

Original Research Paper

Genetic Variations in Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) Ibadan, Nigeria, Using Allozyme Markers

Oluwakayode M Coker^{*}, Osamede H Osaiyuwu² Otobong M Isong¹

¹Department of Wildlife and Ecotourism Management, University of Ibadan, Ibadan, Nigeria

²Department of Animal Science, University of Ibadan, Ibadan, Nigeria

Corresponding Author: Oluwakayode Michael COKER, University of Ibadan, Ibadan, Nigeria, E-mail-liftingup2003@gmail.com

Article history; Received: September 5th 2020; revised. September 25th 2020 ; accepted December 4th 2020

Abstract

Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) are two of the eight squirrel species found in Nigeria with overlapping habitats in Southwestern Nigeria. Squirrels are involved in pollination, seed dispersal, vectors of human and domestic animal diseases as well as crop and household pests. These species and the crucial ecosystem services they render are threatened by habitat destruction, human encroachment and the fact that they are being used as a source of protein in Nigeria. Their conservation would be enhanced by availability of accurate genetic information which are scarce for these species. This study seeks to provide baseline data on genetic variation at three allozyme loci in the two species in University of Ibadan, Ibadan, Nigeria. Twenty-nine individual squirrels including eighteen *F. anerythrus* species and eleven *H. gambianus* were used in this study. These were obtained from the wild within the University of Ibadan. Observed and expected heterozygosity (H_o and H_e), Hardy-Weinberg equilibrium (HWE), genetic distance between the species, and inbreeding coefficients were performed using POPGENE and Tools for Population Genetic Analyses (TFPGA). In *F. anerythrus*, H_o and H_e were 0.6092 and 0.5635, respectively, while, for *H. gambianus*, H_o and H_e were 0.6242 and 0.5745, respectively. There were no deviations from HWE in all the tested loci ($p > 0.05$). Nei genetic distance between the species was 0.0070 and the populations showed fairly high level of outbreeding ($F_{ST} = 0.0029 \pm 0.0017$). More robust genetic markers would be required to further ascertain the genetic status of the species.

Keywords: Biodiversity conservation, *Funisciurus anerythrus*, *Heliosciurus gambianus*, protein polymorphism, transferrin.

Introduction

Genetic diversity is the result of evolution occurring over long periods of time (Luo *et al.*, 2005). Evolution operates through a combination of forces including natural selection, mutation, random drift, migration, reproduction, frequency of alleles etc. It occurs due to variation which is maintained through evolution (Allendorf *et al.*, 2013; Kumar and Singh, 2014). These lead to differentiation at both population and species levels (Chauhan and Rajiv, 2010). This adaptive evolutionary ability, if lost, can prove fatal, spelling doom for populations unable to survive changes. The presence of low levels of variation present higher probabilities of becoming genetically inbred with a resulting consequence of reduced fitness (Allentoft and O'Brien, 2010), hence the need to investigate and preserve the amount of genetic diversity within and between species (Chung, 2009; Merilä and Fry, 1998).

While physical characters can be observed easily, genetic variation is uncovered with the development of molecular techniques that are capable of detecting variations directly at the gene level. Following their

emergence over the last 50 years, biochemical and molecular techniques have greatly improved the view of nature while evolving during the process. They have also contributed immensely to the study of the genetic structure of populations using allozymes, mitochondrial DNA and a host of other nuclear DNA markers (Arnaud-Haond *et al.*, 2003; Schlötterer, 2004). Protein variation forms the basic molecular tool for measuring genetic diversity in organisms (Frankham *et al.*, 2002). It has proved effective in documenting some basic baseline data in the study of genetic diversity. Millar and Westfall (1992) opined that allozyme markers are useful in forest genetic conservation, assessing the amount and distribution of genetic variation, and monitoring changes in genetic diversity. Protein electrophoresis enables detection of allelic differences among protein products of hundreds of genes coded for by protein structures. These protein products are commonly known as allozymes (Singh, 2001). Allozyme studies evaluate polymorphisms in blood proteins (Jordan *et al.*, 2005) and the principle of allozyme markers is that protein variants in enzymes can be distinguished by native gel electrophoresis according to differences in size and charge caused by amino-acid substitutions (Schlötterer, 2004; Belletti *et al.*, 2005). Such studies are aimed at characterizing genetic resources of evolutionary importance as well as suggesting effective measures for in-situ conservation of biodiversity. Allozyme variation aids in describing genetic diversity (Belletti *et al.*, 2005). They are useful in examining the status of described species (Darda, 1994).

Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) are two of the eight squirrel species found in Nigeria. Their habitats overlap in Southwestern Nigeria. They form a small fraction of the global squirrel population (family Sciuridae) of approximately 280 squirrel species which belong to 60 genera divided into 5 sub families (IUCN, 2018). Squirrels occupy a large range of geographical land and can be found on all continents excluding Australia and Antarctica. Squirrels are predominantly herbivorous, eating seeds, nuts, fruits, fungi, and other plant matter; however, insects, eggs and occasionally, small vertebrate may be part of the diverse diet of these animals (Jansa and Myers, 2000; Steppan and Hamm, 2006; Thorington and Ferrell, 2006; Lurz, 2011; Brown *et al.*, 2014; IUCN, 2018). Apart from being a source of protein in Nigeria (and other west African countries), squirrels serve some other crucial ecosystem services such as pollination, seed dispersal, vectors of human and domestic animal diseases as well as crop and household pests. (Chakravarthy and Thyagaraj, 2012; IUCN, 2018). Common threats include habitat destruction and human encroachment. Sciurid conservation is enhanced by availability of accurate information on which scientific work can be built (Thorington and Ferrell, 2006; Brown *et al.*, 2014; IUCN, 2018). This information includes genetic information which are scarce for *F. anerythrus* and *H. gambianus*. Coker *et al.* (2020) described the relationships between these two species, using morphological markers and suggested molecular data as more reliable source of information.

Baseline genetic diversity information among and within species is needed for conservation analyses and applications. It is important in assessing the changes that occur in the genetic makeup of organisms with time (Millar and Westfall, 1992). Data on genetic diversity from this study will provide useful information to be applied in different fields including evolution, conservation and management of natural resources as well as genetic improvement programmes (in case of any future consideration for domestication since they are being used as an alternative source of proteins). For the sake of future conservation efforts, intra/inter-specific genetic diversity existing within species needs to be understood before populations are driven towards extinction (Sultana *et al.*, 2014). Currently, information regarding genetic variation of *F. anerythrus* and *H. gambianus* are scarce. This study seeks to provide baseline data and information on the genetic variation at three allozyme loci in *F. anerythrus* and *H. gambianus* in University of Ibadan, Ibadan, Nigeria.

Materials and methods

Twenty-nine individual squirrels including 18 individuals of *Funisciurus anerythrus* species and 11 individuals of *Heliosciurus gambianus* were used in this study. These were obtained from the wild in

January 2019, within the University of Ibadan campus (7°23'47"N and 3°55'0"E). The study area lies within the rain forest vegetation zone having an average annual rainfall of between 1524mm and 2032mm, relative humidity of 74.55% and elevation of ranging from 150m to 275m above sea level (Ishaku and Majid, 2010). About 1-2ml of blood were obtained from each individual, (based on the allowable 1% of body weight) through the femoral vein as described by Head *et al.* (2017) and Parasuraman *et al.* (2010) into EDTA treated bottles. Plasma fractions were separated from erythrocyte fractions by centrifugation. Thereafter, cellulose acetate electrophoresis of albumin, esterase and transferrin were conducted following the procedure described by Riken (2006). The migration for each protein in this study was from the cathode to anode. Details of the protocols observed for each protein are presented in Table 1.

Statistical analysis

Allelic bands for each protein were visually scored based on the migration patterns and was used to calculate the gene frequencies. Genetic distance between the species, allelic frequencies, observed and expected heterozygosity (H_o and H_e), Hardy-Weinberg equilibrium (HWE) and inbreeding coefficients were performed using POPGENE and Tools for Population Genetic Analyses (TFPGA) (Nei, 1977; Miller, 1997; Yeh et al. 1999). Unweighted pair group method with arithmetic mean (UPGMA) was used to construct a dendrogram.

Table 1: Electrophoretic protocols used in the analysis of each protein loci

LOCUS	FRACTION	BUFFER SYSTEM	VOLTAGE	TIME
Transferrin (TF)	Plasma	Tris glycine; pH 8.5	150V	30 minutes
Albumin (ALB)	Plasma	Tris citrate; pH 7.6	180V	30 minutes
Esterase (EST)	Plasma	0.01M phosphate; pH 6.8	140V	30 minutes

Results

Table 2 shows the allelic frequencies and Hardy-Weinberg Equilibrium (HWE) of the two species of Squirrel studied. In *F. anerythrus*, Transferrin (TF) loci revealed three alleles A, B and C at frequencies 0.411, 0.5294 and 0.0588, respectively; Albumin (ALB) loci had two alleles A and B at frequencies 0.5000 each; Esterase (EST) loci had three alleles A, B and C with frequencies 0.3750, 0.500 and 0.1250, respectively. In *H. gambianus*, TF loci had three alleles A (0.500), B (0.4545) and C (0.0455); ALB loci two alleles A (0.4545) and B (0.5455); and EST loci three alleles A (0.3500), B (0.500), and C (0.1500). The chi square test for each locus in both species shows no significant difference from what is expected under HWE conditions (Table 2).

Table 2: Allele frequencies and Hardy-Weinberg Equilibrium at three blood protein loci in Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) in Ibadan, Nigeria

Loci	Allele			HWE	
	A	B	C	Chi-square (χ^2)	Probability (p)
<i>Funisciurus anerythrus</i>					
TF	0.4118	0.5294	0.0588	0.6039	0.8955
ALB	0.5000	0.5000		0.1227	0.7261
EST	0.3750	0.5000	0.1250	0.3455	0.9513
<i>Heliosciurus gambianus</i>					
TF	0.5000	0.4545	0.0455	2.4485	0.4847
ALB	0.4545	0.5455		0.0303	0.8618
EST	0.3500	0.5000	0.1500	3.4508	0.3272

TF-Transferrin; ALB-Albumin; EST-Esterase

The number of alleles (n_a) in both species were 3 (TF), 2 (ALB), and 3 (EST), culminating in allelic diversity (A) of 2.6667 (Table 3). The effective number of alleles (n_e) were expectedly lower than the n_a (ranging from

1.9836 to 2.5316) except at the ALB loci in *F. anerythrus* where they both had the same value (2.0000). The Shannon information index showed that most of the loci were highly informative indicating the polymorphism across the loci with an overall mean of 0.8454 ± 0.1420 and 0.8444 ± 0.1548 in *F. anerythrus* and *H. gambianus*, respectively. Observed heterozygosity (H_o) was higher than the expected heterozygosity (H_e) at all the loci for both species except at the EST loci in *H. gambianus* (Table 3). Average gene diversity was 0.5635 and 0.5745 in *F. anerythrus* and *H. gambianus*, respectively (Table 3).

Table 3: Measures of genetic diversity at three blood protein loci in Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) Ibadan, Nigeria

Locus	Allele			N	n _a /A	n _e	I	Heterozygosity	
	1	2	3					H ₀	H _e
<i>Funisciurus anerythrus</i>									
TF	A	B	C	34	3.0000	2.2061	0.8687	0.6471	0.5633
ALB	A	B		36	2.0000	2.0000	0.6931	0.5556	0.5143
EST	A	A	C	32	3.0000	2.4615	0.9743	0.6250	0.6129
			Mean	34	2.6667	2.2225	0.8454	0.6092	0.5635
			St. Dev	2.0000	0.5774	0.2312	0.1420	0.0478	0.0493
<i>Heliosciurus gambianus</i>									
TF	A	B	C	22	3.0000	2.1802	0.8455	0.7273	0.5671
ALB	A	B		22	2.0000	1.9836	0.6890	0.5455	0.5195
EST	A	A	C	20	3.0000	2.5316	0.9986	0.6000	0.6368
			Mean	21	2.6667	2.2318	0.8444	0.6242	0.5745
			St. Dev	1.1547	0.5774	0.2776	0.1548	0.0933	0.0590

TF-Transferrin; ALB-Albumin; EST-Esterase; N-Sample size; n_a - Number of alleles; n_e - Number of effective alleles; A- Allelic diversity; I-Shannon's information index; H_o - Observed heterozygosity; H_e - Expected heterozygosity

The wright's F-statistics (Wright (1951) is shown in table 4. The average F_{is} , F_{it} and F_{st} were -0.1274, -0.1241 and 0.0029, respectively. Estimated gene flow between the two populations was 86.5312 (Table 4) Nei's genetic distance between the two species was 0.0070 (Figure 1)

Table 4: Wright's F-statistics and Gene flow analyses at three blood protein loci in Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus*) Ibadan, Nigeria

Locus	N	F_{is}	F_{it}	F_{st}	N_m^*
TF	56	-0.2631	-0.2553	0.0062	40.0922
ALB	58	-0.1056	-0.1033	0.0021	120.5000
EST	52	-0.0219	-0.0214	0.0005	479.5000
Mean	55	-0.1274	-0.1241	0.0029	86.5312
S.E.	1.7638	0.0707	0.0685	0.0017	

* N_m —Gene flow estimated from $F_{st} = 0.25(1 - F_{st})/F_{st}$; TF-Transferrin; ALB-Albumin; EST-Esterase; N- Sample size

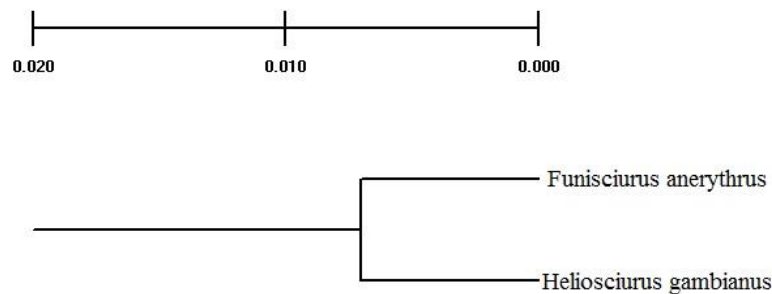


Figure 1: Dendrogram showing similarity between the two squirrel species (*F. anerythrus* and *H. gambianus*) in Ibadan, Nigeria based on three blood proteins analysis

Discussion

Results of this study revealed polymorphism across all the three loci examined in both species. A total of eight (8) alleles were found at the transferrin, albumin and esterase loci. In a study conducted by Cothran *et al.*, (1997) the TF locus was observed to be monomorphic in the *Spermophilus tridecemlineatus* (a species of ground squirrels) with the “A” allele fixed in all studied populations. The locus was however polymorphic in *S. spilosoma* with the “B” allele being most predominant in the species. The esterase locus was observed to be highly polymorphic with six genotypes controlled by three alleles occurring in both species. Previous studies by Cothran *et al.* (1977) and Arbogast *et al.* (2005) supports the highly polymorphic nature of the esterase loci as their results show esterase occurring in various molecular forms with as many as six alleles controlling various genotypes with varied levels of expression. At the albumin locus, two alleles (A and B) controlling three genotypes were observed in both species. Similar results were obtained in a study of rock squirrel (*Spermophilus variegatus*) (Gustafson-Ropski *et al.* 1989) where the albumin locus was equally observed to be diallelic. The chi square values show no significant deviations from HWE at any of the loci in both species, indicating the absence of the influence of evolutionary forces, such as mutation, migration and selection. There seems to be random mating within the two species. High levels of genetic variability suggest that neither of the species in this study have experienced historical bottlenecks capable of reducing genetic variation necessary for continuity of adaptive evolutionary processes.

According to Frankham *et al.* (2010), expected heterozygosity is often reported for outbreeding species because it is less sensitive to sample size than observed heterozygosity. The expected heterozygosity in all the tested loci together with the gene diversity measure in this study are indicative of healthy outbreeding species (populations).

Nei (1972) states that in a variety of species, the genetic distance between them is approximately 1.0 for interspecific comparisons, around 0.1 for subspecies and 0.01 for local races. Considering the results from this study, genetic distance of 0.007 between the two species (*F. anerythrus* and *H. gambianus*) is far below expectation. This may be due to the sample size and limitation of protein markers.

The most commonly used measure of summarizing structure within and between populations are the F statistics developed by Wright (Wright, 1951; 1978). F statistics partition genetic variability as measured by levels of heterozygosity into components of within population and between population variations (McVean, 2001). The negative values of F_{IS} in all the loci is an indication of excess heterozygosity, though small effective population size could also contribute to this (Allendorf *et al.*, 2013). The fixation index (F_{ST}) between both species at all loci are closer to zero than 1, as such both species in this study have allele frequencies that are not too divergent from each other. This rules out inbreeding within each species.

Conclusion

Conservation of natural population is largely dependent on genetic diversity. *F. anerythrus* and *H. gambianus* species are being hunted and used as a source of protein in Nigeria and are being persecuted due to their involvement as disease vectors and crop/household pests. Habitat destruction and human encroachment are also threats to their conservation. Despite these a considerable level of genetic diversity still exists among them. The absence of significant deviations from the Hardy Weinberg Equilibrium is indicative of random mating and the absence of other evolutionary forces. Inbreeding is also not occurring in both species. It is recommended that DNA marker be used to unravel the degree of genetic polymorphism within and between these species of squirrel.

Acknowledgment

We like to appreciate Mr E. O. Fijabi of the Department of Animal Science, Faculty of Agriculture, University of Ibadan, Nigeria for helping out during the data collection.

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