variability across its range, greater than most other African mammals, both in body size and weight, coloration and horn size [21]. As a result, it has previously been assigned to as many as 52 distinct subspecies divided across two species [21,24]. This number has decreased considerably as our understanding of the species has increased. Today, two [25], three [21] or four [26,27] subspecies are commonly recognized (Table 1). The forest buffalo (*S. c. nanus*), which occurs in the West and Central African rainforests, shows adaptation to forest life [22] having a smaller size, unobtrusive swept-back horns and a characteristic red to reddish brown color. The Cape buffalo (*S. c. caffer*) of the South-East African savannas is about twice the size of the forest buffalo, with large downward curved horns and a brownish to black color. The third and fourth subspecies—not recognized by all authorities—are the West African savanna buffalo (*S. c. brachyceros*) and the Central African savanna buffalo (*S. c. aequinoctialis*), which are more difficult to distinguish morphologically. They occupy the Sahelian savanna, characterized by a low net above ground primary production compared to the East and Southern African savannas (±15% lower) [28]. The two latter subspecies seem to be morphologically intermediate between the Cape and forest buffalo [29]. The evolutionary relationships between these four putative subspecies are unclear, having therefore led to the taxonomic controversy. This uncertainty is corroborated by the observation of intermediate phenotypes in contact zones between all four subspecies [21] and of at least one reported cross between *S. c. caffer* and *S. c. nanus* in captivity, these being the two subspecies with the more divergent morphological characteristics [30]. Moreover, differences in karyotypes between the nanus and the caffer phenotypes have been reported, with *S. c. caffer* having 52 chromosomes and *S. c. nanus* having 2n = 54 to 56 chromosomes [30–32]. The extreme morphological variability within the species therefore allows us to test the congruence between phylogenetic-based and morphological-based classification.

Although several genetic studies on African buffalo have already been conducted, these were generally focused at smaller spatial scales, mainly in East and Southern Africa (*S. c. caffer*), using various molecular markers such as mitochondrial DNA, autosomal microsatellites, Y-chromosomal microsatellites and the histocompatibility complex (MHC) *DRB3* gene [33–44]. Partial exceptions include the studies by Van Hooft [36,37], which include a few samples (n = 14) from Central Africa. They highlighted a clear differentiation between *S. c. caffer* and *S. c. nanus*/*S. c. brachyceros*. No significant genetic differentiation was found between *S. c. nanus* and *S. c. brachyceros* [37], but these results were not conclusive due to a limited sample size. A truly pan-African genetic study of the African buffalo has not been undertaken yet.

In the present study, a detailed genetic analysis of the African buffalo across its geographic range was performed using mitochondrial *D-loop* (control region) sequences. The control region is the most variable part of the mammalian mtDNA genome [45]. This study provides an unprecedented sampling scheme for buffalo, representing 43 study localities in 17 countries. We combined newly derived sequences from faeces and tissue biopsies from South, West and Central Africa with published sequences from East and Southern Africa. A total of 255 West-Central and 511 East-Southern buffalo mtDNA sequences were analyzed. Sequence data were analyzed with regard to demographic changes, phylogeography and evolutionary history. Specifically, we aimed to I) test whether there is a correspondence between the distinct morphological phenotypes and the genetic lineages, and II) infer the phylogeographic history that has led to the observed distribution of genetic and phenotypic variation.

**Results**

As the taxonomic status of the subspecies is still subject to controversy (see above), we defined each of the four putative subspecies as recognized by East [27] and Kingdon [26] and adopted by the IUCN (2004) as an ecophenotype, i.e., *nanus* ecophenotype (forest-dwelling buffalo from West Africa), *brachyceros* ecophenotype (savanna buffalo from West Africa), *aequinoctialis* ecophenotype (savanna buffalo from Central Africa) and *caffer* ecophenotype (Cape buffalo from East and Southern Africa).

**Population Differentiation**

1. **Network analysis and genetic distance.** Two lineages that made geographic sense were retrieved with the minimum spanning network (H1 and H2; Figure 1), following a South-Eastern/West-Central separation, with eight mutational steps separating the two lineages. Within these lineages, no finer geographic structure was evident, with samples from the various localities being paraplythetic. The first lineage (H1) is composed of South-Eastern (SE) African populations and contains 143 haplotypes, mainly of the *S. c. caffer* ecophenotype (136 haplotypes). This predominantly South-Eastern lineage also includes seven haplotypes of West-Central (WC) African origin. With the exception of the Namibian samples, haplotypes from Southern Africa (Zimbabwe, Botswana and South Africa) occupied the tip position in the network. About 45% of the Ugandan haplotypes occupied a central position in the network. The second geographic lineage (H2) consisted mainly of West-Central (WC) African samples, including samples from three ecophenotypes: *S. c. brachyceros*, *S. c. nanus* and *S. c. aequinoctialis*. These ecophenotypes were not monophyletic and were distributed throughout the H2 lineage. H2 included 94 distinct haplotypes, including seven of SE African origin.

The minimum spanning network is characterized by a clear star-like topology for the H1 lineage, and there is a tendency toward the same pattern for the H2 lineage. Distances separating SE African haplotypes were less than among the WC African haplotypes, reflecting the relatively low nucleotide diversity observed in SE Africa. Within each lineage, there was a low structural per country affinity of haplotypes.
2. Genetic variation between populations and between subspecies. At the continental scale, African buffalo display high levels of genetic diversity, as reflected by both haplotype and nucleotide diversities (h = 0.98, π = 5.50%; Table 2). As suggested by our previous results, a partition within two lineages at the continental scale has been revealed, with a high and significant proportion of the variation accounted for by the between-lineage component (F_{CT} = 0.42). At the population level, low overall sequence divergence among the populations within each lineage was observed, ranging from 0.1% to 1.3% in H1 and from 0.1% to

<table>
<thead>
<tr>
<th>Weight (kg)</th>
<th>S. c. caffer</th>
<th>S. c. nanus</th>
<th>S. c. aequinoctialis</th>
<th>S. c. brachyceros</th>
</tr>
</thead>
<tbody>
<tr>
<td>700–900 [26,74]</td>
<td>&lt;320 [26,74]</td>
<td>intermediate</td>
<td>intermediate</td>
<td></td>
</tr>
<tr>
<td>Dress</td>
<td>blackish [78]</td>
<td>reddish [74,76]</td>
<td>brown/black [75]</td>
<td>brown/black [75]</td>
</tr>
<tr>
<td>Body length (cm)</td>
<td>&lt;340 [26,74]</td>
<td>&lt;120 [26,74]</td>
<td>intermediate</td>
<td>intermediate</td>
</tr>
<tr>
<td>Length of skull (mm)</td>
<td>503±17.9 [46]</td>
<td>426±10.7 [46]</td>
<td>500±18.2 [46]</td>
<td>455±22.9 [46]</td>
</tr>
</tbody>
</table>

Table 1. Morphological characteristics, including weight in kilogram (kg), dress color, body length in centimetre (cm), width of horn and length of skull in millimetre (mm) of the four recognized subspecies of African buffalos (out of literature).

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Figure 1. An unrooted minimum spanning tree (MST) of Syncerus sp. showing genetic relationship among D-Loop region haplotypes detected in this study. The sizes of circles are proportional to haplotype frequency and length of lines is proportional to the number of nucleotide substitutions separating the haplotypes, except for the dotted lines, where the numbers of mutational steps joining the circles are indicated above the connecting branches. Circles were colored according to geographical sample origin: Green/Red/Blue: H1 South and East Africa; Turquoise/Pink: H2 West Africa; Yellow: H2 Central Africa; Black: Namibia. Asterisks represent nanus ecophenotype samples. CAR: Central African Republic; DRC: Democratic Republic of the Congo.
doi:10.1371/journal.pone.0056235.g001
Table 2. Genetic diversity and demographic test for both genetic lineages (H1 and H2), as well as for each ecophenotypes.

<table>
<thead>
<tr>
<th></th>
<th>h</th>
<th>h SD</th>
<th>h p-value</th>
<th>ρ</th>
<th>ρ SD</th>
<th>ρ p-value</th>
<th>Fu’s Fs</th>
<th>Fu’s Fs p-value</th>
<th>τ</th>
<th>τ p-value</th>
<th>SSD</th>
<th>SSD p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 (i.e., Ecophenotype caffer)</td>
<td>0.980</td>
<td>0.002</td>
<td>0.001</td>
<td>4.58</td>
<td>0.001</td>
<td>0.001</td>
<td>−24.016</td>
<td>0.003</td>
<td>6</td>
<td>0.003</td>
<td>0.850</td>
<td>0.001</td>
</tr>
<tr>
<td>H2 (i.e., three other ecophenotypes)</td>
<td>0.981</td>
<td>0.002</td>
<td>0.001</td>
<td>6.42</td>
<td>0.002</td>
<td>0.001</td>
<td>−23.902</td>
<td>0.001</td>
<td>13</td>
<td>0.005</td>
<td>0.170</td>
<td>0.002</td>
</tr>
<tr>
<td>Ecophenotype aequinoctialis</td>
<td>0.997</td>
<td>0.010</td>
<td>0.001</td>
<td>6.95</td>
<td>0.004</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecophenotype nanus</td>
<td>0.890</td>
<td>0.001</td>
<td>0.001</td>
<td>5.77</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ecophenotype brachyceros</td>
<td>0.961</td>
<td>0.007</td>
<td>0.001</td>
<td>5.78</td>
<td>0.002</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

h: haplotype diversity; ρ: nucleotide diversity expressed in percentage; SD: standard deviation; ρ: expansion times; r: Harpending’s raggedness index; SSD: sum of squared deviations using ARLEQUIN and DnaSP software.

doi:10.1371/journal.pone.0056235.t002

Table 3. Population pairwise $F_{ST}$ between the twenty-eight populations significantly differentiated calculated with ARLEQUIN software.

|       | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    | 15    | 16    | 17    | 18    | 19    | 20    | 21    | 22    | 23    | 24    | 25    | 26    | 27    | 28    |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| h     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| h SD  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| h p-value |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| ρ     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| ρ SD  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| ρ p-value |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

The significance level is shown above the diagonal, *indicates P<0.05, **indicates P<0.005, ***indicates P<0.0005; ns, non-significant. From line 1 to 11: H2 lineage (S. c. nanus, S. c. brachyceros and S. c. aequinoctialis); from line 12 to 28: H1 lineage (S. c. caffer) bold/italic written value: ecophenotype.
2.9% in H2. According to the pairwise $F_{ST}$ values (Table 3), lower $F_{ST}$ values between populations within each lineage (SE is WC) can be observed, compared to higher $F_{ST}$ values between populations between each of the lineages. Fixation indexes between populations between lineages ($F_{ST}$) were estimated to be 0.52, and among populations within lineages ($F_{SC}$) to be 0.17 (Table 4). This corroborates the absence of structure observed within lineages in the network and evolutionary trees. Moreover, pairwise $F_{ST}$ values between the *brachyceros/aequinoctialis* ecophenotypes and the *nanus* ecophenotype of Western Africa were quite low, ranging from 0.02 to 0.12, which is lower than between most population pairs within these ecophenotypes (Table 3 and 5).

Nucleotide diversity is lower for H1 (4.6%) than for H2 (6.4%), except for the Campo-Ma’an population (Table 6 and 2). The highest nucleotide diversity was observed in the *auquinoctialis* ecophenotype populations (7%), being on average 1.4 times higher than in the *caffer* ecophenotype populations. The largest distances, up to 2.9% (higher $F_{ST} = 0.47$ (Table 5)), were found among forest buffalo populations (*S. c. nanus*). The *nanus* ecophenotype also displays the lowest haplotype diversity, being on average 1.3 times lower than in the other populations.

**Demographic Trends**

The mismatch distribution analysis showed a predominantly unimodal pattern for both lineages (Figure 2). In addition, Fu’s $F_{S}$ values as well as the raggedness index were significantly negative (Table 2) ($H1$: $p = 0.007$; $H2$: $p = 0.001$) which suggests a demographic expansion for both lineages. According to the $\tau$ values ($\tau_{H1} = 6$; $\tau_{H2} = 13$), demographic expansion for H1 was estimated as starting approximately 48 000 YBP (Year Before Present), and H2 approximately 104 000 YBP.

**Coalescence Based Analyses**

The phylogenetic analyses performed with the program BEAST confirmed the clear separation into two distinct geographical lineages, namely H1 and H2, as revealed by the minimum spanning network.

For a better visual clarity of the BEAST results, the global tree is drawn concerning the taxonomic status of these subspecies before using complementary nuclear markers. According to these genetic results, *S. c. nanus* would include *S. c. brachyceros* and *S. c. aequinoctialis* subspecies following standard nomenclature rules (*S. c. nanus* Boddaert 1785, *S. c. brachyceros* Gray 1837 and *S. c. aequinoctialis* Blyth 1866). This is further substantiated by the low amount of genetic differentiation between *S. c. brachyceros*, *S. c. aequinoctialis* and *S. c. nanus*. Although inferences based on DNA data are somewhat hampered because of the time required for complete lineage sorting to occur, our data unequivocally show that the separation between *S. c. brachyceros*, *S. c. nanus* and *S. c. aequinoctialis* is not taxonomically equivalent to the split between all of these and *S. c. caffer*. We note that these are preliminary genetic results as they are based exclusively on single-locus mtDNA. No final conclusion can be drawn concerning the taxonomic status of these subspecies before using complementary nuclear markers.

**Discussion**

**Continental Phylogeographic Structure and Taxonomic Status of the Buffalo**

Our study, based on the mtDNA genetic structure points to a genetic discontinuity between the WC (mainly lineage H2) and the SE (mainly lineage H1) populations ($F_{ST} = 0.42$). Van Hooft [37] found similar results on the basis of mitochondrial and Y-chromosomal markers, with a smaller sampling from WC Africa (14 samples). These lineages or haplogroups are not equivalent to populations, but the clear separation between them suggests that they are the results of a significant divergence and hence represent Management Units (or MUs; Moritz 1994). The lineages correspond to the two subspecies distinguished by all authorities: *S. c. caffer* for the SE populations, and *S. c. nanus* for the WC populations. According to these genetic results, *S. c. nanus* would include *S. c. brachyceros* and *S. c. aequinoctialis* subspecies following standard nomenclature rules (*S. c. nanus* Boddaert 1785, *S. c. brachyceros* Gray 1837 and *S. c. aequinoctialis* Blyth 1866). This is further substantiated by the low amount of genetic differentiation between *S. c. brachyceros*, *S. c. aequinoctialis* and *S. c. nanus*. Although inferences based on DNA data are somewhat hampered because of the time required for complete lineage sorting to occur, our data unequivocally show that the separation between *S. c. brachyceros*, *S. c. nanus* and *S. c. aequinoctialis* is not taxonomically equivalent to the split between all of these and *S. c. caffer*. We note that these are preliminary genetic results as they are based exclusively on single-locus mtDNA. No final conclusion can be drawn concerning the taxonomic status of these subspecies before using complementary nuclear markers.
The absence of genetic structure within H2 based on the analysis of the mitochondrial DNA - contrasting with extreme phenotypic variability - also suggests that buffalo may rapidly adapt (in evolutionary terms) to different ecological conditions, with ecophenotypes not being reproductively isolated. Finer scale resolution of the connectivity between the three ecophenotypes of WC Africa should be investigated using more sensitive molecular markers as microsatellites or single nucleotide polymorphism. Besides, based on our results, the fourth species described in Groves and Grubb [46], Syncerus mathewsi, a population restricted to the forested mountainous area of Virunga volcanoes, would correspond to an intermediate between the nanus and the caffer ecophenotypes (contact region close to the Rift Valley), with morphological intermediate characteristics, as also proposed by Grubb [47] himself. Further investigations using neutral nuclear markers within the contact region would permit clarification of this last assumption.

Although the morphology, behavior and respective habitats of S. c. caffer and S. c. nanus are very different, the amount of genetic differentiation is typical of that of subspecies, especially when compared to the range of \( F_{ST} \) values observed for other large African bovids (Table 7). The most parsimonious explanation for this pattern is that an ancient allopatric separation between an African bovid (Table 7). The most parsimonious explanation for this pattern is that an ancient allopatric separation between an Eastern and a Western population formed the two observed lineages. This is further supported by earlier findings based on Y-chromosomal microsatellite loci, which indicated that while S. c. caffer was monomorphic for a single allele, the same allele was not present in S. c. nanus [37]. Nevertheless, gene flow between those two lineages, even if low, was indicated by a few haplotype infidelities in the network and the phylogenetic analyses. This gene flow was estimated to be in the order of five mitochondrial genomes per generation since these lineages diverged. The difference in chromosome number between the nanus and the caffer ecophenotypes, due to a Robertsonian fusion of two acrocentric pairs (S. c. caffer: 52 and nanus ecophenotype: 54–56 chromosomes) [30–32], may have also contributed to a low gene flow by decreasing hybrid fertility [48] and may be a consequence of their divergence in allopatry. Negative effects of the variation of chromosome number on fertility have been observed in mice, cattle and humans [49,50].

The phylogeographic study of the African Buffalo

The separation into two lineages, distributed in WC and SE Africa respectively, is a pattern observed in many other savanna African mammal species, as for example within the roan antelope (Hippotragus equinus) or the bushbuck (Tragelaphus scriptus) [9,12]. Convergent phylogeographic patterns could indicate the existence of common African refugia for various African savanna species during climatic oscillations [18], proposed to be located in West, East, Southern and South-West Africa [51]. Buffalo populations in Uganda and in the Central African Republic displayed the highest genetic diversity found in the species and were the most likely candidates for the tree root location (Figure 3), suggesting that these are good candidate areas for historic refugia within this species. As the two lineages are found around this region, it may also be considered as a hybrid or overlapping zone between populations of the two lineages, explaining the higher genetic diversity observed. The same phylogeographical pattern is observed within the kob (Kobus kob- based on nuclear DNA and mtDNA) with an overlapping region located around Northern Uganda [7]. It was proposed based on divergent phenotype and life-history adaptations of the kob subspecies that populations went isolated within refugia in West and East Africa during the Pleistocene, with subsequent dispersal leading to secondary contact and hybridization between lineages around the present-day Uganda. Similar refuge location were also proposed for the hartebeest (Alcelaphus buselaphus- based on mtDNA), the topi (Damaliscus lunatus- based on mtDNA) and the roan antelopes (Hippotragus equinus- based on both nuclear DNA and mtDNA) [1,6,7,9]. Based on the concordant pattern of intraspecific structure in African mammals, we propose that the two lineages division observed in different savanna species has arisen as a consequence of Pleistocene climate oscillations in the absence of an obvious present-day geographical barrier. More arid conditions observed during glacial periods would have promoted isolation of populations in refugia, with expansion during interglacial wet periods [8], with secondary contact and hybridization between lineages when overlapping occurred. We thus propose that the African buffalo survived unfavorable periods during the Pleistocene in at least one refuge located in Western and one refuge located in Eastern Africa, with overlapping around the present-day Uganda.

Even if the buffalo would show strong philopatric behavior [21,52,53], its high nucleotide diversity and low differentiation between populations suggest a capability to disperse widely over evolutionary time scales. The combination of a relatively large effective population size and the possible ancestral nature of the H2 lineage as discussed hereafter may explain its higher nucleotide diversity. Furthermore, it is interesting to note that the overall nucleotide diversity reaches 6%, which, except for the Grant’s gazelle and the bushbuck, is high in comparison to other large African mammals, even if buffalo populations are reported to have declined in numbers due to outbreak of rinderpest during the last century (elephant (Loxodonta africana), 1.4%; bushbuck (Tragelaphus scriptus), 11.7%; roan antelope (Hippotragus equinus), 1.6%; waterbuck (Kobus ellipsiprymnus), 4.1%; impala (Aepyceros melampus), 2.8%; Grant’s gazelle (Nanger granti), 10.9%) [9,12,54–57]. Same high level of genetic variation was found in previous studies investigating the impact of such severe population bottlenecks on the genetic variability of buffalo populations, indicating that rinderpest pandemic had very little impact on the genetic diversity of the African buffalo in terms of allelic or haplotype diversity [35,43,55]. The nanus ecophenotype populations are in comparison relatively small and isolated, as indicated by low haplotype diversities and high between-population genetic distances. Pairwise \( F_{ST} \) values between the nanus ecophenotype populations were about three times as large as those between populations within the other ecophenotypes (see bold/italic written \( F_{ST} \) value in Table 3),

| Table 5. Pairwise \( F_{ST} \) computation between ecophenotypes. |
|-------------------|-------------------|-------------------|
| Ecophenotype | nanus | brachyceros | aequinoctialis |
| Ecophenotype brachyceros | 0.12 | | |
| Ecophenotype aequinoctialis | 0.02 | 0.08 | |
| Ecophenotype caffer | 0.51 | 0.45 | 0.44 |

doi:10.1371/journal.pone.0056235.t005

Phylogeographic study of the African Buffalo
Table 6. Sample table summarizing their origin, number per park, subspecies affiliation based on morphological data, number of haplotypes, and haplotype and nucleotide diversities with their associated standard deviation estimated with ARLEQUIN and DNaSP software.

<table>
<thead>
<tr>
<th>Country</th>
<th>To Nind</th>
<th>Locality within each country</th>
<th>spp</th>
<th>Local Nind</th>
<th>Local Nhap</th>
<th>h</th>
<th>SD</th>
<th>π (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sierra Leone</td>
<td>1</td>
<td>Gola Forest A</td>
<td>1</td>
<td>1</td>
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<td></td>
<td></td>
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<tr>
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<td>Mole B</td>
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<td>13</td>
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<td>0.037</td>
<td>5.42</td>
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<tr>
<td></td>
<td></td>
<td>Kpetus A</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>5</td>
<td>Arly, Pama, Singou B</td>
<td>5</td>
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<td>19</td>
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<td>Mount Elgon D</td>
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<tr>
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<td>Tsavo D</td>
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<td>Hwange D</td>
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<td>Chobe D</td>
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<td>4.53</td>
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<td>41</td>
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<td>Mother from Okahandja E</td>
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<td>1</td>
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<td>AVERAGE</td>
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<td></td>
<td>0.871</td>
<td>0.051</td>
<td>4.525</td>
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</table>

The nineteen samples excluded from the analysis are not quoted in this table (A S. c. nanus; B S. c. brachyceros; C S. c. aequinoctialis; D S. c. caffer; E Unknown affiliation; spp: subspecies; h: Haplotype diversity; π: nucleotide diversity expressed in percentage; CAR: Central African Republic; DRC: Democratic Republic of the Congo). The samples from the Benoue National Park and the Ngoto forest were morphologically intermediate between the S. c. nanus and the S. c. brachyceros/S. c. aequinoctialis subspecies respectively, indicated by the both capital letter.

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indicating that forest buffalo populations show signs of fragmentation and of genetic drift, reflecting the importance of rainforest as a biogeographical barrier to gene flow. Observations of forest-dwelling buffalo (*nanus* ecophenotype) confirm our assumption [21,58,59]. These studies showed a high correlation between forest buffalo presence and clearings, necessary for feeding and for water accessibility [23]. This dependence on open places has been identified as a limiting factor in movement of individuals. Our results support the fact that rainforest acts as a major biogeographic barrier limiting gene flow.

**Evolutionary History and Demographic Trends**

According to the IM analysis, although this result was not significant, the split parameter suggests that the *S. c. caffer* subspecies originates from the isolation of a subpopulation of the ancestral population. Fossil records from the late Pleistocene are in agreement with this. Indeed, the close resemblance of the Pleistocene-dated fossils to the actual West-Central African buffalo suggests that the H2 population existed before the H1 population [52,60]. Our results therefore suggest a recent origin of the Cape buffalo (*S. c. caffer*), which possibly derived from a stock of savanna buffalo originating from West-Central Africa (H2 lineage) [52].

The coalescent approach with migration suggested a Middle to Late Pleistocene divergence (150–300 kyr), which is contemporaneous with previous studies of other African species [1,6]. It should be noted that we are restricted to making inferences going back to the MRGA (the “mitochondrial Eve”) of the buffalo mtDNA, which is most likely much younger than the species itself, unfolding only part of the buffalo history.

We also found evidence under the IM model of an important expansion in both lineages since their Pleistocene divergence.
A

H1

Uganda
Eastern Africa (Kenya/Tanzania)
Southern Africa (Botswana, Zimbabwe, South Africa)
Central Africa/Sahel (CAR, Chad, DRC)
Western Africa/Sahel (Sierra Leone, Burkina Faso, Ghana, Benin, Niger)
Western Central Africa (Cameroon, Gabon)
Southwestern Africa (Namibia)

* narus ecophenotype

30,000 years

B

H2

30,000 years
which is supported by the star-like topology of the haplotype network of the H1 lineage and the negative Fu’s $F_{S}$ index for both lineages. This is in agreement with two earlier studies based on S. c. caffer, using an alternative coalescent approach with Bayesian skyline plots [61,62]. The expansion time, calculated based on the $\tau$ values, was estimated as starting at approximately 48 000 YBP for the H1 lineage, consistent with a previous study on the Cape buffalo by Van Hooft [37]. Expansion time for the H2 lineage was estimated at approximately 104 000 YBP. The more recent expansion of the H1 compared to the H2 lineage could be related to the development of open grassland on the East-Southern part of the continent at the end of Pleistocene. Paleoclimatic indications support our assumption. Indeed, Eastern and Southern Africa experienced an extremely arid period between 135 and 90 kyr, far more severe than the conditions occurring during the Last Glacial Maximum (35–15 kyr). Aridity decreased progressively after 95 kyr, until it reached near modern conditions around 60 kyr [63]. This period approximately coincides with the expansion signal for the H1 lineage. The development of large savanna-type grasslands could have allowed southward colonization by providing suitable habitat for the buffalo populations [64,65]. Van Hooft [37] also proposed that this expansion could have followed the
extinction of a buffalo-like species, the giant long-horned buffalo (*Peloveris antiquus*), which dominated the African savanna until the late Pleistocene, as demonstrated by fossil data [52,66,67], a hypothesis also in agreement with our results.

Nevertheless, recent radiometric studies on fossil records support the presence of buffalo in Southern Africa around 542 kyr (95% CI: 435–682 kyr) [68], which indicates that some *Syncerus*-like species occupied Southern Africa earlier than we could infer based on mtDNA. Our signal of expansion toward Southern Africa could thus be a signal of re-colonization, which supports the hypothesis of the importance of the refugia located in Eastern and Western Africa. Southern Africa could have witnessed multiple colonization-extinction events, following habitat suitability.

Lineage H2 on the other hand was inferred to have expanded earlier, at approximately 104 000 YBP. More varied and open habitats prevailed after 1.8 Ma in subtropical Africa [69], with more pronounced vegetal changes after 220 kyr associated with climatic shifts [70,71]. Paleorecords support the probable persistence of a rainforest zonal belt in both dry and wet periods before 220 kyr, with large westward expansions of *Podocarpus* forest during favorable periods [70]. After 220 kyr, a more pronounced reduction of scattered occurrences of the rainforest was recorded.

The development of savanna habitat in Western Africa, replacing forest habitat, could have promoted the expansion of the Western populations of savanna buffalo, whose expansion signal approximately coincides with the savanna development.

Major Colonization Events and Migration Routes

The Eastern region around the present-day Uganda appears to have played a prominent role throughout the history of the H1 lineage. Many internal branches had high posterior probabilities for the geographical state of Uganda, and the earliest occurrence of a non-Ugandan branch in H1 was 83–103 kyr (Figure 3B), roughly 170 000 years after the divergence of H1 and H2. This supports an important conclusion: a primary refuge for *S. c. caffer* located in Eastern Africa appears to have played an important role in its history, when climatic conditions were unfavorable. Migration from a core Eastern refuge into other parts of SE Africa was found to have happened several times, as these other locations are positioned at multiple separate external branches, dated independently, on internal Ugandan branches, probably related to the climatic oscillation registered during Pleistocene. Further support is found in the network reconstruction, which reflects the same pattern, with Southern African haplotypes forming sub-groups positioned at tip positions, without being
monophyletic. Our analysis of the quantification of the strength of connectivity between geographical states in the tree, which attempted to identify the historically most important migration routes, identified strong support for a Uganda-Tanzania and a Tanzania-Kenya link, but not for a Uganda-Kenya link. Thus, a central role of Tanzania in this Southern expansion was also highlighted.

The H2 lineage showed a less clear geographical pattern with an earlier geographical diversification from a core population in Chad, Cameroon or the Central African Republic (CAR) (i.e., Western refuge). The genealogy of H2, with its longer branch lengths, indicates that H2 underwent a demographic expansion earlier than H1, a fact corroborated by the mismatch distribution profiles. For H2, Cameroon and Gabon hosting forest buffalo ecophenotypes form migratory cul-de-sacs connected to CAR, indicating that these populations are the result of a unique migration route from CAR, which is different from the migration route between CAR and the Western savanna buffalo populations. This is consistent with Figure 3A, where the same three Central African forest buffalo populations mainly occur together in lineages distinct from the Western savanna buffalo of Niger, Chad, Ghana and Benin. This interpretation suggests that the forest buffalo ecophenotype, rather than being the ancestor of all living African buffalo as Kingdon [26] proposed, may be an advanced form derived from the WC savanna ecophenotype. This is also supported by the fact that the forest ecophenotype appears to be non-monophyletic, indicating different separate migration route endpoints. Furthermore, the work of Bekhuis [23], based on the study of the diet of the forest buffalo in Cameroon, concluded that the ancestral niche of the buffalo ancestors more likely corresponds to a savanna-rainforest gradient or mosaic than to a true rainforest, in agreement with the observation that most Pleistocene buffalo fossils resemble S. c. brachyceros [72]. CAR was clearly an important link in the westward dispersal of buffalo from a presumed Central African origin, as evidenced by the high number of strongly supported rates between CAR and other H2 locations in addition to the inferred link between CAR and Uganda.

Interestingly, the one available sample from South-Western Africa (Namibia) was positioned well within H2. This shows an important migration connection between West-Central and West-Southern Africa, which has been proposed before [37] and is supported by morphological studies, reporting the existence of a dwarf-buffalo-like-population in Angola [21]. Nevertheless, as this deduction is based on only one sample from Antwerpen Zoo with mother from Okahandja [37, this study], it should be regarded as tentative and to be further investigated by increasing the sampling from this region.

In summary, the pattern in Figure 4 and Figure 3 suggests that the split between two major lineages of buffalo occurred in Western Africa, probably around the present-day CAR. One lineage (H1) apparently remained in Eastern Africa for a long time before expanding in population size and range, and the other (H2) expanded earlier along two separate routes into the African forest belt and the Western Sahel region, respectively. Overall, we propose the following phylogeographic scenario: the ancestor of all living buffalo lived in Western Africa. This ancestral population became separated (for unknown reasons) around 100–300 kyr into two isolated populations, one of which (ancestors of H2) started expanding westward at an early time, around 100 kyr or earlier. The other (ancestors of H1) retained its core population in Eastern Africa, probably being unable to colonize arid Southern Africa until about 50–80 kyr, where it expanded South and East, possibly after adapting to an arid savanna environment or after the decline of an obvious competitor, Pelorus antiquus [32,66,67]. A recent colonization of Southern Africa by S. c. caffer is supported by the lack of true Cape buffalo characteristics, i.e., the sweeping horns and pronounced horn boss, in all buffalo fossils from this region [32,72]. This tentative phylogeographic scenario is in agreement with the wealth of fossil and molecular data currently available on the African buffalo.

Conclusions

Our main finding is that African buffalo should be partitioned into two MUs (i.e., Management Units as defined by Moritz [73]), putatively named the South-Eastern African buffalo (S. c. caffer) and the Western African buffalo (S. c. nana), which has important implications for the conservation of the species. We found little genetic structure distinguishing the three morphologically distinct ecophenotypes of the Western African buffalo, hence we posit that these ecophenotypes merely represent rapid adaptations to local habitat variations without reproductive isolation. Our results also suggest that the forest buffalo ecophenotype may be an adapted form derived from the West-Central savanna ecophenotype. The more important phenotypic variability observed in West-Central Africa could be the result of an earlier origin of the Western lineage than of the Cape buffalo. More genetic clusters in the Western lineage that very recently diverged may also exist and could be identified using finer genetic markers. We have demonstrated that extensive sampling from the whole distribution of a species makes it possible to infer important aspects of historical migration, refugial areas and taxonomic subdivision.

Materials and Methods

Sample Collection and Laboratory Methods

The protocols for animal sampling used for our study did not induce pain or distress, according to the Animal Care Resource Guide, and thus correspond to USDA category C. Indeed, capture relied on the live capture technique of large mammals covered in the American Society Mammalogists Guidelines. Procedures were not more invasive than peripheral blood sampling or peripheral tissue sampling. Chemical immobilisation was only performed to

**Table 7. Maximum fixation indices (FCT) per species for mtDNA between subspecies or genetic lineages reported in various Bovinae and African mammals.**

<table>
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<th>Species</th>
<th>FCT mtDNA</th>
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<td>Giraffe, Giraffa camelopardalis</td>
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<td>Roan antelope, Hippotragus equinus</td>
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<td>Kob, Kobus kob</td>
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<td>African buffalo, Syncerus caffer</td>
<td>0.42 [This study]</td>
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<td>Impala, Aepyceros melampus</td>
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<td>Bonobo, Pan paniscus</td>
<td>0.31 [78]</td>
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<td>Savanna elephant, Loxodonta africana</td>
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<td>Hippopotamus, Hippopotamus amphibus</td>
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doi:10.1371/journal.pone.0056235.0007
facilitate the procedure. Animals were released in favourable condition that enabled them to avoid predators, seek shelter, and survive inclement weather.

A set of 787 buffalo samples from West (Ghana, Burkina Faso, Benin, Niger, Sierra Leone, Chad), Central (Cameroon, Central African Republic, Democratic Republic of Congo, Gabon), East (Uganda, Kenya, Tanzania) and Southern Africa (Zimbabwe, Botswana, South Africa, Namibia, Angola) was analyzed. Details and location of the samples are shown in Table 6 and Figure 6. This extensive sampling covers almost the entire distribution of the African buffalo and comprises samples of all putative subspecies. Sample access was facilitated by a large campaign of epidemiological monitoring, but also because buffalo is a prestigious hunting trophy.

Blood, hair, tissue and dung samples were kept in ethanol solution and stored at ambient temperature, except the dung samples from Ghana, Sierra Leone and Benin, which were collected on Whatman FTA cards [80,81]. Total DNA was extracted using either a DNeasy tissue kit (QIAGEN) or a Puregene kit (QIAGEN), following the manufacturer’s instructions. The Puregene protocol was modified slightly for use with Whatman FTA cards. DNA was released from the cards by soaking 1.25 cm² of the card in 240 µl water and heating this to 90°C for 10 minutes. Following this, 120 µl of the solution was transferred to a new tube together with 480 µl Puregene Cell Lysis solution and 3 µl of a 20 mg/ml proteinase K solution. The remainder of the protocol followed the manufacturer’s instructions.

The D-loop hypervariable region (HVR) and adjacent regions were amplified in the majority of samples using forward L0 (5′-CCCAAGCTGAAAATTCCTACTAACTA-3′) and reverse S0 (5′-TCTAGGCTTTTCAGTGCCTGGTCTT-3′) primers [82]. In the samples from Ghana, Sierra Leone, Benin, Cameroon and Gabon, DNA amplification took place with forward primer Dsca1 (5′-AGATAAAAGAAGCCCTCCTCCACG-3′) and reverse primer Dsca2 (5′-CGGCTCATAGCTGATGTCCTGC-3′) [35]. In general, DNA amplification was carried out as described in Heller [43] with polymerase chain reaction (PCR) products sent to MACROGEN Inc (Capillary electrophoresis on a 3730XL) or [43] with polymerase chain reaction (PCR) products sent to Macrogen (including standard deviations) using ARLEQUIN and DNAsp [92].

1. Population differentiation. The hierarchical distribution of genetic variance among and within populations was assessed using an analysis of molecular variance (AMOVA) on the basis of individual nucleotide frequencies. We also performed pairwise comparisons of individual nucleotide frequencies between populations using Wright’s F-statistics, as implemented in ARLEQUIN v.3.1 [90]. The groups and populations for AMOVA and Wright’s F-statistics were defined according to their geographic position, including the national park or game reserve where it was collected (see Table 6), except for the neighboring populations in the Chobe-Hwange and the Masai Mara-Serengeti-Maswa ecosystems that were not significantly differentiated from one another. The statistical significance of the F-statistics was assessed using 1000 random permutations. For all populations where the number of included samples exceeded four, we calculated the number of haplotypes, haplotype diversity h and nucleotide diversity π [91] (including standard deviations) using ARLEQUIN and DNAsp [92].

2. Demographic trends. Past demographic history of each of the buffalo lineages was inferred by a pairwise mismatch distribution analysis between individuals [93], comparing the distribution of observed pairwise nucleotide differences, with the expected distribution in an exponentially expanding population. To assess the statistical significance of the distribution, we examined the sum of square deviations (SSD) between the observed and expected mismatch and Harpending’s raggedness index r computed under a population growth-decline model in ARLEQUIN. The P-value of the test was approximated based on the fraction of times the real data showed a lower value than the simulated data. The timing of demographic expansion can also be roughly estimated by the mode of mismatch distribution τ expressed as τ = 2 μt, where t is the expansion time in number of generations and μ is the mutation rate for the whole sequence [94]. Generation time was fixed within a range of five to seven years, based on the estimates of O’Ryan [34] and Prins (personal communication), and the mutation rate per site was fixed to 32% per million years for the D-loop as estimated by Shapiro [95].
Demographic history was also inferred by testing departure from neutrality using Fu's \( F_{S} \) and Tajima D statistics [96] in DNaSP.

3. Coalescence based analyses. BEAST v1.6.1 [97] was used to reconstruct the colonization history of buffalo populations. Recent developments in the software allow one to simultaneously infer the genealogy and ancestral geographic state at each node in the tree [98]. Furthermore, the method allows identification of statistically significant diffusion rates between geographic states (i.e., historical migration patterns) through a proper Bayes factor test rather than by parsimony analysis [98]. We chose to define the country of origin as the geographical states contained within the software as we were interested in spatial patterns at a larger scale (i.e., above the level of the sampling location) (Table 6). This yielded 17 distinct states. We imposed normalized inverse-distance

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simulations. We used a geometric heating scheme with
41%, respectively, from their average value across three
limits of the 95% confidence interval deviated no more than 27%
certainty in our findings. The estimates of the mean and the
splitting, irrespective of the length of the runs. However, we got
was not always achievable, especially for the time of population
effective sample size (ESS) for estimates exceeded 50. The latter
obvious trends in the trend line plots and ensured that the lowest
program for a sufficient length of time so that there were no
over which the posterior density is non-trivial. We ran the
distributions were chosen that included all or most of the range
2006–2009). Finally, Bayes factors (BF) [98] were calculated to
estimate the diffusion rates between states throughout the trees.
We imposed a threshold value of BF = 3 to determine the
significance of the diffusion process connecting the location states.
An isolation-with-migration (IM) model for two closely related popula-
tions or ecophenotypes was applied using the IM program
[101,102]. The IM model presents seven demographic parame-
ters, scaled by the mutation rate: current effective population sizes,
ancestral effective population size, migration rate in both di-
rections, time of population splitting and a splitting parameter. The latter
parameter, not included in newer versions of IM (IMA and IMA2),
estimates what fraction of the ancestral population formed each of
the two current populations or ecophenotypes. We used a burn-in
of 500 000 steps followed by a run of 12–15 million steps. Prior
distributions were chosen that included all or most of the range
over which the posterior density is non-trivial. We ran the
program for a sufficient length of time so that there were no
obvious trends in the trend line plots and ensured that the lowest
effective sample size (ESS) for estimates exceeded 50. The latter
was not always achievable, especially for the time of population
splitting, irrespective of the length of the runs. However, we got
similar estimates for all parameters in all simulations, providing
confidence in our findings. The estimates of the mean and the
limits of the 95% confidence interval deviated no more than 27%
and 41%, respectively, from their average value across three
simulations. We used a geometric heating scheme with $h_1 = 0.8$
and $h_2 = 0.9$, applying metropolis coupling using a 10 chain
geometric heat mode with 45 chain swap attempts per step. The
final prior values used for respectively current population size,
ancestral population size, migration rate, lower limit divergence
time, and upper limit divergence time were $q_{ancestral} = 70, q_{current} = 0,$
$m = 0.12, a = 7$ and $t = 40$, respectively. The splitting parameter $s$
was included, migration rates were estimated in both directions
and the HKY substitution model was applied.

Due to computational constraints, 360 samples were randomly
selected, 180 from West-Central and 180 from South-Eastern
Africa. Three simulations with 360 samples were performed, each
using a different random subset of samples and a different random
seed number. All populations within a specific group were
represented with a random selection of samples, while equalizing
sample size per population as much as possible resulting in the
following scheme: West Africa (90 samples), Cameroon and
Gabon (54 samples), Chad, Central African Republic and
Democratic Republic of Congo (Eastern part of Central Africa
close to the lineage border, 36 samples), Uganda (Western part of
East Africa close to the lineage border, 36 samples), Kenya and
Tanzania (54 samples) and Southern Africa (90 samples).
Countries close to the border between two lineages were defined
as distinct groups to get maximum sample representation from the
presumed center of the divergence.

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Conceived and designed the experiments: NS CB DC RH PVH PC JM.
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