Molecular characterization of giant African land snails using polymerase chain reaction - random amplified polymorphic DNA fingerprinting

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Abstract Random amplified polymorphic DNA (RAPD) markers were used to characterize the genetic diversity and relatedness among different populations of Archachatina marginata, a highly relished source of protein in West Africa. Sixteen (16) accessions comprising nine (9) black and seven (7) albino-bodied forms were collected randomly from three different locations in Nigeria and genetic differentiation and morphometric studies conducted. In the genetic analysis, a total of 84 reproducible bands were produced using three (3) oligonucleotide primers. Of these, 79 amplified bands (94.04%) were found to be polymorphic with an average of 28 bands per primer while the remaining 5 were monomorphic loci. Similarly, an analysis of morphological traits resulted in the division of the entire population into 2 major groups based on the geographical distribution that generally reflected expected trends between the genotypes. Among all the morphometric characters, the highest mean value was observed in shell spire (23.38) while the lowest mean value was observed in stripes on the blossom end (1.31). In conclusion, average linkage cluster analysis revealed a high level of genetic diversity and heterogeneity among the snail accessions.

Keywords Archachatina marginata; Genetic variation; Heterogeneity; RAPDs

Introduction

Archachatina marginata, commonly known as the giant African land snail, appears to be the largest terrestrial gastropod found anywhere in the world with a relatively long, conical shell. Commonly ubiquitous in eastern Africa (Mead, 1961), it may have originated from the coastal areas of Kenya and Tanzania, from where it may have spread through anthropological transport to other parts of the continent and is presently distributed all over sub-Saharan Africa (Raut and Barker, 2002) with major colonies in West and East Africa (Venete and Larson, 2004). Apart from Africa, the giant land snail has been accidentally introduced to many parts of the world for different purposes including the preparation of folk medicine and food (Raut and Barker, 2002). This giant terrestrial snail is commonly found in areas having tropical climate with warm, mild year-round temperature and high humidity (Venete and Larson, 2004). As in other non-native species, A. marginata populations generally increase dramatically after introductions (Craze and Mauremootoo, 2002), which has caused damage to plants and is, therefore, considered a significant pest of agricultural crops in some climes (Smith and Fowler, 2003).

In parts of West Africa, these snails are considered the best local delicacies and as such are highly prized. Giant African land snail meat is tender, tasty and highly nutritious with no cholesterol. Compared to that of other animals, the meat which is generally low in fat (0.05 – 0.08%) contains high levels of protein (37 - 51%), iron content (45 - 59mg/kg) and almost all the essential amino acids required by man (Adelye, 1996). In West African folk medicine, the bluish fluid obtained when the meat has been removed from the shell is believed to be good for the development of infants. It is also believed in some traditional settings that snail meat contains pharmacological properties of value in counteracting high blood pressure, heart-related diseases, hypertension and rheumatism
(Abere and Lameed, 2008). The high iron content of snail meat is considered important in the treatment of anaemia and in the past, the meat was recommended as a means of combating ulcers and asthma in many local communities. In addition to the nutritional value of the meat, recent studies have demonstrated that a glandular substance from edible snails causes agglutination of certain bacteria, which could cause ailments such as whooping cough.

Though the meat is usually light black in colour, lately a completely different variety of giant African land snail, with albino body pigmentation, has become quite commonly noticeable in many parts of the forest zone of Nigeria, especially in the south eastern part, where the gastropods have, naturally, become quite a source of curiosity to many people. Intriguingly, these albino snails appear to be predominating, as they are not consumed as food due to the common belief that they are poisonous and there is something undesirable or freakish about them (personal observation).

To exploit microbial, plant or animal genetic resources it is important that a detailed knowledge of the amount of genetic variation that exists within the species is known. Molecular markers, which help in the detection of differences in the genetic information carried by different individuals, are highly useful and can be used as valuable tools to assess and exploit genetic diversity, varietal classification, and germplasm identification of the genetic variability that exists within species for purposes of their improvement. Of the different molecular markers currently available, random amplified polymorphic DNA (RAPD) fingerprinting, in which random fragments of DNA are amplified from DNA samples using short, arbitrary primers, is highly preferred by many researchers as one of the effective methods for identification of genetic diversity amongst populations of same species (Jordan and Godboy, 2000; Tsuda and Ide, 2004). Besides that, RAPD markers also show levels of polymorphism similar to isoenzyme markers and can also target and amplify a large number of loci (Bartish et al., 1999; Lez et al., 2002).

Although RAPD markers have their limitations and drawbacks, they are considered as one of the most effective genetic markers used in recent times as they serve as powerful tools for discerning different gene loci within plant and animal germplasm and for studying evolutionary relationships between species. This technique requires no prior knowledge of the genomic DNA as it needs only small amount of DNA (Hadrys et al., 1992). Moreover, polymorphism can be detected in closely related organisms using RAPD markers.

Generally, there is paucity of information on the genetic diversity of giant African land snails. A search of the literature shows that an assessment of genetic diversity amongst giant African land snail varieties by DNA-based markers has not been undertaken by any research group. Consequently, the current study was designed to determine if there were genetic differences amongst the black and albino-bodied forms of giant African land snails seen in different parts of the lowland humid tropics in southern Nigeria using RAPD fingerprinting.

1 Materials and Methods

1.1 Sample size and morphometric analysis

Sixteen (16) giant African land snails consisting of nine (9) samples with meat that was black and seven (7) that was albino were procured from three different locations (Ekpoma in Edo State, 6.75° N, 6.13° E; Udua Akpananem in Akwa Ibom State, 5.03° N, 7.92° E; and Calabar in Cross River State, 4.95° N, 8.33° E) of Nigeria. The snails were kept alive and maintained under moist conditions to ensure their survival. They were fed with fresh leaves, fruits and succulent plant parts for two weeks before being subjected to morphometric and genetic analyses.

Morphometric evaluation of shell variations (size, shape and colour) was performed using multivariate methods. The shell of each snail was described by five measurements using veneer caliper (MITUTOYO 500-752-10 Digital caliper IP67 ABS 6’’/150MM; Spain). The shell characteristics evaluated in the current study included the height of shell, width of shell, spire length, aperture height, and aperture width and was conducted according to Madec and Bellido (2007). These five direct measurements of the shell characteristics of the snails was performed to determine whether the species under study can be
reliably distinguished using morphometric methods on the basis of the striking polymorphisms exhibited by their shells.

**1.2 DNA extraction and quantification**

DNA was extracted from the meat of each sample according to the CTAB method of Saghai-Maroof et al. (1984), with minor modifications containing 2% (v/v) β-mercaptoethanol (added just before use) and 2% (w/v) polyvinylpyrrolidone, followed by RNase treatment for the removal of contaminating RNA. The purity of the extracted DNA was examined by agarose (0.8%) gel electrophoresis while its concentration was determined by UV-visible spectrophotometer (UV-1601, Shimadzu, Japan).

**1.3 Screening of RAPD primers and polymerase chain reaction (PCR) amplification**

Twenty of the 50 RAPD primers ordered from Eurofins Genomics™ (https://www.eurofinsgenomics.eu/media/962761/rapd_10mer_kits_sequences.pdf) were randomly selected and screened for polymorphism on template DNA from land snail accessions. Three of the screened primers (OPB03, OPB04 and OPB08), developed by Hadris et al. (1992), showed significant polymorphism (as outlined in Table 1) and were used for further analysis (Figure 1).

![Figure 1 RAPD banding pattern of the sixteen (16) accessions of the snails](image)

**Table 1 Oligonucleotide primers selected for the study**

| Serial number | Primer | Sequence       |
|---------------|--------|----------------|
| 1             | OPB-03 | 5’AGACGTCCAC3’ |
| 2             | OPB-04 | 5’GGAAGTCGCC3’ |
| 3             | OPB-08 | 5’GTCCACACGG3’ |

Polymerase chain reaction amplification was performed in 25μl reaction mixtures containing 1x Taq, 100 ng genomic DNA, 3mm MgCl₂, 250μM of each dNTPs, 0.2 nm primer and 2 units of Taq polymerase (BioGene, USA) using MJ Research PTC-200 Thermal Cycler (GMI Inc., Ramsey, Minnesota, USA). The cycling conditions consisted of 1 cycle of 94°C for 5 min (initial denaturation), followed by 45 cycles of 94°C for 30 sec (denaturation), 35°C for 1 min (annealing), and 72°C for 2 min (polymerization), with a final extension of 7 min at 72°C. A negative control without the snail genomic DNA was kept for amplification along with each primer to check the quality of the primer and to avoid the possibilities of contaminations and primer dimers. The amplification products (10μl) were loaded in an ethidium bromide-added agarose gel (1.2%) for electrophoresis (at 125V) in 1x Tris-boric acid-EDTA buffer. The electropherograms were documented using gel Alpha Imager documentation analysis system (Alpha Innotech, USA). DNA bands were compared with 100 and 1500 bp DNA standard markers (BioGene, USA). The amplifications were performed twice with genomic DNA isolated independently to confirm the reproducibility.

**1.4 Data Analysis**

Basic statistical analyses method for morphological variations including the range, mean and their standard deviation were calculated to assess morphometric variation using the standard statistical procedures as described by Snedecor and Cochran (1994).

For the PCR-RAPD fingerprinting, all positive amplicons were treated as separate characters and scored for the presence (1) or absence (0) of bands. Polymorphism Information Content (PIC) was calculated based on the number of bands/primer, using the formula PIC = 1 – Pi/2, where Pi is the frequency of the ith band.

Cluster analysis and Principal Coordinate Analysis (PCA) were carried out in NTSYS (Numerical Taxonomy and Multivariate Analysis System) version 2.01i. Genetic similarities based on Dice coefficient were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. A
dendrogram was constructed by using UPGMA (Unweighted Pair Group Method with Arithmetic mean) to group individuals into discrete clusters.

2 Results
2.1 Morphological variations
Basic statistical information including the range, mean and standard deviation of each of the five direct measurements of the shell characteristics of the snails are shown in Tables 2 and 3. A wide range of sizes was found among the snails procured from Akwa Ibom and Cross River States, respectively. For example, the height of shell differed significantly amongst the samples from these two localities with standard deviations of 0.55 and 0.45, revealing greater heterogeneity amongst them. Such significant differences were also seen in the width (with standard deviations of 0.48 and 0.38) and aperture height of the shell (with standard deviations of 0.36 and 0.44, respectively), for the same samples.

| Variable                  | Akwa Ibom State (n = 4) | Edo State (n = 5) | Cross River State (n = 7) |
|---------------------------|-------------------------|-------------------|---------------------------|
| Height of shell (HS)      |                         |                   |                           |
| Range                     | 5.0 – 6.0               | 9.0 – 10          | 3.8 – 5.2                 |
| Mean                      | 5.48                    | 9.4               | 4.37                      |
| SD                        | 0.55                    | 0.38              | 0.45                      |
| Width of shell (WS)       |                         |                   |                           |
| Range                     | 1.3 – 2.2               | 3.8 – 4.3         | 1 – 2                     |
| Mean                      | 1.93                    | 4.08              | 1.39                      |
| SD                        | 0.48                    | 0.22              | 0.38                      |
| Spire length of shell     |                         |                   |                           |
| Range                     | 0.1 – 1.3               | 2.6 – 3.2         | 0.3 – 1.3                 |
| Mean                      | 0.8                     | 2.84              | 0.71                      |
| SD                        | 0.50                    | 0.23              | 0.27                      |
| Aperture height of shell  |                         |                   |                           |
| Range                     | 0.6 – 1.4               | 3 – 3.5           | 0.2 – 1.4                 |
| Mean                      | 1.1                     | 3.14              | 0.64                      |
| SD                        | 0.36                    | 0.28              | 0.44                      |
| Aperture width of shell   |                         |                   |                           |
| Range                     | 0.1 – 0.3               | 0.5 – 0.6         | 0.1 – 0.2                 |
| Mean                      | 0.18                    | 0.52              | 0.13                      |
| SD                        | 0.10                    | 0.05              | 0.05                      |

Note: Range and mean (mean) values are given in centimeters; n = number of accessions

Table 3 Mean values of the characteristics within each group of *Archachatina marginata* accessions

| Cluster | Location of collection | Shell height (cm) | Shell width (cm) | Spire height (cm) | Aperture height (cm) | Aperture width (cm) |
|---------|------------------------|-------------------|------------------|-------------------|----------------------|---------------------|
| 1.      | C/River                | 4.23              | 1.13             | 0.6               | 0.63                 | 0.13                |
| 2.      | C/River                | 4.57              | 1.73             | 0.87              | 0.67                 | 0.13                |
| 3.      | Edo                    | 10                | 4.3              | 3.2               | 3.5                  | 0.5                 |
| 4.      | Edo                    | 9.25              | 4.03             | 2.75              | 3.05                 | 0.53                |
| 5.      | A/Ibom                 | 5.63              | 2.13             | 1.03              | 1.23                 | 0.2                 |

The accessions in Clusters 1 and 2 were collected from Cross River State and their calculated means for the five characteristics were not significantly (p>0.05) different from one another. Those collected from Edo State are in Clusters 3 and 4 while those from Akwa Ibom State are in Cluster 5 (Table 3). One accession in Cluster 3 (B52) with light brown skin seems to be a hybrid between the pigmented and non-pigmented snails in Groups 1 and 2 and they tend to have the highest mean values in the five characteristics examined.
2.2 Genetic diversity of Archachatina marginata

The RAPD banding patterns of the sixteen (16) accessions are illustrated in Figure 1. The 3 primers used for PCR – RAPD fingerprinting were able to amplify the DNA from fifteen (15) out of the sixteen (16) snail accessions examined. A total of 84 bands were observed from these 15 accessions using the 3 primers. Out of the amplified products, 79 were found to be polymorphic with an average of 28 bands per primer. The number of bands produced per primer ranged from 26 (for OPB 03) to 30 (for OPB 04). Of these, the percentage of polymorphic bands was 85.90% (Table 4).

| Primer   | Sequence 5′-3′ | Total number of bands | Number of polymorphic bands |
|----------|----------------|-----------------------|-----------------------------|
| OPB-03   | CATCCCCCTG     | 26                    | 23                          |
| OPB-04   | GGACTGGAGT     | 30                    | 30                          |
| OPB-08   | GTCCACACGG     | 28                    | 29                          |
| TOTAL    |                | 84                    | 79                          |

2.3 RAPD polymorphism resulting from OPB 03

Twenty-six (26) scorable RAPD fragments were generated from amplification of genomic DNA of A. marginata (N = 16) with OPB-03. Three (3) fragments accounting for 11.54% of overall bands were monomorphic and were fixed in all investigated samples. Twenty-three (23) fragments accounting for 88.46% of overall RAPD bands generated by OPB 03 were polymorphic (Figure 1).

2.4 RAPD polymorphism resulting from OPB04

Thirty (30) scorable RAPD fragments were generated from the amplification of genomic DNA of A. marginata (N = 16) with OPB-04. All the thirty fragments accounting for 100% of overall bands were polymorphic and fixed in all investigated samples (Figure 1).

2.5 RAPD polymorphism resulting from OPB08

Twenty-eight (28) scorable RAPD fragments were generated from amplification of genomic DNA of A. marginata with OPB 08. Two (2) fragments accounting for 7.14% of overall fragments were monomorphic; twenty-six (26) fragments accounting for 92.86% were polymorphic and fixed in all investigated samples (Figure 1).

2.6 Genetic relationship

The genetic relationship among the accessions was examined by UPGMA cluster analysis. The UPGMA dendrogram (Figure 2) of the populations was constructed based on Nei’s unbiased genetic distance matrix. The dendrogram indicated that the five populations were divided into two groups. One group consisted of the individuals from populations A7 and A8, and the other six populations were clustered in another group that can be further divided into two sub-clusters. A1 population was clustered into one separate sub-group, and the other five populations (A2, A3, A4, A5 and A6) constituted the other sub-group.

The relationship between genetic distance and corresponding geographical distance among populations was tested by Mantel’s Test, and the result shows that there was no significant correlation (r = -0.1345, p = 0.71). This result showed that geographical distance was not the main reason for the genetic differentiation observed amongst the A. marginata populations evaluated here.
The dendrogram obtained from the average linkage cluster analysis showed that the accessions studied were grouped into five clusters which can be classified into two main groups (A and B). The first main group A consists of Accessions C32, C33, C23 and C21 in Cluster 1, C11, C14, and C12 in Cluster 2 and B52 in Cluster 3. These accessions were characterized by lack of pigmented skin and brown shell colour. While accessions in Cluster 1 completely lacked the albino pigmentation (that is, the light yellow skin colour), those in Cluster 2 were all albinos with the light yellow skin colour. Although accession B52 fell into this group, it was characterized by light brown skin pigmentation. The second main group B consists of accessions A22, A33, A42 in Cluster 4 and B22, B32, B42 and B11 in Cluster 5, respectively. This group was characterized by strong black pigmentation in skin and had black shell colour. While accessions in Cluster 4 generally had brown skin colour, those in Cluster 5 had black or brown skin colour.

3 Discussion

Information on sizes of populations and levels of genetic diversity of species of interest is important for the development of the appropriate natural resource management and conservation schemes for different groups of plants and animals. Land snails, including A. marginata, typically live in discrete populations often isolated from one another with low dispersal ability (Denny, 1980; Fearnley, 1993). This suggests that they are prone to effects of population differentiation with reduced gene exchanges between them, leading presumably to strong local differentiation (Schilthuizen and Lombaerts, 1994; Penninger et al. 1996). Moreover, habitat fragmentation and instability of human-disturbed environments may impose severe restrictions on gene flow and increase random genetic drift. Extinction and recolonization dynamics in local populations may also modify the distribution of genetic variability, leading to a decrease or an increase of variation among populations (Schilthuizen and Lombaerts, 1994; Ruckelshaus, 1998).

In the present study, morphometric analysis of A. marginata was carried out using 5 morphological characters (shell height, shell width, spire height, aperture height, and aperture width), and biogeographic differentiation was observed in all the samples examined with samples from Akwa Ibom and Cross River States, respectively, showing great heterogeneity in the width and aperture height of the shell, thus resulting in major differences in the shell shapes between the different snail accessions. On account of this, molecular approaches were also applied to characterize the genetic diversity of A. marginata. In this case, RAPD-PCR was used to identify DNA segments exhibiting high evolutionary rates using 3 different primers. A large number of scorable RAPD fragments (84) were obtained from only fifteen (15) samples using 3 selected primers and 79 of the fragments were polymorphic. The percentage of polymorphic bands ranged from 50 to 94.04%. These results are in tandem with those reported previously by Tassanakajon et al. (1998) during their examination of genetic variation in wild black tiger shrimp and Thaewnon-ngiw et al. (2003), who studied the genetic diversity of introduced golden apple snail in comparison with four native apple snails in Thailand by RAPD analysis. The morphometric and genetic diversity of A. fulica from 10 geographical locations in Thailand and Malaysia was examined by Pattamarnon (2004) using RAPD, RFLP and SSCP analysis. Seventy two (72) polymorphic fragments were generated across all investigated samples (n = 215) using RAPD while RFLP revealed limited genetic diversity and lack of genetic heterogeneity. The low number of RAPD patterns and the low percentage of polymorphic RAPD fragments found in A. fulica compared with those found in A. marginata in the current study and other local species described above are further confirmations of the status of high genetic diversity of A. marginata. A point of great significance from these results is the fact that inter- and intra-population genetic diversity can enhance adaptation to a particular habitat and also expand the boundary of colonization and distribution, enabling a species to survive in a wide variety of conditions (Williamson, M. (1996). Consequently, the high genetic diversity observed amongst A. marginata accessions collected from the southern part of Nigeria can possibly be related to environmental differences. Another point of interest from the data presented here is the fact that though one of the primers used in the
current study (OPB04) generated interesting results on genetic diversity and population differentiation of A. marginata, however all the three (3) primers used could not amplify the genomic DNA from one of the samples (that is, A11), perhaps on account of the presence of polynucleotides or polysaccharides in the DNA of the said sample.

In conclusion, the genetic and morphometric analysis of 16 accessions of A. marginata collected from three (3) different locations in southern Nigeria using RAPD-PCR and average linkage cluster analysis revealed high level of genetic diversity and heterogeneity. Of the 84 scorable bands that were generated in the RAPD analysis using OPB03, OPB04, and OPB08 primers, 79 of the bands (accounting for 94.04% of the total number of bands) were polymorphic. The OPB04 primer gave 30 RAPD fragments exhibiting significant genetic differentiation across all samples. At the population level, this primer revealed significant genetic differences between the samples from Cross River State (albino-bodied form) and each of the remaining samples from Akwa Ibom and Edo States (black), respectively. This study is the first to elucidate genetic diversity in African land snail species that are prevalent in Nigeria. Given that the RAPD markers used in this study showed genetic variability among a small sample of A. marginata found in the southern part of the country, it would mean that with the use of greater resolution markers including single nucleotide polymorphisms (SNPs) and simple sequence repeat markers (SSRs) on a greater number of diverse samples, the full range of diversity in African land snail accessions in Nigeria could be determined.

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