MOLECULAR PHYLOGENY OF THREE SPECIES OF LAND SNAILS (STYLOMMATOPHORA AND ACHATINIDAE), ARCHACHATINA MARGINATA (SWAINSON, 1821), ACHATINA ACHATINA (LINNAEUS, 1758), AND ACHATINA FULICA (BOWDICH, 1822) IN SOME SOUTHERN STATES AND NORTH CENTRAL STATES IN NIGERIA

Michael Olufemi AWODIRAN1*, Matthee Conrad ARTHUR2, Awopetu Joel IDOWU1
1Department of Zoology, Obafemi Awolowo University, Ile-Ife, Nigeria
2Stellenbosch University, South Africa
*Corresponding author e-mail: michfemi@yahoo.com
Received 11 October 2015; accepted 4 November 2015

ABSTRACT
Partial sequences of mitochondrial gene cytochrome oxidase sub unit 1 (CO1) and ribosomal RNA 18S nuclear encoding gene of 43 individuals belonging to two genera in order Stylommatophora and the family Achatinidae (Archachatina and Achatina) were obtained to investigate molecular phylogeny in the family. The CO1 was found to be highly variable while the 18S was found to be highly conserved yielding invariable sequences. Several primers were also tested both for the mitochondrial and nuclear genomes but CO1 produced the best results giving single and clear bands. Four main genetic phylogroups/clades were identified within the Bayesian tree constructed and all the four clades were supported by bootstrap values of 100% and also supported by bootstrap values above 79% in the NJ. Two unidentified species used in the analysis were found in the basal clade which may mean that they were of ancient origin. This study provides preliminary and novel insights on the molecular phylogeny of the snails’ species, though there is still a need to collect samples of other species in this family to provide a more robust phylogenetic relationship of achatinid snails in Nigeria.

KEY WORDS: mtDNA,, cytochrome oxidase sub unit 1, phylogeny, taxonomy, polymorphism.

INTRODUCTION
The family Achatinidae is generally believed to be made up of about 13 genera among which are Archachatina and Achatina which are the most conspicuous (Mead, 2004). These two genera contain land snails that are generally referred to as Giant land snails. There are three species of these giant snails that are native to Africa (Raut & Barker, 2002) and are the largest of all snails on earth. These are Achatina fulica (Bowdich, 1822), Achatina achatina (Linnaeus, 1758) and Archachatina marginata (Swainson, 1821) (Stylommatophora, Achatinidae).

Traditional classification and taxonomic studies of these snails are based on morphological and soft tissues data analysis. Generally, as it is true of many
invertebrates, mollusks include poorly known groups, for which delimitation and relationships between species are not clear. Taxonomic inferences in mollusks are often hindered by a lack of morphological diversification between different lineages, as occurs in different cryptic species or species showing overlapping variability (Wilding et al., 2000; Liu et al. 2003; Elejalde et al., 2007).

The taxonomy of the Achatinidae is presently in a state of flux (Owen & Reid, 1986), partly because so many species are named from shell characters only (Bequaert, 1950; Owen & Reid, 1986), and partly because few people have collected large samples that enable the range of variation to be appreciated. This confusion is further pronounced by the striking polymorphism in body colouration shown by the family Achatinidae (Owen & Reid, 1986).

Though Wade et al. (2001) used DNA sequences from *A. marginata* collected in Nigeria to investigate comprehensive molecular phylogeny of stylommatophoran snails; there has not been much emphasis on the molecular investigation of the Nigerian achatinids. While several workers have carried out extensive studies on the phylogeny of the achatinid snails in other places (Mead, 1950; Tillier, 1989; Tillier et al., 1996; Bequaert, 1950; 2004, Van Bruggen, 2004), there is a great dearth of information on the achatinids snails of Nigeria.

The present study therefore investigates the molecular relationships among three known species and two unknown species of the family Achatinidae across the southern and North central states of Nigeria (Fig 1). We complete the molecular phylogeny of these snails towards carrying out the eventual investigation of the phylogeographical distribution of these species of snails in Nigeria.

**MATERIALS AND METHODS**

**Specimens examined.** We examined a total of 53 individuals representing three known species and two unknown species of the family Achatinidae. *A. marginata* was collected from eight localities across the sampled areas (Fig.1) while *A. achatina* was collected from two sampling localities due to its restricted distribution. Specimens of *A. fulica* and the two unknown species were collected from National Centre for Genetic Resources and Conservation NACGRAB, Ibadan, Nigeria.

**DNA extraction.** DNA was extracted from foot muscle of each specimen using the Promega Wizard SV Genomic DNA Purification System, following the manufacturer’s protocol. Polymerase chain reaction (PCR) is used to amplify the mitochondrial genes cytochrome oxidase subunit 1COI (using primers LCO1490 (5’-GGT CAA CAA ATC ATA AAG ATA TTG-3’ and HCO2198(5’-T AA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Folmer et al., 1994). The PCR conditions include denaturation at 95°C, 5 min.; 40 cycles in all, annealing at 48°C, 45s and 1 min amplification for 72°C with final extension step of 10 min at 72°C for COI. 18S 7R
primers were also used for the amplification of partial nuclear DNA sequences while
the PCR conditions include denaturation at 95°C, 5 min.; 40 cycles in all, annealing at
50°C, 45s and 1min amplification for 72 C with final extension step of 10 min at 72
C. Two other nuclear DNA primers; for ATP Synthetase subunit α and ATP
Synthetase β with varying annealing temperatures were tested to see if they could be
used to generate sequences for analysis. Reaction products were run on 1.5% agarose
gels and stained with ethidium bromide for about two hours at 120V to verify positive
amplifications and products were visualized under ultraviolet (UV) light using a UV
transiluminator. The gel bands of DNA were excised and the DNA extracted and
purified using the Promega Wizard SV Gel and PCR Clean- Up System. Amplicons
were sequenced using ABM PRISM Dye Terminator Cycle Sequencing Reaction Kit,
Perkin-Elmer. Sequencing was performed on a capillary ABI 3100 automated
sequencer, housed in the Department of Genetics, University of Stellenbosch. The
sequences were deposited in GenBank with accession numbers KT290283 -
KT290319.
AWODIRAN et al: Molecular phylogeny of three species of land snails (Stylommatophora and Achatinidae), Archachatina marginata (Swainson, 1821), Achatina achatina (Linnaeus, 1758), and Achatina fulica (Bowdich, 1822) in some southern states and north central states in Nigeria

Out-group selection. An outgroup Amphidromus species (family Camaenidae) was selected from the GenBank with the accession number AY148562 (NCBI). Other closely related species could have been used but their sequences were not derived from CO1 primers.

Phylogenetic analysis. Sequences obtained were aligned using the Bio Edit Sequence Alignment Editor -BioEdit v.5.09. (Hall, 1999) and adjusted manually. The phylogenetic trees were constructed by both parsimony and Neighbor-Joining method based on the maximum likelihood distance using PAUP* 4.0b10 (Swofford, 2002). Sequence distance values were computed by the TrNEf model for the construction of neighbor Joining (NJ) tree (Tamura & Nei, 1993). Reliability of the nodes was ascertained by 1000 bootstrap replicates. The parsimony analysis was performed with a bootstrap with 1000 pseudoreplicates which included the following:
(a) heuristic search with 100 addition sequences, tree bisection reconnection (TBR) with 100 random additions, character optimization criteria, gaps were treated as missing, branches collapsed (creating polytomies) if maximum branch length is zero. Multistate taxa interpreted as uncertainty while Starting trees were obtained via stepwise addition.
(b) Character-status summary also included: Of 616 total characters: all characters were of type 'unord'. All characters have equal weight while 345 characters were constant, 63 variable characters were parsimony-uninformative. The numbers of parsimony informative characters were 208.
(c) 'MulTrees' option in effect, topological constraints not enforced while trees were unrooted.

Bayesian analysis was performed using the MR BAYES v3.0 package (Huelsenbeck & Ronquist, 2001). In order to get the best model of nucleotide substitution, AIC criteria was carried out using MODEL TEST, Version 3.06 (Posada and Crandall, 1998) with the programs from PAUP* 4.0b 10 (Swofford, 2002). HKY+G model was used on the data matrix and rate variation across sites was modeled using gamma distribution, with a proportion of sites being variants.

RESULTS AND DISCUSSIONS
A 615 base pair fragment of the CO1 locus was successfully amplified and sequenced for 43 specimens while 450 base pair of the 18S 7R locus for the nuclear gene was also sequenced. Table 1 shows the number of specimens of each species used for DNA analysis.

Phylogenetic relationships among the snails species under this study were analyzed by both the maximum-parsimony and neighbour joining methods and the trees were found to be almost similar (Fig 2). The most appropriate substitution model with an estimate of invariable sites (I = 0) and a discrete approximation of the gamma distribution shape parameter (α = 0.4119)
The Bayesian analysis recovered the same tree as the maximum parsimony and is shown in Fig 3.

The results of various primers tested are shown in Table 2. Cytochrome oxidase sub unit 1 (CO1) is the only primer tested that produces data that are analyzable. 18S primers produced good PCR results with single bands but the sequences were invariable. Other primers did not yield any appreciable results in PCR products and amplification quality (Table 2).

**TABLE 1: Snail populations Achatinidae used for DNA analysis collected in some southern states and north central states in Nigeria.**

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>LOCALITY</th>
<th>ZONE</th>
<th>NO OF SPECIMEN USED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archachatina marginata</td>
<td>ASEJIRE (AS)</td>
<td>RAIN FOREST</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>IBADAN (IB)</td>
<td>RAIN FOREST</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>IFE (IF)</td>
<td>RAIN FOREST</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>GBONGAN (GB)</td>
<td>RAIN FOREST</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>OKADA (ED)</td>
<td>NIGER DELTA (LOW LAND RAIN FOREST)</td>
<td>4*</td>
</tr>
<tr>
<td></td>
<td>SABONGIDA ORA (EDO L)</td>
<td>NIGER DELTA (LOW LAND RAIN FOREST)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>ILORIN (IL)</td>
<td>NIGER DELTA (LOW LAND RAIN FOREST )</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>LOKOJA</td>
<td>DERIVED SAVANNA</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GUINEA SAVANNA</td>
<td></td>
</tr>
<tr>
<td>Achatina achatina</td>
<td>IBADAN (IB)</td>
<td>RAIN FOREST</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IFE (IF)</td>
<td>RAIN FOREST</td>
<td>3</td>
</tr>
<tr>
<td>Achatina fulica</td>
<td>IBADAN (NACGRAB)</td>
<td>RAIN FOREST</td>
<td>3</td>
</tr>
<tr>
<td>UNKNOWN SPECIES I</td>
<td>IBADAN (NACGRAB)</td>
<td>RAIN FOREST</td>
<td>3</td>
</tr>
<tr>
<td>UNKNOWN SPECIES II</td>
<td>IBADAN (NACGRAB)</td>
<td>RAIN FOREST</td>
<td>3</td>
</tr>
</tbody>
</table>

- Other specimens were sequenced but were shorter in length and so could not be used.

**TABLE 2: List of primers tested and their results of populations Achatinidae used for DNA analysis collected in some southern states and north central states in Nigeria.**

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
<th>REFERENCE</th>
<th>PCR result</th>
<th>amplification quality</th>
<th>sequence product</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCO 1490</td>
<td>GGT CAA CAA ATC ATA AAG ATA TTAG</td>
<td>TAA ACT TCA GGG TGA CCA AAA AAT CA</td>
<td>FOLMER, et al., 1994</td>
<td>+</td>
<td>single band</td>
<td>good</td>
</tr>
<tr>
<td>HCO 2198</td>
<td>GCGAAAGCATTTGCCAA GAA</td>
<td>GCATACAGACCT GTTATGC</td>
<td>WHITING et al., 1997</td>
<td>+</td>
<td>single band</td>
<td>good</td>
</tr>
<tr>
<td>18 S</td>
<td>GAGCAGTGGATTAGTGA TATTAGGGGT</td>
<td>CTTTCTCACAAGT</td>
<td>JARMAN et al., 2002</td>
<td>-</td>
<td>no band</td>
<td>none</td>
</tr>
<tr>
<td>ATPS A</td>
<td>GAGCAGTGGATTAGTGA TATTAGGGGT</td>
<td>CTTTCTCACAAGT</td>
<td>JARMAN et al., 2002</td>
<td>-</td>
<td>no band</td>
<td>none</td>
</tr>
<tr>
<td>ATPS B</td>
<td>CGTGAGGGAAYGATT HTACCATGAGTGA</td>
<td>TACTTGCCTGGNGGT DCCRCGGGCACGG GC</td>
<td>JARMAN et al., 2002</td>
<td>-</td>
<td>no band</td>
<td>none</td>
</tr>
</tbody>
</table>
AWODIRAN et al: Molecular phylogeny of three species of land snails (Stylommatophora and Achatinidae), *Archachatina marginata* (Swainson, 1821), *Achatina achatina* (Linnaeus, 1758), and *Achatina fulica* (Bowdich, 1822) in some southern states and north central states in Nigeria.

Bootstrap
Fig 2: Molecular phylogeny of five species of achatinid snails (a) Neighbour joining tree (b) parsimony tree
AWODIRAN et al: Molecular phylogeny of three species of land snails (Stylommatophora and Achatinidae), *Archachatina marginata* (Swainson, 1821), *Achatina achatina* (Linnaeus, 1758), and *Achatina fulica* (Bowdich, 1822) in some southern states and north central states in Nigeria

Fig 3: Phylogenetic relationships (Bayesian tree) among five species of achatinid snails (three species are known while the other two are unknown) from four different vegetation zones in Nigeria.

Four main genetic phylogroups/clades were identified within the Bayesian tree constructed and all the four clades were supported by bootstrap values of 100% and also were supported by bootstrap values above 79% in the NJ. Clade 1 contains the *Archachatina* sub populations (8 sub-clades a-h). 1a and b contains *A.marginata* mostly of Ife while 1c cannot be defined as it is made up of *A.marginata* from two
populations (Ife and Ibadan), while the unknown species is also contained in this subclade. 1d is made up of \textit{A.marginata} from Ilorin and Sabongida Ora (Edo 1). 1e subclade contains three individuals from Lokoja and one from Ife and with this is also \textit{Achatina achatina} from Ife. 1f subclade is wholly made up of two specimens from Sabongida Ora and 1g also of two individuals from Sabongida Ora population.

From the foregoing, clade 1 obviously shows homogeneity of the \textit{A.marginata} populations of the rainforest zone of the south west Nigeria. Lokoja and Sabongida Ora (Edo1) populations have potential to separate. Clade 2 has two individuals of two different unidentified species which were also found in clade 4. Clade 3 has both \textit{A. marginata} (from Ife and Ibadan) and \textit{Achatina achatina} from both Ife and Ibadan. The two unidentified species which were found in clade 4 (basal) probably means that they are the most ancient in evolution among the species understudy.

In this study, the phylogenetic relationships of three known species and two unidentified species of land snails in the family achatinidae was carried out on the basis of the nucleotide sequence of the mitochondrial gene for cytochrome oxidase subunit 1. Mitochondrial genes have been found to be useful as a molecular marker because it provides easy access to an orthologous set of genes with little or no recombination and rapid evolution (Ballard & Rand, 2005; Elejalde et al., 2008). The sequence length of the mitochondrial CO1 gene used in the analysis was fairly long (615 bp) and compared with what was obtained in some other studies of phylogenetic relationships of snails (Kojima et al., 2001; used 516 bp; Jorgensen et al, 2008 employed the use of 626 bp; while Kojima et al., 2006 used 489 bp of CO1).

The nuclear sequences obtained from 18S primers could not be used in the analysis because they were not variable being very highly conserved (Jorgensen et al., 2008) while other nuclear primers tested did not yield any appreciable results. Though, Jarman et al., (2002) used ATPS α and β nuclear primers for some molluscan species these two primers could not work for achatinid snails’ used in this study. This work has therefore shown CO1 gene as a reliable phylogenetic marker for the land snails employed in this study. Many previous studies have established cytochrome c oxidase 1 as a reliable molecular marker (Folmer et al., 1994, Zhang & Hewitt, 1997, Hershler et al., 1999; Liu et al., 2001).

Also, the preliminary analysis of all the five species in the family achatinidae revealed interspecific differences among nucleotide sequences within the analyzed region of the mitochondrial DNA; the tree topologies showed the five species to be distinct enough to be separate into different taxa. The \textit{Archachatina} clade (Clade 1) showed that all samples of this species from various localities across the sampled area are monophyletically derived with very high bootstrap probabilities (100%). There is probably a very low rate of divergence among the individuals of the different localities. This may be due to very low rate of evolution among the species studied as a
AWODIRAN et al: Molecular phylogeny of three species of land snails (Stylommatophora and Achatinidae), Archachatina marginata (Swainson, 1821), Achatina achatina (Linnaeus, 1758), and Achatina fulica (Bowdich, 1822) in some southern states and north central states in Nigeria

result of little or no genetic differentiation and absence of environmental heterogeneity of the sampled area.

The two unidentified snail species form a sister clade with the Archachatina clade implies a close relationship with Archachatina marginata. The presence of Achatina achatina in the clade 3 indicated that it is sufficiently different from Archachatina to be placed in a different genus. This finding agrees with the traditional classification based on morphological parameters and soft tissues (Mead, 1950; Bequaert, 1950; Mead, 2004; Van Bruggen, 2004). Moreover, Wade et al. (2001) showed Archachatina marginata and Achatina as sister relatives.

Similarly, Achatina fulica and the second unidentified snail species formed a basal clade implying very ancient origin but distinct from others. However, it was not possible to compare the findings of this study with those of previous workers because their studies on the family Achatinidae were based on land snails peculiar to their environment. Moreover, Fontinallia (2010) opined that despite huge amount of morphological data available on the Achatinidae, no systematic molecular approach has yet been attempted to correlate molecular data with the morphological data, in particular, among various genera and species of the family Achatinidae. The present study agrees with the finding of previous workers supporting monophyly of the family Achatinidae (Wade et al, 2001, Wade et al., 2007; Fontinallia, 2010) .The limited natural range of the Achatinidae to sub Saharan Africa (Raut & Barker, 2002) and the existence of fossils no earlier than the Pleistocene (0.01-1.8 MYA) (Zilch, 1959-1960; Solem, 1979) suggesed that the achatinids are a relatively recent group, although Raut & Barker (2002) believed them to be much older than the fossil record.

Given the preliminary and novel insights on the molecular phylogeny of the snails’ species studied in this work there is need to collect samples of other species in this family to provide a more robust phylogenetic relationship of achatinid snails in Nigeria. Further work also needs to be done using mtDNA barcode to identify the two unidentified species.

ACKNOWLEDGEMENT

Our research group wants to acknowledge the financial support from African Network of Scientific and Technological Institutions (ANSTI) for research visit to Stellenbosch University, South Africa and the supply of three species of snails by the National Centre for Genetic Resources and Conservation (NACGRAB), Ibadan, Nigeria. We also appreciate the three anonymous reviewers of this manuscript for their constructive feedback and insightful suggestions, which greatly improved this article.

REFERENCES


AWODIRAN et al: Molecular phylogeny of three species of land snails (Stylommatophora and Achatinidae), Archachatina marginata (Swainson, 1821), Achatina achatina (Linnaeus, 1758), and Achatina fulica (Bowdich, 1822) in some southern states and north central states in Nigeria