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Vol. 15(25), pp. 1306-1314, 22 June, 2016
DOI: 10.5897/AJB2015.15154
Article Number: 103271B59045
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity in two populations of *Limicolaria aurora* (Jay, 1839) from two ecological zones in Nigeria

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Received 7 December, 2015; Accepted 6 May, 2016

Limicolaria aurora **belongs to the group of land snails commonly called garden snails. This study seeks to use shell morphology and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to examine gross morphological differences in populations of *L. aurora* from New Bussa (guinea savannah) and Benin City (tropical rain forest) in Nigeria and possibly delimit the populations into sub species. A total of one hundred and ten specimens of *L. aurora* made up of fifty five individuals from each of the two ecological zones were collected randomly for the study. Data on shell parameters including: height of shell (SH), width of shell (SW), aperture height (AH), aperture width (AW), spire length (SL), and first whorl length (1WL) measured on each snail were subjected to one way analysis of variance (ANOVA). Principal component analysis (PCA) and canonical variates analysis (CVA) were performed on the data using PAST statistical software. DNA which was extracted from the muscular tissues of the foot of eight individuals from each location using cetyltrimethylammonium bromide (CTAB) method was subjected to RAPD-PCR. Amplification of the DNA was done using five primers (OPB-12, OPB-18, OPH-08, OPD-11 and OPS-13). Analyses showed significant differences (P<0.05) in *L. aurora* populations within and between the vegetation zones revealing great heterogeneity in the populations. Both PCA and CVA clusters did not separate the populations into distinct sub-populations. SH was the most variable morphological characteristic and consequently the most suitable for the separation of *L. aurora* specimens into distinct populations. All the primers used in the amplification of the DNA produced polymorphic bands. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster diagram revealed two major clades within the snail populations with about 74% similarity. The study showed that RAPD-PCR analysis is more suitable for delimiting populations of *L. aurora* than morphometrics and that the basis for gross morphological differences in these populations might not only be environmental but also genetic factors.**

Key words: Achatinidae, biodiversity, environmental factors, morphometrics, phylogenetics, shell, sub-populations, variation.

INTRODUCTION

The genus *Limicolaria* belongs to the group of land snails of the family Achatinidae. The genus consists of snails that are commonly referred to as garden snails. The species of this genus that have been reported in the West African sub region include *Limicolaria aurora* and *Limicolaria flammea*. The genus *Limicolaria,* along with other achatinids has been reported to be serious crop pests and when introduced to a new environment can unbalance local ecosystem. Many achatinids have attained pest status even within their native range when

the habitat is modified for human habitation and farming (Raut and Barker, 2002).

L. aurora however, is not only agriculturally disastrous, but can as well be advantageous; the snail meat can be a good source of protein in fish feeds (Madu et al., 2006). The shell could be ground and used as a source of calcium carbonate in the formulation of animal feed. Land snail shells serve as a calcium source for various organisms that feed on them, especially for eggshell formation, muscle contraction, and osmo regulation. The alkalinity of the crushed shell is also useful in reduction of soil acidity (Graveland and van der Wal, 1996; Hotopp, 2002).

Moreover, in land snails, shell forms (morphology) often provide relevant morphometric data used in taxonomy and phylogenetic inference as well as in population biology. Shell morphometry is a useful tool in mollusc taxonomy and ecology. It has been used to discriminate between species, to recognize intraspecific morphological variation and to associate shell variations with environmental conditions and geographical distribution (Chiu et al., 2002; Wullschleger and Jokela, 2002; Pfenninger et al., 2003).

The extent of genetic diversity in natural population results from an interplay between forces generating local genetic differentiation and forces generating genetic homogeneity. Hence, the level of population genetic variation can be influenced by such processes as founder events, genetic drift, mutation, recombination, migration (gene flow) and selection. These forces may also interact with other factors, such as life-history traits, breeding system, dispersal and other ecological and evolutionary processes to determine the patterns of genetic structuring that are observed in the field (Gow et al., 2004).

In South Western Nigeria, biodiversity information of some achatinid snails exist (Oke and Odiete, 1996; Oke and Alohan, 2006; Oke, 2007; Oke et al., 2007, 2008; Oke and Chokor, 2010; Oke, 2013). However, genetic diversity data is lacking on most of the land snails. This study therefore combines the morphological analysis of the shell with random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) data to understand the basis for gross morphological differences in *L. aurora* populations and their possible delimitation into sub species.

MATERIALS AND METHODS

A total of 110 specimens (55 specimens from each location) were randomly collected for the study. The specimens collected were identified on the field according to Crowley and Pain (1970).

Specimens of *L. aurora* were collected from New Bussa (guinea savannah) and Benin City (tropical rain forest). New Bussa is a city in Niger state, Nigeria. The city sits at longitude 4°31'E (4.5167°E) and latitude 9°53'N (9.8833°N), while it is located at 40 km south of the original Bussa town. The city is located 499 feet above sea level. The climate is sub-humid, classified as tropical savannah and the average annual precipitation is 1,109 mm.

Benin City is the capital city of Edo State, Nigeria, sits at longitude 5°37'39"E (5.6275°E) and latitude 6°20'06"N (6.3350°N). Benin City is 259 feet above sea level. Climate is tropical with average annual precipitation of 2,025 mm. Figure 1 shows the map of Nigeria with the study areas.

Morphometric studies

The shell of each snail was described by six parameters as shown in Figure 2. The parameters measured are: height of shell (SH), width of shell (SW), spire length (SL), aperture height (AH), aperture width (AW), and 1st whorl length (Awodiran et al., 2012).

Measurements of each morphometric character were transformed to shell width (SW) to remove size-effect by growth allometry using Reist (1985) as described in Gunawickrama (2007) methods. Width corrected data were then analyzed by multivariate statistical method. Principal component analysis (PCA) and canonical variates analysis (CVA) were performed on the data using the software, PAST (Hammer et al., 2001). To find out the morphometric factors that can discriminate among the two populations, PCA was used in which factor loadings based on eigen values were used to determine the morphometric factors.

RAPD studies

DNA extraction

Total genomic DNA was extracted from foot tissue of the snails. Muscular foot sample was collected from the snails using a new razor blade. Different razor blade was used for each of the specimens. This was preserved in 80% ethanol and kept in the refrigerator till use. After which 0.1 g of the tissue sample was later collected from each of the preserved samples. DNA was extracted from these tissues using a CTAB protocol (Bucklin, 1992). DNA samples were then stored in a refrigerator. DNA concentration of all samples was measured on a spectrophotometer at 260 and 280 nm and the DNA purity was determined. The quality of DNA was detected by Agarose gel electrophoresis. Genomic DNA was used in PCR amplification using RAPD markers. A negative PCR controls was run to overcome one of the major limitation of RAPD marker which is the generation of artefact fragments and bands that appeared consistently in the negative controls were removed from the final analysis. Also, the dominance marker limitation was managed by estimating eight samples per locus for each primer coupled with the large number of polymorphism derived from the analysis. Code sequence and nucleotide lengths used in RAPD studies are shown in Table 1.

PCR reaction mix

The reaction mix was carried out in 20 µl final volume containing 50 to 60 ng genomic DNA, 0.1 µM of the primers, 2 mM $MgCl_2$, 125 µM of each deoxy Nucleotide Triphosphate (dNTP) and 1 unit of Taq DNA polymerase. The thermocycler profile has an initial denaturation temperature of 94°C for 3 min, followed by 45 cycles

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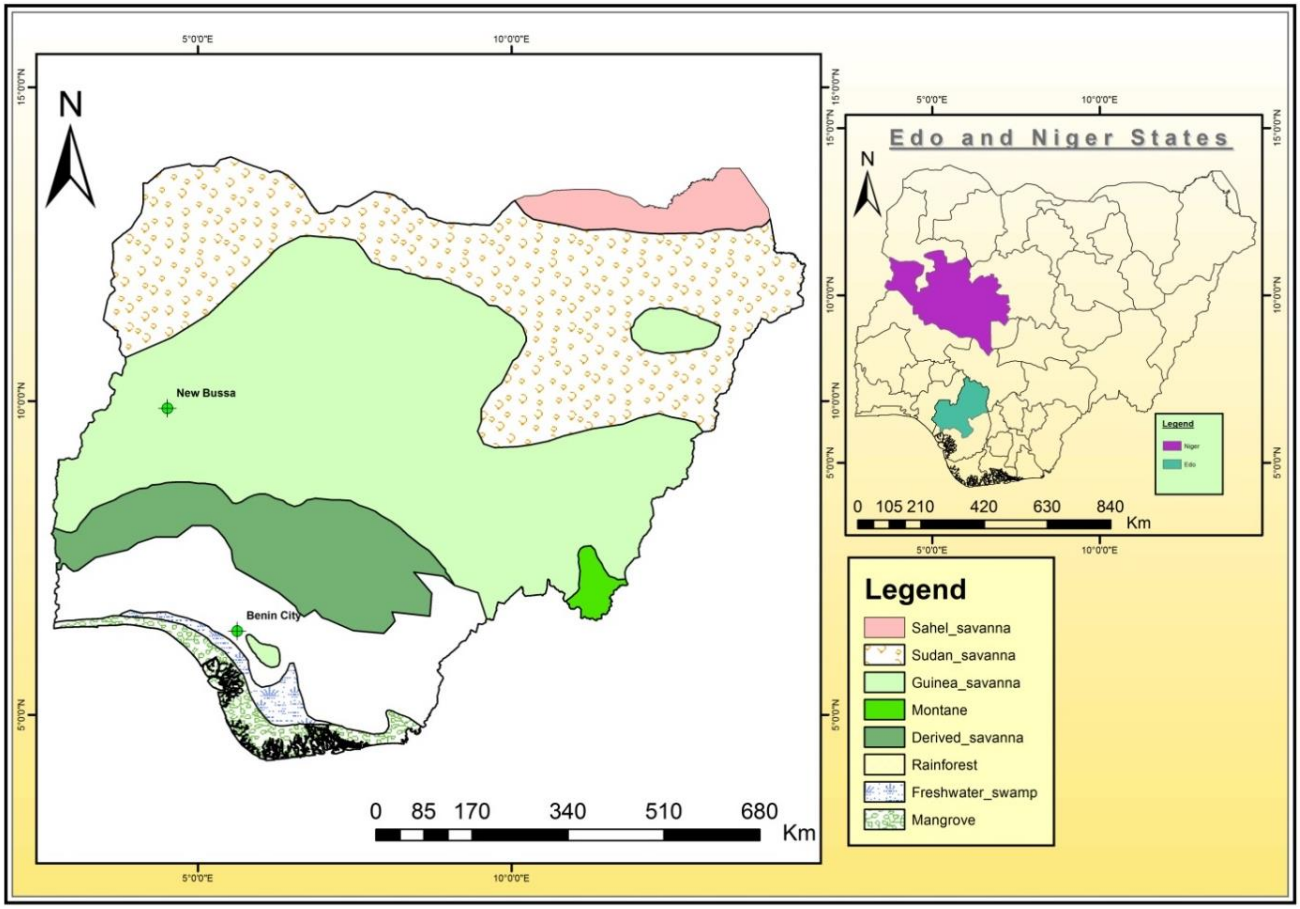

Figure 1. Map of Nigeria showing the study areas of Benin City (Edo State) and New Bussa (Niger State).

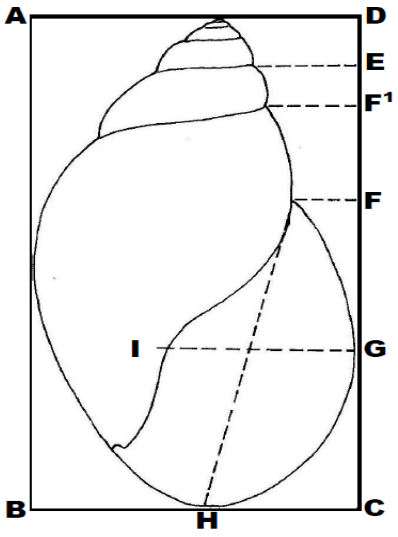

Figure 2. Measurements of shell morphology used in morphometric analysis (AB=Height of shell, SH; BC=Width of shell, SW; DF= Spire length, SL; FH= Aperture height, AH; GI=Aperture width, AW; and EF1=1st whorl length, 1WL).

of denaturation temperature of 94°C for 20 s, annealing temperature of 37°C for 40 s and primer extension temperature of 72°C for 40 s, followed by final extension temperature at 72°C for 5 min.

Gel electrophoresis

PCR amplicon electrophoresis was carried out by size fractionation on 1.4% Agarose gels. Agarose gels were prepared by dissolving and boiling 2.8 g agarose in 200 ml 0.5X Tris Boric Ethylenediamine Tetra-acetic Acid (TBE) buffer solution. The gels were allowed to cool to about 50°C and 10 µl of 5 mg/ml ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, the PCR amplicon was loaded in the well created. Electrophoresis was done at 100 V for 2 h. The DNA was visualized and photographed on UV light source.

Band scoring and data analysis

Each gel was analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring can be done based on the banding profiles which is clear and transparent. The scores were then pooled for constructing a single data matrix. Purity of the DNA was carried out using spectrophotometer by measuring absorbance at 260 and 280 nm. The RAPD banding profiles were

Table 1. Primer sequences and the percentage polymorphism.

S/N	Primer name	Sequence	No. of polymorphic loci	No. of monomorphic loci	Total No. of Loci	Polymorphism (%)
1	OPB – 12	CCTTGACGCA	8	1	9	89
2	OPB – 18	CCACAGCAGT	10	1	11	91
3	OPH – 08	GAAACACCCC	8	1	9	89
4	OPD- 11	AGCGCCATTG	6	nil	6	100
5	OPS – 13	GTCGTTCCTG	9	nil	9	100
Total	-	-	41	3	44	

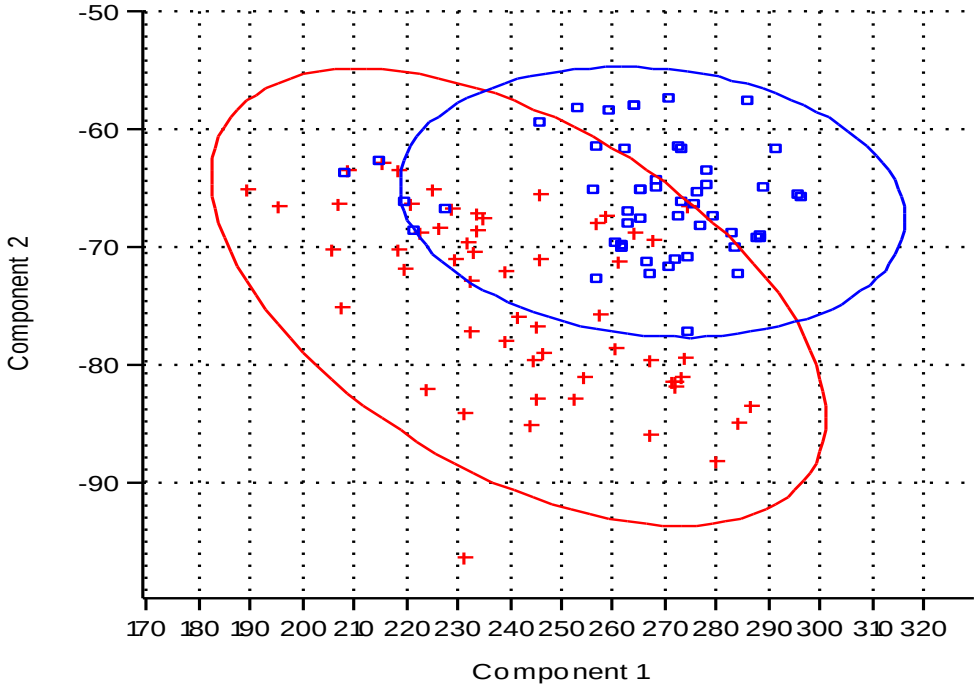

Figure 3. Principal component analysis of shell measurements of *Limicolaria aurora* from the two populations showing overlap of data between populations from New Bussa and Benin city (+ represents New Bussa, while □ represents Benin City).

visually scored for all the DNA samples and for each primer. Similarity coefficients were calculated across all the possible pair wise comparisons of snail samples among populations, using the formula:

$$Sxy = \frac{2nxy}{nx + ny}$$

where nxy is the number of common bands shown in both individuals x and y, and nx and ny are the total numbers of bands observed in individual x and y, respectively (Nei, 1978). As a means of providing a visual representation of genetic relationships, a dendrogram was constructed based on the similarity coefficient values (1 - Sxy) between pairs of snail samples. The NTSYS-PC software program was used to estimate genetic similarities with the Jaccard's coefficient (Rohlf, 2000) and a dendrogram was constructed using the Unweighted Pair Group Method of Arithmetic Averaging (UPGMA) employing the Sequential, Agglomerative, Hierarchical and Nested clustering module (SAHN).

RESULTS

Morphometric studies

Figure 3 is the PCA diagram of the morphometric measurements of *L. aurora* from the two locations studied. The clusters produced overlapped. Figure 4 shows the relationship between shell characteristics and the loadings on PCA. This shows that Height of Shell (E) is the characteristic responsible for most of the variation among

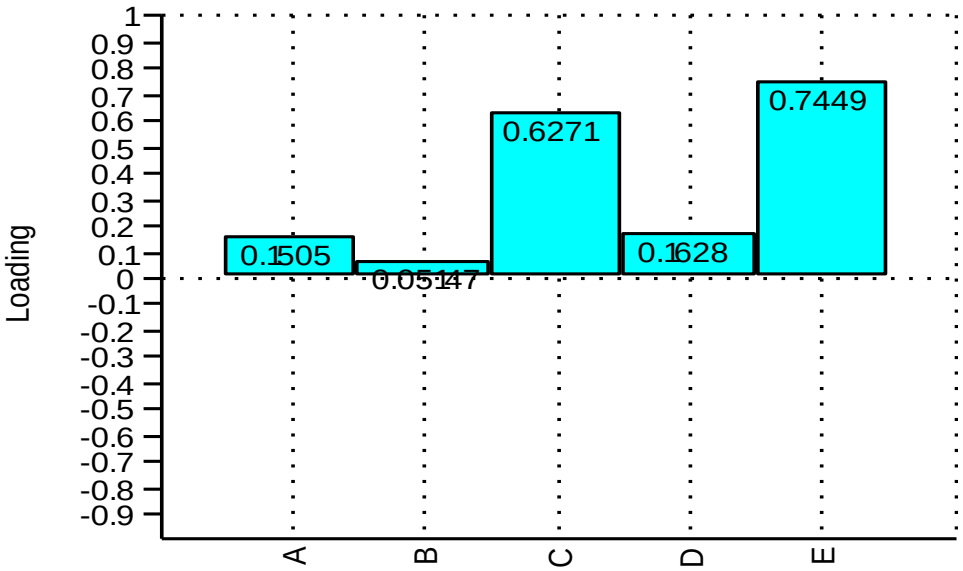

Figure 4. Snail shell characteristics and their loadings on PC1 of the principal component analysis showing height of shell as the character most responsible for variation among the populations studied. A: Aperture height (AH); B: aperture width (AW); C: spire length (SL); D: 1st whorl length (1WL); E: height of shell (SH).

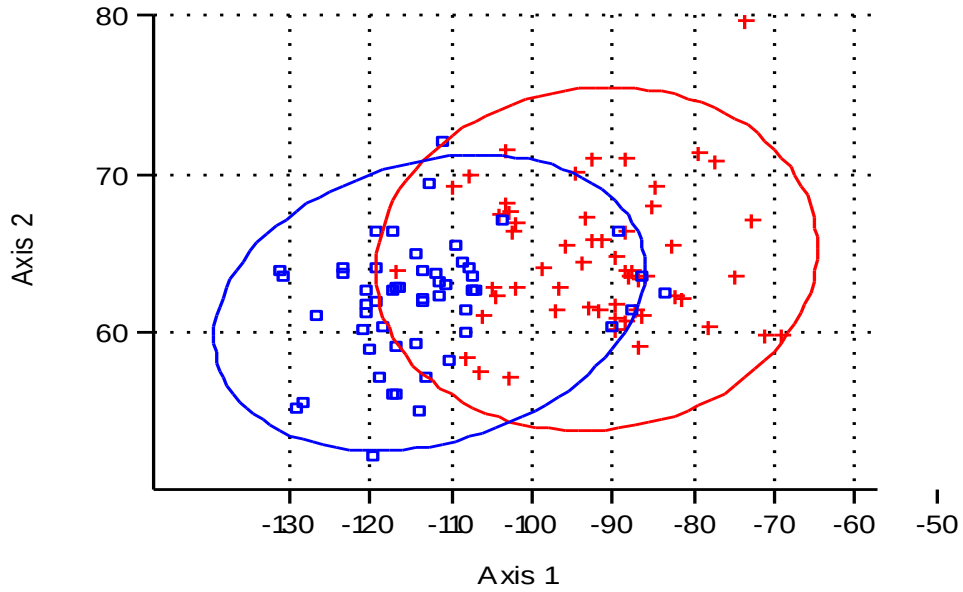

Figure 5. Canonical variates analyses of shell measurements of *L. aurorafrom* New Bussa and Benin City showing homogeneity of characters (+ represents New Bussa, while □ represents Benin City).

the populations of *L. aurora*, followed by spire length (C) while aperture width (B) is the characteristic which contributes least to the variation. CVAs were carried out to determine if there are significant differences among the populations of *L. aurora*. Figure 5 shows the CVAs of the shell of *L. aurora.* The CVA plots showed overlapping of clusters of specimens from the locations studied.

Data for these characteristics were also compared in box plot (Figure 6). The box plots represent summaries of measurements of the two highest loading characters (that is, SH and SL) and when subjected to ANOVA, these measurements were not significantly different ($P > 0.05$)

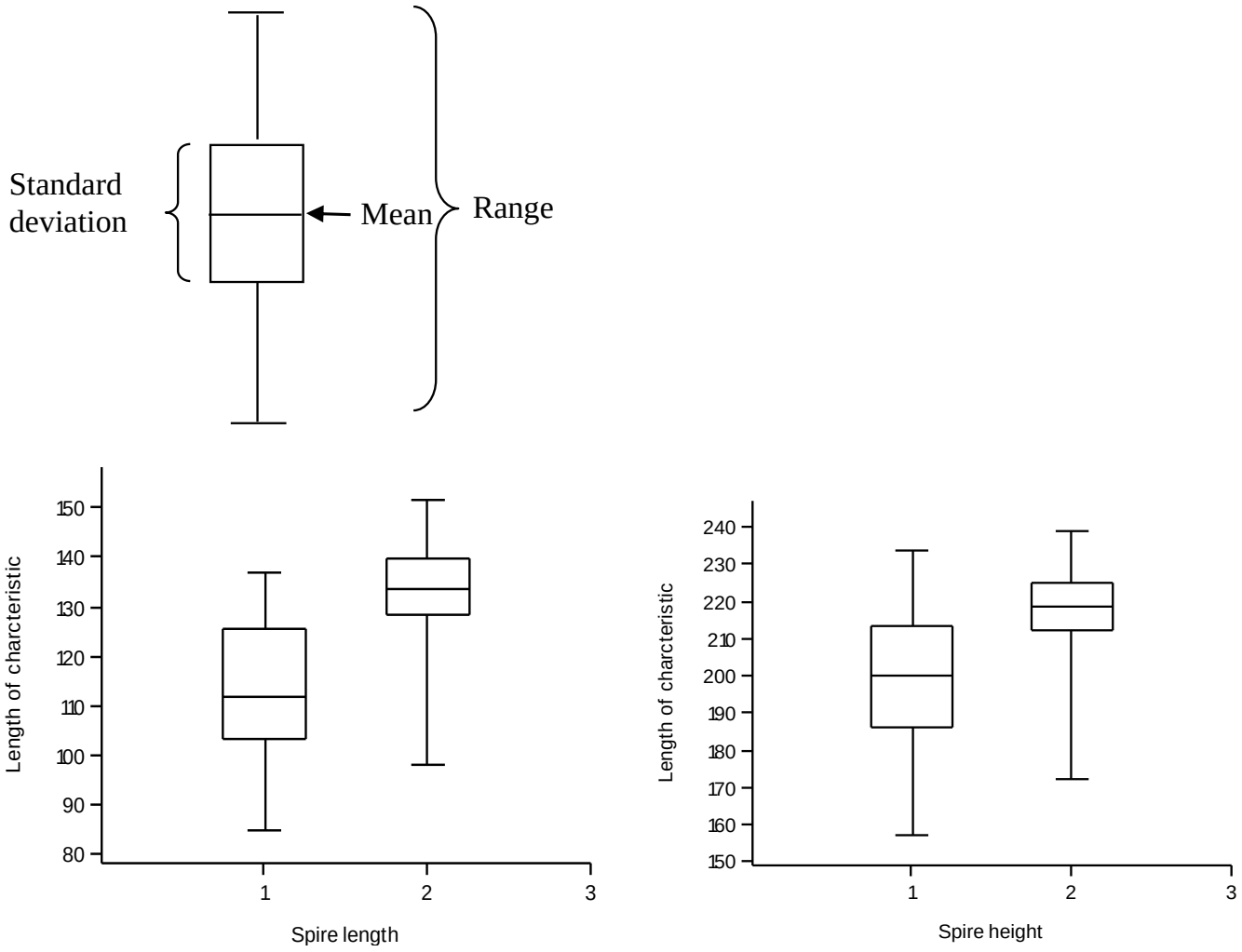

Figure 6. Box plots of morphometric characteristics *for Limicolaria aurora* from 1= New Bussa and 2=Benin City.

for both SH and SL.

RAPD result

The primers used for this study, their sequences as well as their percentage polymorphism are shown in Table 1. All the primers produced good RAPD amplifications with varying bands. Forty four loci were generated from the five primers, of these 41 (93%) were polymorphic, while three (7%) were monomorphic. The average number of loci per primer is nine.

The total number of RAPD bands produced by the primers for the two locations was 178. Samples of *L. aurora* from New Bussa has the highest band score of 103 (65% polymorphism), followed by Benin City population with the band score of 75 with 96% polymorphism (Tables 2 and 3). There was no band that was population specific. OPB-18 primer generated the highest number of bands among all the primers, while OPB-11 produced the least number of bands. The UPGMA cluster diagram revealed two major genotypic groups within the snail populations with about 74% similarity (Figure 7). The first clade consists of all the samples of *L .aurora* from New Bussa, while the second clade consists of all the samples from Benin City.

DISCUSSION

Morphometric studies

Morphological homogeneity was evident between the two populations of *L. aurora* as shown by overlapping of the clusters produced from both PCA and CVA. CVA did not separate the specimens into two distinct populations as shown by the overlapping of clusters (Figure 5). Among the shell characteristics measured, SH was the most variable and consequently the most suitable for the separation of the populations. Another significant

Table 2. Estimates of genetic variation.

Population	Number of polymorphic loci	Number of monomorphic loci	Total	Proportion of polymorphic loci (%)
New Bussa	17	9	26	65
Benin City	26	1	27	96
Combined	41	3	44	93

Table 3. Total band scored in each population.

Population	Total band score
New Bussa	103
Benin city	75
Total	178

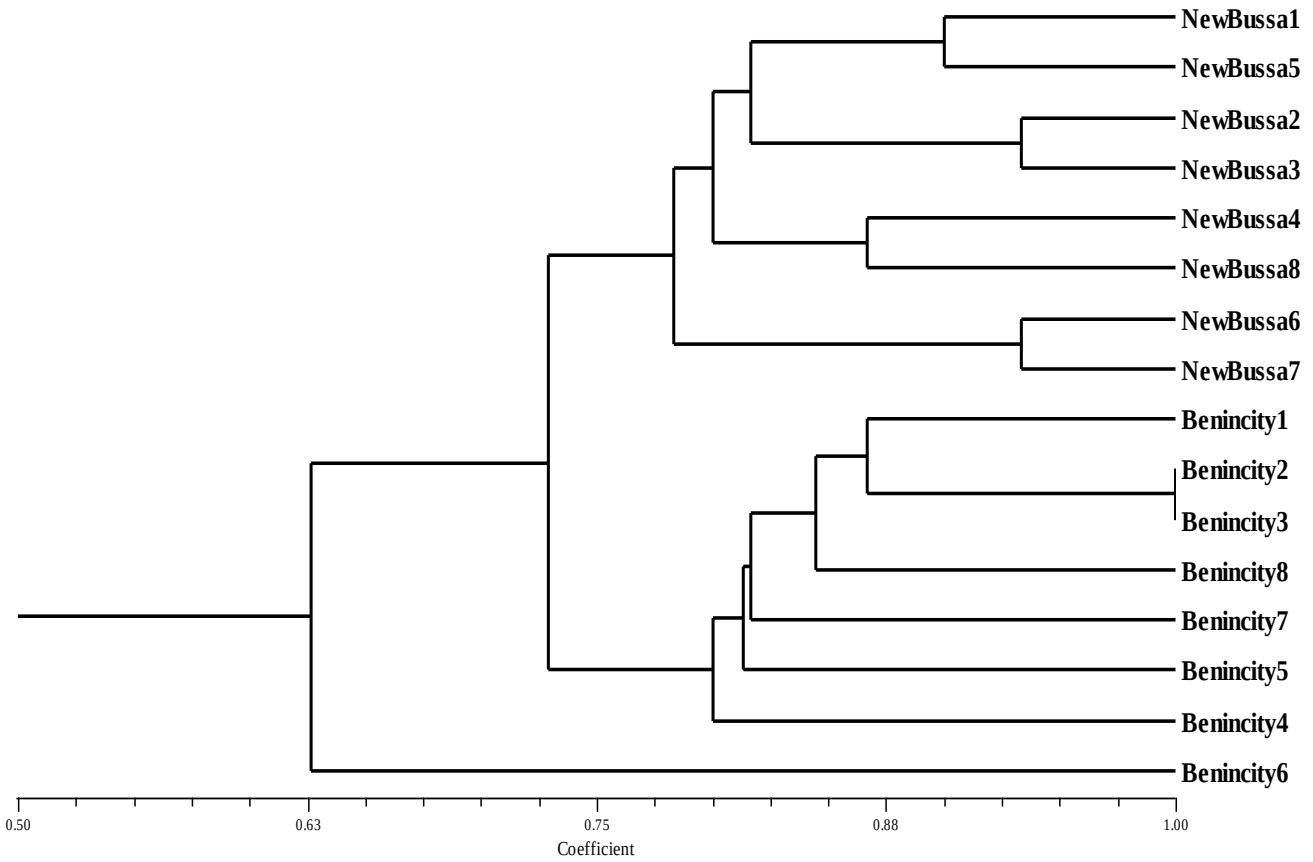

Figure 7. UPGMA dendrogram summarizing the data on differentiation between *Limicolaria aurora* populations according to RAPD analysis.

diagnostic characteristic was the SL.

Conchological variations observed on the shells of the *L. aurora* from different geographical locations may be due to many possible factors including genetic, biotic and abiotic factors. Since *L. aurora* exhibited morphological differences among populations, it is argued that the difference or variations in size can be attributed to environmental conditions as well as genetic factors. Dela et al. (2010) argued that variation in snail shells is not only genetic, but also affected by the growth rate and population density of the snails. It may also be possible that the diversity within populations observed could be

due to the numerous introduction and reintroduction of several gene pools of snails to the study areas by local snail marketers.

The variations in the snail's shell morphology could also indicate phenotypic plasticity or genetic differentiation. Plasticity influences the evolution and adaptive responses of organisms, because it can alter the relationship between the phenotype and the genotype (Trussell and Etter, 2001).

It is also possible that since the snails were collected from different geographical locations which have varied climatic and environmental factors, these may have direct effects on the snail shell shapes.

RAPD studies

Percentage polymorphism was high in the two populations studied. This was 96% in Benin City and 65% in New Bussa (Table 2). Analysis of the proportions of polymorphic loci and band sharing based similarity indices for within-location samples indicates that a relatively high level of genetic similarity exists in New Bussa population than in Benin City population, hence higher genetic diversity in Benin City population than New Bussa population. The higher within location genetic similarity and lower level of frequency of polymorphic loci and gene diversity estimates for New Bussa population could be an indication of comparatively closer relationship among individuals within the location.

Aestivation stage of the organism, humidity and temperature range in a habitat of the land snail are factors that were argued to have influenced variations in land snail (Vinic et al., I998; Albuquerque et al., 2009). Environmental stress such as drought could possibly have influenced genetic diversity in New Bussa with lower annual rainfall compared to Benin City. It may relate to reduced genetic variability in the population, genotype-specific survivorship, that is, individuals with particular genotypes which are tolerant to drought might survive while less tolerant genotypes might be wiped out, hence resulting in reduced genetic variability and diversity.

Moreover, breeding experiments have shown that a large part of the variation leading to the characterization of some morphotypes is genetically determined (e.g. the 'giant' form *Helix aspersa maxima* or the 'conical' form *Helix aspersaconoidea*), but within a given form, the variation in the trait itself is sometimes subject to a strong environmentally induced component (Madec and Guiller, 1993; Madec et al., 1998).

Genetic drift and natural selection are the two primary evolutionary mechanisms that cause population differentiation (Hufford and Mazer, 2003). Natural selection by ecological factors will result in development of ecological adaptation or ecotypes. It remains to be determined whether the observed population differentiation in this study resulted from any natural selection.

The UPGMA cluster diagram revealed two major clades. The first cluster comprises of New Bussa specimens, while the second cluster comprises mainly specimens from Benin city. UPGMA cluster separation of the specimens into two distinct groups reveals that the two populations are genetically distinct from each other. Estimate of genetic distance between the populations is 0.9245.

In conclusion, DNA based analysis unlike morphometrics employed in this study differentiates the populations of *L. aurora* under study into two sub populations which may imply that the basis of gross morphological differences in these populations might not be due to environmental factors only but also genetic. The RAPD analysis differentiates the populations better, revealing two distinct groups. According to Thorpe and Sol-Cave (1994), the average genetic distance for conspecific populations is 0.05 (range: 0.02 to 0.07), the high genetic distance (0.9245) between the populations in this study revealed that the two populations of *L. aurora* are not categorized in conspecific value, hence a subspecies of *L. aurora* is suspected. Further studies involving the use of DNA sequence analysis is needed to maximize the efficiency of this study.

Conflicts of Interest

The authors declare no conflict of interest.

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