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Mitochondrial D-loop diversity of grasscutter (*Thryonomys swinderianus* Rodentia: Hystricomorpha) in Ghana

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ABSTRACT

Attempts are being made to domesticate the grasscutter (*Thryonomys swinderianus*) for commercial production in Sub-Saharan Africa to cater for the protein needs of the people and to satisfy the craving for bushmeat, thereby reducing habitat destruction through hunting. The objective of this study was to determine the genetic diversity of grasscutter populations in Ghana. DNA was extracted from roots of hair samples collected from 84 grasscutters from three agro-ecological zones in Ghana, namely Guinea Savanna (n = 17), Forest (n = 22), and Coastal Savanna (n = 45). Mitochondrial D-loop was sequenced and the diversity was determined across the zones. Out of 26 haplotypes found, 15 were obtained from Guinea Savanna, 7 from Forest and 13 from Coastal Savanna. Haplotype diversities were 0.978, 0.853 and 0.875 respectively for Guinea Savanna, Forest and Coastal Savanna zones. Analysis of molecular variance (AMOVA) revealed significant differentiation between Forest and Savanna populations (F_{ST} = 0.14, p < 0.05). Network analysis indicated two clusters, one of which consisted of only Savanna haplotypes. Population neutrality tests showed that Forest and Coastal Savanna populations had been stable while the Guinea Savanna zone population had undergone an expansion (Fu's F_S = −7.132, p < 0.05). The results of this study demonstrated that the Ghanaian populations of grasscutters are highly diverse but are less distinctive.

Keywords: Agro-Ecological Zones; Rodent; D-Loop; Genetic Variation; *Thryonomys swinderianus*

1. INTRODUCTION

The grasscutter (*Thryonomys swinderianus*), also known as the cane rat (or *aulacode* in Francophone West Africa), is a hystricomorph rodent which mainly inhabits the Sudan and the Guinea savanna [1]. It is an herbivorous species found in grassland areas and wooded savanna and is particularly widely distributed at places where its most preferred grasses for feeding are available [2]. In farming areas, it is often regarded as a major crop pest, destroying cassava, maize, sugarcane, and in plantations feeding on young cocoa, coconut and oil palm [2,3]. Wild grasscutters are distributed throughout Ghana, except in rain forests, but they occur in secondary forests and cropped areas leading to continuous expansion of their habitat due to forest clearance for agricultural activities [3].

Surveys in Ghana have revealed that grasscutter meat is the most preferred by "chop bar" operators (cooked food vendors) and consumers [4,5]. Grasscutters are thus hunted aggressively to the point of using poisonous baits and fire to the peril of wildlife habitats, the environment and consumers. The meat is eaten by all classes of people with no religious prohibitions [6], and is also exported to continental Europe and the United States, where it is sold mainly to West Africans living in those regions [7].

Grasscutter domestication started in the 1970s but efforts over the years have met with little success. Rearing attempts suffered from high mortality due to the aggressive nature of the species, referred to as "berserk behavior" [7]. In any domestication process, selection for de-

sirable traits is of great importance to ensure ease of handling and for profitable production in the case of the grasscutter, which is being developed as a mini-livestock in Sub-Saharan Africa to alleviate poverty and to cater for the protein needs of the people. Various aspects of grasscutter biology have been studied in order to better manage the species under domestic conditions. These include reproduction [8-13], nutrition [14,15], parasites and diseases [16-18]. However, much remains to be done on the genetics of the grasscutter production, especially at the molecular level.

Mitochondrial DNA is known to be maternally inherited, and non-recombinant with a high mutation rate [19-21]. The D-loop is a hypervariable, non-coding part of the mitochondrial genome that is approximately 1 kb long and less than 7% of the total mitochondrial genome of most mammals [21]. It is also known to regulate transcription and replication [22]. The above characteristics of the mitochondrial DNA, specifically the D-loop, make it an excellent marker for population genetics studies. Also, because of easier amplification due to the presence of multiple copies within a cell, mitochondrial DNA has been used extensively to study populations and to trace maternal lineages. Sequence analysis of the D-loop as a dominant marker can efficiently reveal genetic structure and differentiation among populations [23,24]. These genetic diversity measures of populations nevertheless serve as useful information for conservation.

Management of populations for conservation requires baseline information such as genetic structure and diversity. Even though the grasscutter is not expected to be endangered in the foreseeable future, increasing our understanding of the population dynamics and genetic diversity of the species is necessary for monitoring. The objective of this study was therefore to assess the genetic diversity among grasscutter populations inhabiting three agro-ecological zones in Ghana. As far as we are aware, no genetic diversity study about the grasscutter has been reported in the literature. This paper therefore reports for the first time, the mitochondrial D-loop diversity of grasscutter populations in Ghana.

2. MATERIALS AND METHODS

2.1. Sample Collection

A total of 84 hair samples were collected from grasscutters in three agro-ecological zones in Ghana; Coastal Savanna (n = 45), Forest (n = 22) and Guinea Savanna (n = 17) (**Figure 1**). The different zones differ in terms of climatic conditions (e.g. rainfall pattern and temperature) and vegetation type and therefore each was considered to be habitat for a separate population of grasscutters. All samples except semi-domesticated individuals, were collected from centrally located bushmeat markets in the different agro-ecological zones. These bushmeat markets were thought to cover wide range and samples were representative of each zone because animals traded were hunted from both far and near. The exact locations could however not be ascertained. Out of the 45 Coastal Savanna samples, 21 were obtained from semi-domesticated unrelated individuals which were kept by farmers. DNA was extracted from the root of 15 - 20 hair pieces of each sample using Instagene Matrix (Bio-Rad Laboratories, USA), quantified using NanoDrop Spectrophotometer (Thermo Scientific, USA) and stored at −30˚C until ready for use.

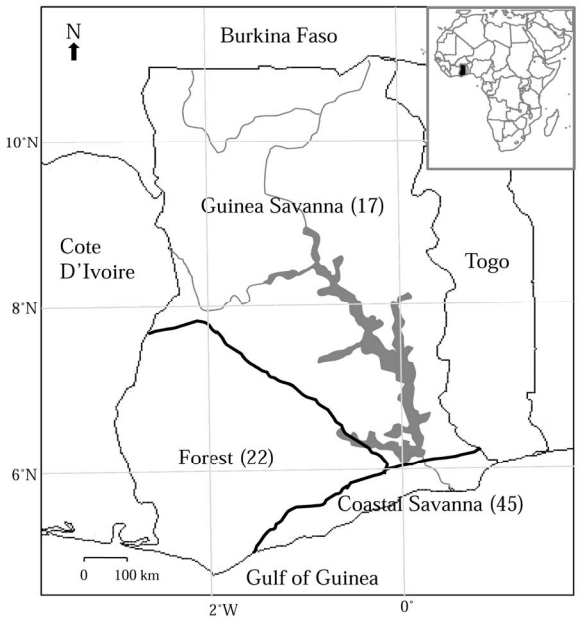

Figure 1. The map of Ghana showing three agro-ecological zones. Numbers in parenthesis indicate number of samples collected in each zone.

2.2. PCR Amplification and Sequencing

Mitochondrial displacement loop (D-loop) was amplified in a PCR using CRmtDF (5'-CCAACTCCCAAAGCTGATGT-3') as the forward primer, and CRmtDR (5'-GGCACCAACATCATCACAAA-3') as the reverse primer. These primers were designed with a registered sequence of the grasscutter (Accession no. AJ301644) [25] using Primer 3 software and could amplify 501 bp of the D-loop. The PCR mixture contained 0.75 U of *LA-Taq* DNA polymerase (TaKaRa, Shiga, Japan), PCR buffer, 400 µM of each dNTP, 0.4 µM each of forward and reverse primers, 0.1 µg of T4 Gene 32 Protein (Nippon Gene, Japan) and 20 ng of template DNA in a total volume of 15 µl. PCR cycling conditions consisted of an initial denaturation of 95˚C for 2 min, followed by 35 cycles of 95˚C for 30 sec, 55˚C

for 30 sec, 74˚C for 1 min and a final extension of 74˚C for 10 min. Aliquots of 5 µl of the PCR products were electrophoresed on 1.5% agarose gel to check amplification. DNA bands were visualized after Ethidium Bromide staining under UV light, and expected size was determined in relation to a DNA size standard. The remaining aliquots were purified using High Pure PCR purification kit (Roche, Manheim, Germany) and sequenced using Big Dye Terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers protocol and electrophoresed on an ABI PRISM 3130xl sequencer (Applied Biosystems).

Both forward and reverse complements of the reverse sequences were aligned to get a consensus sequence using MEGA version 5 [26]. Primer sequences were then deleted to obtain 501 bp of the D-loop spanning from position 15,661 to 16,161 of the mitochondrial genome [25], and covering the rapidly evolving extended-termination associated sequences (ETAS) domain and part of the central domain (CD) of the D-loop [21].

2.3. Data Analysis

Arlequin ver. 3.5 [27] was used to determine the number of haplotypes, haplotype diversity and nucleotide diversity in each agro-ecological zone. Haplotype diversity indicates genetic diversity within populations and nucleotide diversity is estimated as the function of the number of polymorphic sites and the frequency of transition or transversion and insertion or deletion within the population [23]. A haplotype network was constructed using outputs from TCS ver. 1.21 [28] and Network software ver. 4.6 (www.fluxus-engineering.com). Analysis of molecular variance (AMOVA) was conducted in Arlequin, considering genetic distance between haplotypes and their frequencies to determine the variation among and within populations. It was assumed that the three populations formed one group and therefore the pooled flat genetic structure in Arlequin was chosen. In order to increase the level of accuracy, 10,000 random permutations were performed. The gamma value was set at 0 as this is proposed when mutation rates cannot be assumed to be uniform [27]. This is usually the case for mtDNA D-loop and most especially for rodents because they have short generation intervals [29,30]. The significance (*p*-value) of the fixation index, which is analogous to the *F*-value in the conventional analysis of variance, was estimated as the probability that a random value is greater than or equal to the observed value. To determine which populations were genetically different from each other, pairwise F_{ST} values which show genetic distance between given pair of populations were computed [31].

According to Harpending [32], a mismatch distribution is a measure of the distribution of pairwise differences among non-recombinant DNA sequences in a population. This distribution is multimodal, smooth and has a peak for populations that have undergone expansion but erratic or ragged for populations in equilibrium [32-34]. Past demographic events are known to leave footprints in the DNA sequence of individuals in a population. To get a glimpse of the demographic history of the grasscutter populations, mismatch distributions (10,000 bootstrap replicates) were determined and Tajima's *D* and Fu's F_S neutrality indices were computed under the infinite site model as implemented in the Arlequin software [27]. The number of simulated samples under the infinite site model was 10,000.

To examine the relationship between geographic distance and genetic distance (equilibrium between gene flow and genetic drift), isolation-by-distance analysis was conducted as implemented in GenAlex ver. 6.41 [35]. Coordinates between pairs of sampling sites were converted into geographic distances, and a Mantel test was performed to assess the correlation between geographic distance and pairwise F_{ST} values.

3. RESULTS

3.1. Haplotype Diversity

Guinea Savanna samples had 21 polymorphic sites whereas Coastal Savanna and Forest zones had 15 and 12 polymorphic sites, respectively. In all, there were 23 variable sites including five singletons and 18 parsimonious informative sites. All polymorphic sites were transitions except for one transversion at position 16,125 in a Guinea Savanna zone haplotype. Out of a total of 26 haplotypes, 15 were from Guinea Savanna, seven from Forest and 13 from the Coastal Savanna (**Table 1**). The sequences were deposited in Genbank under the accession numbers AB675385 to AB675410. The Forest and Coastal Savanna zones shared three haplotypes whilst five haplotypes were shared between Coastal Savanna and Guinea Savanna. Forest and Guinea Savanna also had three haplotypes in common. In all, only two haplotypes were found to be common to the three agro-ecological zones. Haplotype diversities were 0.978, 0.853 and 0.875 for Guinea Savanna, Forest and Coastal Savanna zones respectively (**Table 2**). Even though there were fewer samples from the Guinea Savanna zone, the Guinea Savanna zone was found to have the highest haplotype diversity compared to the Forest and the Coastal Savanna zones.

Nucleotide diversity was 0.012 for both Guinea Savanna and Coastal Savanna zones which is almost twice as that of the Forest zone (0.007) (**Table 2**). AMOVA results indicated that 85.79% of the total variation was within populations whilst the remaining 14.21% was

Table 1. Haplotypes and their frequencies in each population.

Accession no.	Haplotype[a]	Zone			
		Guinea Savanna	Forest	Coastal Savanna	Total
AB675402	G1	1	-	-	1
AB675403	G2	1	-	-	1
AB675404	G3	1	-	-	1
AB675405	G4	1	-	-	1
AB675406	G5	1	-	-	1
AB675407	G6	1	-	-	1
AB675408	G7	1	-	-	1
AB675409	G8	1	-	-	1
AB675410	G9	1	-	-	1
AB675388	F1	-	3	-	3
AB675390	F2	-	1	-	1
AB675391	F3	-	1	-	1
AB675392	C1	-	-	1	1
AB675393	C2	-	-	1	1
AB675394	C3	-	-	1	1
AB675396	C4	-	-	9	9
AB675397	C5	-	-	1	1
AB675398	C6	-	-	1	1
AB675401	C7	-	-	1	1
AB675386	GF	1	5	-	6
AB675387	FC	-	5	9	14
AB675395	GC1	3	-	9	12
AB675399	GC2	1	-	5	6
AB675400	GC3	1	-	2	3
AB675385	GFC1	1	5	3	9
AB675389	GFC2	1	2	2	5
Total		17	22	45	84

[a]G1 - G9 indicate Guinea Savanna zone specific haplotypes, F1 - F3 are Forest zone specific haplotypes, C1 - C7 are Coastal Savanna zone specific haplotypes, GFC denotes common haplotypes, GC denotes haplotypes shared between Guinea Savanna and Coastal Savanna zones, FC denotes haplotypes shared between Forest and Coastal Savanna zones and GF denotes haplotypes shared between Guinea Savanna and Forest zones.

Table 2. Genetic diversity and indices of population neutrality across the three agro-ecological zones.

Zone	*n*	No. of haplotypes	Haplotype diversity, *h*	Nucleotide diversity, π	Harpending's raggedness, *rg*	Tajima's *D*	Fu's F_S
Guinea Savanna	17	15	0.978 ± 0.031	0.012 ± 0.007	0.022 (0.613)[b]	0.189 (0.619)	−7.134 (0.002)
Forest	22	7	0.853 ± 0.037	0.007 ± 0.004	0.034 (0.886)	0.560 (0.745)	0.989 (0.706)
Coastal Savanna	45	13	0.875 ± 0.024	0.012 ± 0.006	0.057 (0.690)	1.442 (0.942)	0.839 (0.673)

[b]*p*-values are indicated in parenthesis.

among populations (**Table 3**). The fixation index resulting from the AMOVA analysis, which gives an indication of population differentiation, was found to be highly significant ($p < 0.001$) even though the larger part of the variation was within populations. This indicated that the populations are less structured. In terms of pairwise F_{ST}, Guinea Savanna and Coastal Savanna had the lowest F_{ST} value (0.052) indicating that they are the closest, while Forest and Coastal Savanna had the highest value (**Table 4**). The results indicated significant genetic differentiation ($p < 0.05$) between Forest and Coastal Savanna and also between Forest and Guinea Savanna. There was however no significant differentiation between Guinea Savanna and Coastal Savanna zone haplotypes.

Figure 2 presents a haplotype network showing two clusters, one of which is fairly simple and consists of only Savanna haplotypes (Cluster A), and the other which harbors the two common haplotypes, consists of haplotypes from all zones, and is more complex (Cluster B). Such a network shows the relatedness of the different haplotypes based on nucleotide substitutions. It can be seen that the Savanna haplotypes in Cluster A are more closely related to each other than Forest haplotypes. Haplotypes from both savanna zones can be seen in both clusters whereas the Forest haplotypes are found only in the complex cluster. This is indicative of the presence of two haplo-groups within the two savanna zones.

3.2. Mismatch Distribution and Population Neutrality

Figure 3 shows mismatch distributions of the three populations, with the observed mismatch distribution being multimodal for all populations, though not significant. Harpending's raggedness index, *rg*, Tajima's *D* and Fu's F_S are presented in **Table 2**. For both *rg* and *D*, none of the zones was found to be significant ($p > 0.05$). These statistical tests therefore indicate that the populations were under neutral selection. Fu's F_S was however significant for Guinea Savanna zone ($p < 0.05$), which is an indication of a recent past expansion event in this population [36]. A Mantel test conducted to assess the equilibrium between genetic drift and gene flow, was found to be non-significant ($Rxy = 0.022$, $p > 0.05$).

Table 3. Results of analysis of molecular variance (AMOVA).

Source of variation	d.f.	Sum of squares	Variance component	% variation	F_{ST}	*p*
Among populations	2	29.46	0.47	14.21	0.14	0.0002
Within populations	81	229.57	2.83	85.79		
Total	83	259.03	3.3	100		

Table 4. Matrix of pairwise F_{ST} values for the three agro-ecological zones.

	Guinea Savannah	Forest
Guinea Savanna	-	
Forest	0.078 (0.018)[c]	-
Coastal Savanna	0.052 (0.090)	0.225 (0.000)[c]

[c]Significant *p*-values.

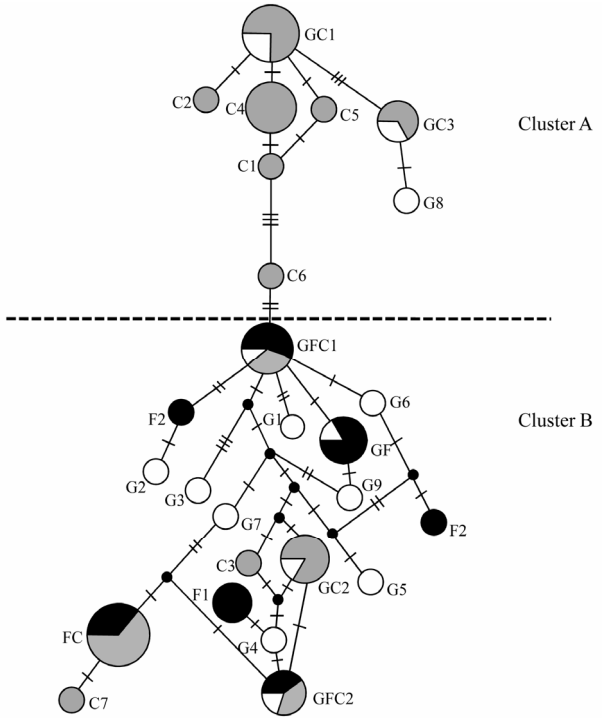

Figure 2. Network among haplotypes. G1 - G9 indicate Guinea Savanna zone specific haplotypes (open circles), F1 - F3 are Forest zone specific haplotypes (black circles), C1 - C7 are Coastal Savanna zone specific haplotypes (gray circles), GFC denotes common haplotypes, GC denotes haplotypes shared between Guinea Savanna and Coastal Savanna zones, FC denotes haplotypes shared between Forest and Coastal Savanna zones and GF denotes haplotypes shared between Forest and Guinea Savanna zones. The proportions of individuals from each agro-ecological zone in the common and shared haplotypes are shown as slices. The size of the circle is proportional to the total number of individuals of that haplotype. The cross-bars show the number of substitution between haplotypes whilst the black nodes indicate missing haplotypes.

4. DISCUSSION

In this study, it is not unexpected that 26 different haplotypes were found coupled with high haplotype diversities (0.85 - 0.97) across the three zones, owing to the fact that the D-loop has a high mutation rate [21]. These results are comparable to a previous report on a South American hystricomorph rodent, *Microcavia australis* [37] which indicated 0.70 - 0.93 for haplotype diversity (*h*) and 0.0006 - 0.0095 for nucleotide diversity

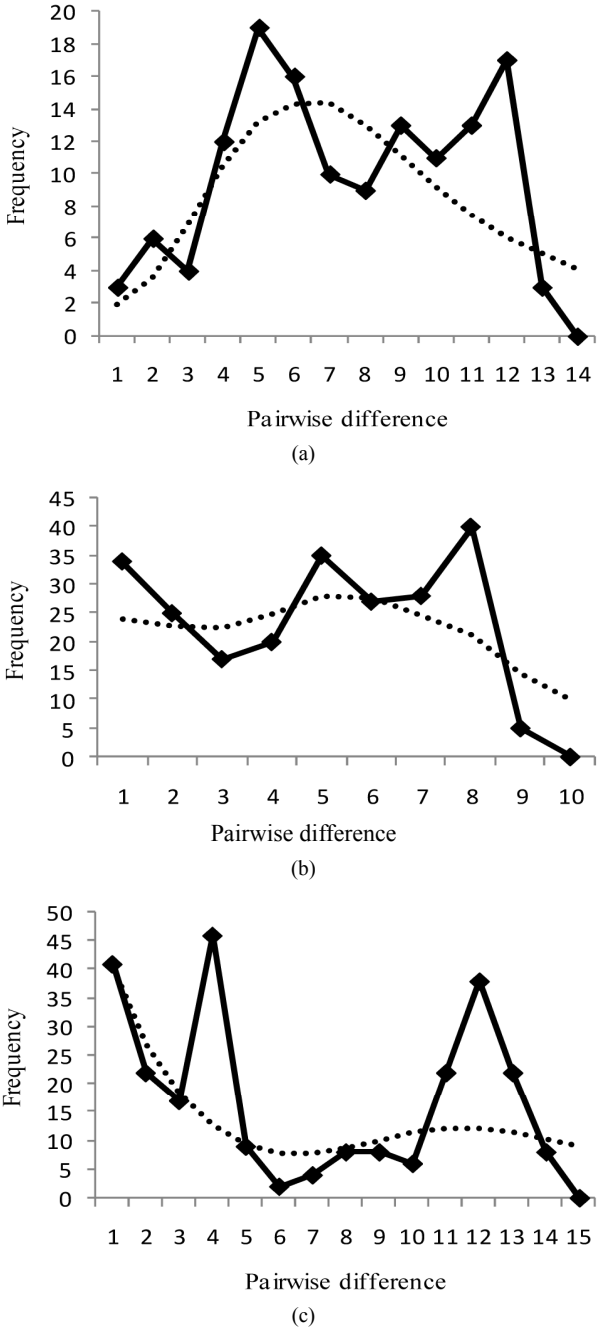

Figure 3. Harpendings mismatch distribution showing observed and simulated frequencies of pairwise differences for each population. (a) Guinea Savanna zone; (b) Forest zone; (c) Coastal Savanna zone. Observed frequency and simulated frequency are shown by thick line and dotted line, respectively.

(π). The results of our study are however higher than that of [38] on *Laonastes aenigmamus* in Laos (h = 0.79). According to [39], lack of gene flow diminishes genetic diversity. Also, genetic drift, dispersal pattern and vicariance events such as habitat fragmentation may all influence genetic structure and diversity of mammals. For instance [37] found highly differentiated highland and lowland populations of *M. australis* with almost all haplotypes being unique to populations, indicating very restricted levels of gene flow between the populations. Similar results were obtained by Ojeda [40] on another rodent, *Tympanoctomys barrerae*, in the same region.

The possible reason for the higher haplotype diversities could be that there are no or limited barriers to gene flow as grasscutters are good swimmers [2] and can cross water bodies which might be perceived as barriers to dispersal. It has been observed that grasscutters in Ghana continue to expand their habitats in the Forest zone due to forest clearance for farming activities [2]. This possible dispersal could account for the two common haplotypes found among the three zones. We hypothesize that there is unrestricted gene flow among the populations especially between the Guinea Savanna and Coastal Savanna zones. More extensive sampling focusing on social structure will however be necessary to test this hypothesis. Another possible reason for the high haplotype diversities could be the very high population of grasscutters which existed in the recent past or presently exist in the country. This assertion could be supported with the fact that grasscutters can be found in almost every part of Ghana [3]. Grasscutters are predominantly savanna species [1] which thrive on grasses and succulent stems, hence the name "grasscutter". It is probable that the Forest population of grasscutters arises out of a recent colonization of the Forest zone by grasscutters from the Guinea Savanna zone, hence the genetic distance between Forest and Guinea Savanna is closer than the Forest and Guinea Savanna than Forest and Coastal Savanna populations. According to [41], lack of equilibrium between genetic drift and gene flow or absence of isolation by distance, which is the case in this study, may be due to a recent colonization event, in turn linked to human impact in the Forest zone. Agriculture-related activities such as forest clearance, bush burning and cultivation of crops result in expansion of grassland areas within the Forest zone and ultimately forest fragmentation. These farmlands and grassland areas have become suitable habitats for the grasscutter.

Non-significant population differentiation between the two Savanna zones suggests that there is direct gene flow between the Guinea Savanna and the Coastal Savanna zones. This genetic distance reflects a dispersal pattern; possibly from the Guinea Savanna zone to the Coastal Savanna zone. This dispersal pattern might be a result of limited availability of feed resources due to harsh climatic conditions in the Guinea Savanna zone, characterised by relatively higher temperatures and a single rainy season between July and October [42]. Longer periods of aridity in this zone coupled with higher temperatures, may cause grasses to dry up. More so, the Guinea Savanna zone experiences frequent bushfires as a result of bush burning related to farming activities or as a hunting

practice. This reduces the amount of feed resources available, forcing groups of individuals to migrate to places where feed resources are readily available.

The Ghanaian populations of grasscutters, except for those in the Guinea Savanna zone, have been stable as observed from the mismatch distributions. [32] noted that populations that have undergone expansion show mismatch distributions that are multimodal, but populations that are stable have erratic distributions. None of the three zones was significant for Harpending's raggedness index, *rg* and Tajima's *D*. The results of mismatch distribution from the three populations reflect a multimodal distribution (**Figure 3**), including the Guinea Savanna. The difference is however found in the Fu's F_S test where the Guinea Savanna population displays a significant negative value ($p < 0.05$), suggesting a demographic expansion event. It is known that Fu's F_S has more statistical power than Tajima's *D* [43] and it is sensitive to other demographic events such as population bottlenecks and genetic hitchhiking [36]. Results should however be interpreted cautiously because the different tests have different sensitivities [22]. According to [44], populations with bottlenecks show low variation in mitochondrial DNA. A population bottleneck might not be the likely reason for the significant F_S. Genetic hitchhiking is also ruled out because mitochondrial DNA is non-recombinant [21]. A past expansion event could account for this significance and this could be supported by the relatively higher haplotype diversity observed in the Guinea Savanna zone compared to other populations. [36] however, cautioned that one cannot make a definite conclusion by using one gene and that a combination of nuclear and mitochondrial markers may be necessary.

[40] suggested that lack of isolation-by-distance pattern could be due to recent habitat expansion or colonization of new areas and inconsistent gene flow. We did not find any pattern of isolation-by-distance in our study, which supposes that an expansion event could influence this result. It was possible that the recent population expansion portrayed by the Guinea Savanna zone as found in the Fu's neutrality test and a recent colonization of the Forest zone as deduced from the results influenced the population structure and consequently the equilibrium between gene flow and genetic drift.

Within the Guinea Savanna and Coastal Savanna zones, double peaks were observed from the mismatch distribution (**Figure 3**). Even though the population history of the grasscutter in Ghana is not known, this result could be an indication of the coexistence of two haplo-groups in the two savanna zones. This tendency which is also clearly evident from the network analysis (**Figure 2**) is not profound in the Forest zone probably because of habitat limitations. This could also explain why the genetic differentiation between the Guinea Savanna and Coastal Savanna populations is not significant. The two populations may therefore be considered as one Savanna population.

The grasscutter is a "Least Concern" species according to the IUCN Red List [45] and therefore does not require to be targeted for conservation at the present time. It is however envisaged that recent human population expansion in Ghana coupled with rampant hunting, if not checked, might cause this species to be endangered in the future. Also, bushfire associated with the hunting of the grasscutters may destroy the habitat of many wildlife species. This study has therefore shed some light on the genetic diversity of grasscutters in Ghana, and this information may be valuable for future conservation efforts. It has also revealed the possibility that Guinea Savanna zone population of grasscutters has undergone population expansion in the past. It may be worthwhile to confirm these results with microsatellite analysis of the populations since mitochondrial and nuclear markers can sometimes present conflicting results due to different modes of inheritance and rates of evolution [46]. As a preliminary study and the first of its kind in grasscutters, we found high D-loop diversity among grasscutter populations in Ghana. However, a more detailed study focusing on genetic structure and dispersal patterns within each agroecological zone would be necessary to ascertain these results. As domestication of this species is being intensified because of its good prospects as a mini-livestock [4,7,10], information presented in this paper will serve as a baseline for further population genetics studies.

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