Establishment of the Phylogenetic Relationship Between the Black Skinned and White Skinned Snails (*Archachatina marginata*) in the Rainforest Zone of Nigeria

[1]Patani Inemotimineri, [2] Ogundu Uduak Emmanuel, [3] Okoro Victor Mela Obinna, [4] Kadurumba, Ogechi Evangeline
Department of Animal Science Federal University of Technology Owerri, Nigeria.
Correspondent author: Patani Inemotimineri, Email: princesspatani5@gmail.com
Phone no.: 07068883222

DOI: 10.56201/jbgr.v9.no2.2023.pg68.76

Abstract
This study evaluated the phylogenetic relationship between black skinned and white skinned snails (Archachatina marginata) in the rainforest zone of Nigeria. Fourty eight (48) Archachatinamarginata snails were assigned to two treatments using completely randomized design. The two treatmentsconsisted of twenty four (24) white skinned ecotypes and twenty four(24) black skinned ecotypes with 12 replicates each. These snails were taken to laboratory to conduct DNA analysis to evaluate the phylogenic relationship between the two snail ectotypes. The quality of DNA was assessed using gel electrophoresis. Data collected was subjected to statistical analysis using Mega 7.0, DNAsp version 5 and GENPOP software packages. The phylogenetic analysis revealed a 0.2 genetic variation and 0.8 similarities between the snail that is black and the white. From the dendogram, it was also observed that the white snails are the original ancestors of the black since the top of the tree was dominated by the white snails with the black occupying the bottom of the tree.

Keywords*: Establishment, Phylogeny, Dendogram*

I. INTRODUCTION

In recent decades, Nigerian snail farming has drawn a lot of interest (Akinnusi, 2002). Other than arthropods, the most significant group of animals that are molluscs is comprised of snails (Wosu, 2003). Snail meat is consumed by both affluent and poor people, regardless of their income level (Murphy, 2001; Ebenso, 2003). Snail flesh is tasty, healthy, and high in protein. It has a low fat, cholesterol, and salt content and a high mineral content, including calcium, phosphorus, and iron. It has linoleic and linolenic acids, which are important fatty acids (Danladi et al., 2020).
In many coastal communities and countries, the meat from snails, is a preferred meal and an essential source of animal protein, even though they are not direct protein sources (Omole et al., 2017; Fatai 2018). The reduced ability of the traditional protein sources—goats, sheep, cattle, and chickens—to satisfy demand and address the issue of protein shortage is the reason for the rising usage of this non-conventional protein. In some parts of Africa, snails are used extensively in

traditional medicine to prepare cures for conditions like whooping cough, diabetes, asthma, and other conditions (Offiong et al., 2013). Because of their comparatively low cholesterol levels, snails have also been suggested as a treatment for anemia, asthma, and asymptomatic conditions (Wosu, 2003). Snail slime is a thick substance that is secreted by snails and travels along their journey. The slime helps the body move smoothly, repairs broken shells and skin, stops moisture from evaporating, and protect the entire body from harm (Jeff et al., 2017; Nyoagbe et al., 2016).According to Nyoagbeet al. (2016), snail slime's animal protein content is expected with a significant result in traditional medicine. While the shell is used to make jewelry and ornaments, it is also employed in the manufacturing of drugs by pharmaceutical and cosmetic industries (Nyoagbeet al., 2016).

According to Ejidike (2002) and Smith and Fowler (2003), *Achatina achatina*, *Achatina fulica*, and *Archachatina marginata* are the prevalent snail in many countries in Africa. *Archachatina marginata*, is the most and predominant specie of snail commonly reared in Nigeria (Okon et al., 2012b;Venette and Larson, 2004;). This common specie matures within two to four months after *A. fulica* and forms a peristome with a mirrored lip (Ibom et al., 2016b). *A. marginata* has either a red or white parietal callus and columella (Venette and Larson, 2004). Despite the many advantages of *A. marginata* snails, they are nevertheless harvested excessively and indiscriminately from farm, together with deforestation, which has significantly decreased the number of this African giant land snails (AGLS), putting it in danger of extinction as a result of these activities (Etukudo 2017;Ojating and Ogar 2002; Okon and Ibom 2012; Okon et al., 2008).

More so than other parameters, the physiological adaptation to the environment, genetic linkages, and diversity between and within breeds significantly affect the productivity and performance of snails (Okon et al., 2012a). Different populations of snails have arisen as a result of natural selection, individual free mating, and variation in the gene pool. Genetic characteristics like heritability and genetic correlations are estimated using relatedness or variation knowledge in genetic analysis (Falconer and Mackay, 1996).

The relationships that indicate the evolutionary distance between two species' common ancestor are known as phylogenetic relationships. Sampling every species throughout a clade, confirming that they are biologically distinct entities, and knowing their geographic distributions are all ideal prerequisites for a phylogenetic strategy for investigating the origin and continued existence of species diversity (Williams and Reid, 2004). Small and isolated populations may be more vulnerable to extinction due to threats that come with their small size (Puurtinen et al., 2004). This has caused a need for swift study to obtain phylogenetic information on GALS (*A. marginata*) species (white and black skinned).

II. Materials and Methods

Forty eight (48) grower snails obtained from Edo, Bayelsa and Akwa-Ibom state was randomly selected two per replicate in the two treatments and taken to the laboratory to conduct the DNA analysis. The snails were slaughtered by breaking the shell and separating the fleshy edible part from the shell and fluid and then cutting off some portion of the muscular foot to use for the DNA extraction. To extract DNA, tissue was crushed into a smooth, blended form per the manufacturer's instructions, then mixed with the Zymo Research DNA Extraction kit. DNA extractions were kept at -120C in the University of Port Harcourt's Department of Animal Science's Genomic

Laboratory. In order to attain optimal outcomes, 500 µl of beta-mercaptoethanol supplied by the user was incorporated into the Genomic Lysis Buffer at an end-user dissolution of 0.5% (v/v), per 100 ml.

1. Specimen(s) were put into a 2.0 mm ZR Bashing BeadTM Lysis Tube. After adding 750µl of Bashing BeadTM Buffer to the tube, it was securely sealed. (In general, only 50 mg of tissue was sampled because bigger samples will go beyond the spin column's capacity to bind DNA). It is also possible to sample up to 8.5 x 106 cells trapped in 200 µl PBS or up to 400 µl of entire blood.
2. After that, samples were subjected in a bead with a 2 ml container assembly (GenieTM) and processed for 10 minutes at maximum speed. Processing time is dependent on the bead beater and sample input. The use of fast connectivity cell disrupters (FastPrep® -24) can be completed in as little as five minutes.
3. A micro centrifuge was used to centrifuge the ZR Bashing BeadTM Lysis Tube (2.0 mm) for one minute at a force of ≥10,000 x g.
4. The supernatant (about 400µl) allowed to centrifuged at 8,000 x g within60s, and place to a Zymo-SpinTM III-F Filter in a receiving Tube.
5. The filtrate from Step 4 introduced to 1,200µl of Lysis Buffer for Genomicand thoroughly mixed.
6. After transferring 800µl of the mixture from Step 5 into a TM IICR Column1Zymo-Spin in a receiving Tube, it was properly mixed in a centrifuge at 10,000 x g for 60s.
7. After discarding mixture from the Collection Tube, Step 6 was carried out once more.
8. 200µl of the DNA Pre-Wash Buffer was introduced to the Zymo-SpinTM IICR Column in a fresh Collection Tube, at 10,000 x g centrifugation for 60s.
9. the 500µl of g-DNA Wash Buffer was introduced into theTM IICR Column Zymo-Spin, at 10,000 x g centrifugation for 60s.
10. 100 µl of elution DNA buffer (a minimum of 35 µl can be used), immediately was introduced to the column matrix of the TM IICR Column in a sterile 1.5 ml microcentrifuge tube, and. After which the the DNA was extracted by centrifuging at 10,000 x g in 60s.

Using a DNA monogram, the amount and quality of DNA were evaluated; samples containing less than 5 µml were discarded. The quantified DNA was further examined by gel electrophoresis using a 1.5% agarose gel at a constant voltage and 1X TBE for about an hour, utilizing a portable gel hood with a built-in Blue LED (470nm) by Royal Biotech/Biolympics (www.royalbiotech.com). They were photographed in the ultraviolet light and made visible using Ethidium bromide staining. The ladder that is being used is a thermo scientific 100 base pair ladder.

Cytochrome B Primers

Cytb F 5'- CCATCCAACATCTCAGCATGATGAAA -3'
Cytb R 5'- CCCCTCAGAATGATATTTGTCCTCA-3'

The following cocktail mix was applied to the DNA in order to prepare it for PCR.

Table 1. Polymerase Chain Reaction Cocktail Mix

Materials	Proportion
PCR buffer (10×)	2.5
$MgCl_2$ (25mM)	1.0

forward primer (5pMol)	1.0
reverse primer (5pMol)	1.0
DMSO	1.0
DNTPs (2.5Mm)	2.0
Taq (5u/µl)	0.1
DNA (10ng/µl)	3.0
H_2O	13.4
Total volume	25µL

Touch Down PCR Condition

Table 2 Conditions for Touch Down PCR

	9 Cycle			35 Cycles				
Initial den.	Den.	Ann. Tempt	Extension	Den.	Ann. Tempt	Extension	Final extension	Hold tempt
94˚C	94˚C	65˚C	72˚C	94˚C	55˚C	72˚C	72˚C	10˚c
5min	15sec	20sec	30sec	15sec	20sec	30sec	7min	∞

Den = Denaturation; Ann = Annealing; Tempt = Temperature

For about an hour, the amplicons from the aforementioned reaction were loaded onto 1.5% agarosegel at a steady voltage and 1X TBE. They were photographed in the ultraviolet light and made visible using Ethidium bromide staining. The ladder that is being used is a thermo scientific 50 base pair ladder. Using the following primers, the Mitochondria DNA (Cytochrome B) gene was amplified:

Cytb F 5'- CCATCCAACATCTCAGCATGATGAAA -3'

Cytb R 5'- CCCCTCAGAATGATATTTGTCCTCA-3'

Five microliters of nuclease-free water, ten milliliters of PCR premix (Quick-Load 2x Master mix with standard bufferone Taq*), eight microliters of genomic DNA, and one milliliter MC4R each for both primers were added to each 25 microliter Polymerase Chain Reaction (PCR) microtube. A touch-down PCR condition was applied, comprising of initial denaturation at 94˚C for five minutes, nine denaturation at 94˚C cyclesfor fifteen seconds each, of annealing at 62˚C for 20s-, and thirty-seconds extension (72˚C). The next 35 cycles were: 15s at 94˚Cof denaturation, annealing at 58˚C, for 20 seconds of, extension at 72˚C for 30s, and final extension at 72˚C for 7min.The results of the Polymerase Chain Reaction (PCR) were electrophoresed for 40 minutes on a 1% agarose gel with IX TBE and a constant voltage. They were photographed in the ultraviolet light and made visible using Ethidium bromide staining. The qualitative Polymerase Chain Reaction (PCR) products for sequencing was carried out at the institute for tropical Agriculture. Mega 6.0 was used to align DNA sequences (Tamura et al., 2013). DNAsp version 5, Mega 7.0, and the GENEPOP software package were used to determine single nucleotide polymorphism and other sequence variation parameters (Rousset, 2008).Finch TV 1.4.0 and Mega 7.0 software were used to perform the statistical analysis of the aligned sequences.

III. Results and Discussion

Figure 1 represents the dendrogram showing genetic distance between white and black snail while plate 1 shows the gel of cytochrome B bands for 48 snail samples. *A. marginata* snails in the

rainforest zone of Nigeria is highly related as the two were connected closely in the tree, except for only one of the white snails that was not closely linked to the others. The phylogenetic analysis revealed only one cluster with one end having only white skinned *A. marginata* and the other end of the cluster having both white and black skinned *A. marginata*. phylogenetic analysis at the top of the phylogenic tree revealed that both white and blacked skinned *A. marginata* share a most recent common ancestor. The bottom of the tree indicates that the ancestry of both species started with the white skinned *A. marginata*. Additionally, a 0.2 (20%) variation between A. marginata with black and white skin is shown in the above figure. The outcome also demonstrates that there is a stronger genetic connection than variation between *A. marginata* with black and white skin. The scale also shows that there is 3.2, or roughly 16 times the scale, separating the top-skinned *A. marginata* with black skin from the bottom-skinned *A. marginata* with white skin. According to Gonzalez, Aramendia, and Davison (2019), the primary explanation for this variation is believed to be due to selective forces like habitat, predation, climate, and selection. This suggests that compared to black snails, white snails absorb less heat.

The present study's outcomes are consistent with those of Etukudo et al. (2018), who assess genetic diversity in Africa giant land snail by using the marker; Inter Simple Sequence Repeats (ISSR). He stated that—1 and 2—were two clusters majorly found through the phylogenetic assay, with the number 2-cluster being further subdivided into two smaller clusters. On the other hand, A. marginata and A. fulica were grouped together. This may help to explain why A. marginata and A. achatina have different genetic makeups despite having similar sizes. Fagbuaro, Mosuro, Bakare & Odaibo (2002) and Gu et al. (2015) also supported the genetic similarity between *A. marginata* and *A. achatina*. 54 chromosomes was associated with *Achantina. fulica*, 44 chromosomes with A. achatina, and 56 chromosomesfor A. marginata had, according to their report. According to analysis of cellular variability (AMOVA), 100% was recorded as molecular variance within a population, but variation between the populations was only 0.00%. In contrast to unbiased identity genetically0.992 (pop1 and pop2), 0.988 (pop1 and pop3), and 0.985 (pop2 and pop3), Nei unbiased genetic separation was 0.008 (pop1 and pop2), 0.012 (pop1 and pop3), and 0.015 (pop2 and pop3). The study's minor genetic variation may result from random mating, arbitrarily fertilization, mutation (which can produce completely new alleles in a populace), and amplification between chromosomes that are similar during meiosis (which reorganizes alleles throughout an organism's progeny). The results showed that there was a high level of genetic identity, which may have been caused by their close proximity.The Tajima Neutrality test results are shown in Table 3. The results show a significant ($p \leq 0.05$) value (-0.431931) for the Tajima test statistic. This analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 329 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. The results showed low negative values for nucleotide diversity. This result is in variance with the findings of Dauda, Okon & Ibom (2018) who evaluated the genetic diversity of Giant African Land Snails (GALS) in Uyo, and observed a high nucleotide diversity and Tajima Test for Selection among the GALS breeds evaluated. The low negative value obtained in this study implied that the breeds may not adapt to various environmental conditions. This could also be the reason for the high mortality

experienced by the hatchlings because negative Tajima's D test signifies imbalance selection (Fu & Li, 1993).

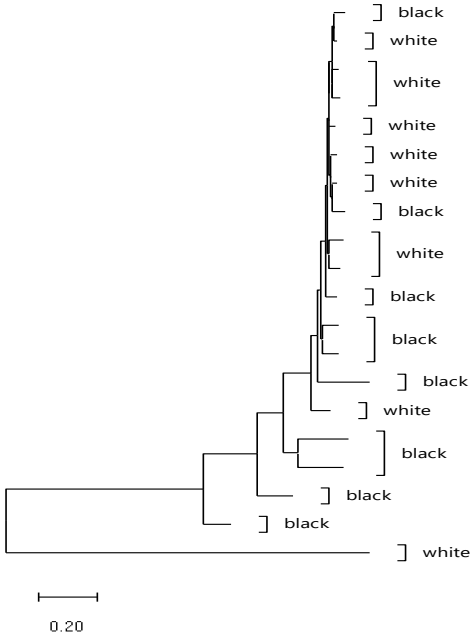

Figure 1.Dendrogram showing genetic distance between white and black snail

Table 3 Results from Tajima's Neutrality Test for black and white skinned snails (*A. marginata)*

M	*S*	P_S	*Θ*	*Π*	*D*
20	292	0.887538	0.250170	0.224236	-0.431931

Key.-- This analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 329 positions in the final dataset. Evolutionary analyses were conducted in MEGA11

Abbreviations: m = number of sequences, n = total number of sites, S = Number of segregating sites, p_s = S/n, Θ = p_s/a_1, π = nucleotide diversity, and D is the Tajima test statistic

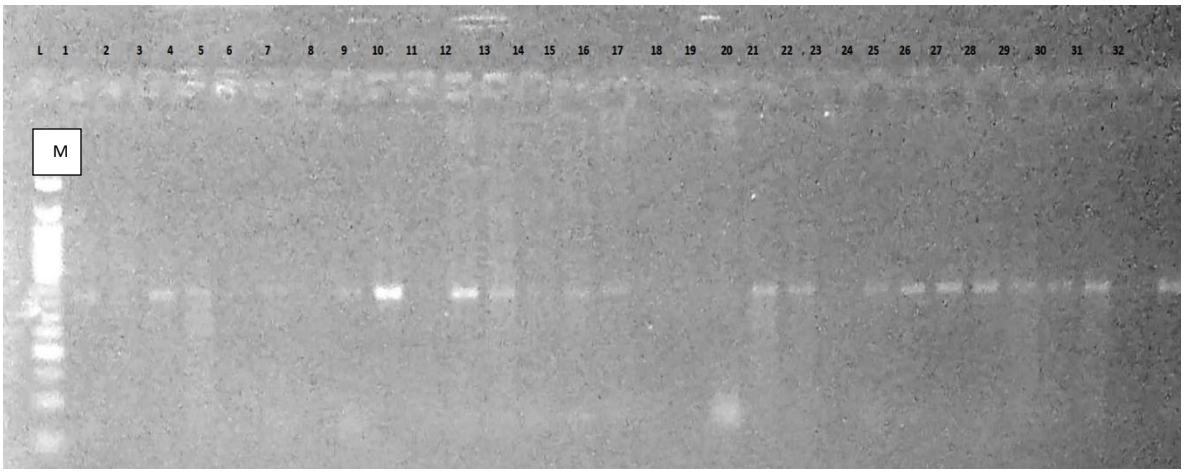

Plate.1Showing gel picture of cytochrome B bands for 48 snail samples. M is 100bp DNA ladder.

IV. CONCLUSION

The study's findings unmistakably demonstrate that African giant land snail *A. marginata*, predominantly found in the rainforest zone of Nigeria, has a limited range of skin colors. Given that specification level of genetic resemblance among the investigated samples, this may suggest that both snails species descended from a similar or the same ancestor.

REFERENCES

Akinnusi O (2002). Introduction to Snail Farming.Triolas Publishing Company, Abeokuta. P 70.

Cagiltay, F., Erkan, N., Tosun, D., Selçuk A. Amino Acid, Fatty Acid, Vitamin and Mineral Contents of the Edible Garden Snail (Helix aspersa). Journal of Fisheries Sciences.com, 2011; 5(4): 354-363.

Danladi J.G, Umezuruike G.O, Amaechi CC, Haruna G.S, Abdullahi M.H, OkohV.I.(2020) proximate and mineral composition of snail slime (Achachinamarginata) world journal of pharmacy and pharmaceutical science volume 9 issue 11, 2304-2512 ISSN 2278-4357

Dauda, A. Okon, B and Ibom, L.A (2018) Genetic Diversity of Giant African Land Snails (GALS) *Proceedings of 6th NSCB Biodiversity Conference; Uniuyo* (144 – 147 pp)

Ebenso IE (2003). A Dietary Calcium Supplement for Edible tropical land snail, Archachatinamarginata in Niger Delta, Nigeria.Livest. Res. Rural Dev. 15(5):82-85.

Ejidike BN (2002). Snail Rearing Practices in southern Nigeria. Proc., 27th Annual Conference of Nigerian Society for Animal Production (NSAP) March 17 – 21, Federal University of Technology, Akure, Nigeria. pp. 307-308.

Etukudo, O. M, B. O. Asuquo, B. O; U. B. Ekaluo, U.B., Okon, B.; Ekerette, E.E; Umoyen, A.I, Udensi, O.U; Ibom, L. A, Afiukwa, A.C and Igwe, D.O (2018) Evaluation of Genetic Diversity in Giant African Land Snails Using Inter Simple Sequence Repeats (ISSR) Markers. *Asian Journal of Advances in Agricultural Research* 7(2): 1-13, 2018; Article no. AJAAR.41088 ISSN: 2456-8864

Etukudo OM, (2017) Genetic diversity of giant African land snails using Inter Simple Sequence Repeat (ISSR) markers. Ph.D Thesis. Department of Genetics and Biotechnology, University of Calabar, Calabar, Nigeria.;120.

Fagbuaro, O, Mosuro AA, Bakare AA, and Odaibo AB. (2002) Cytological studies of *A. marginata* and *Achatina spp*. from South West Nigeria. *Tropical Veterinarian*. 20(2):74-79

Falconer, D. S. and Mackay, T. F. C. (1996) *Introduction to Quantitative Genetics*. 4th Edition Longman, London. 464.

Fatai, A., A. (2018) Snail Farming in Nigeria; Methods and Techniques. [Retrieved online]

Fu, Y. X. and Li, W. H. (1993). Statistical tests of neutrality of mutations. Genetics, 133 (3): 693-709

Gonzalez, D. R., Aramendia, A. C., and Davison, A. (2019). Recombination within the Cepaeanemoralis supergene is confounded by incomplete penetrance and epistasis. *Heredity*. doi:10.1038/s41437-019-0190-6

Gu QH, Cheng QQ, Li XJ, and Zhou CJ. (2015) Novel polymorphic microsatellite markers for Bellamya and their application in population genetics of three species. *Genetics and Molecular Research*. 2015;14(4):15201-15212.

Ibom L.A., PataniI**.,**Okon B. and Okon F. I. (2016b). Phenotypic Variations in reproductive traits of two strains of *Archachtina marginata* snails *(VAr. Ovum and Var. Saturalis)* and their crosses. Proceeding of the 5th annual international conference/workshop on Giant African land snails NetGals. June 5-9, 2016. Pp 36 – 42.

Jeff, T. B., Agu, M. O., Jude, C. O., Ukwubile, C. A. (2017) The Physicochemical, Toxicity and Anti-Diabetic Effect of Costusafer Ker Gawl. (Costaceae) Leaf Methanol Extract and Snail Slime on Alloxan Induced White Swiss Albino Rat. America Association for Science and Technology Journal of Chemistry; 3(4): 23-29.

Murphy B (2001). Breeding and growing snails commercially in Australia.A report for rural industries research and development cooperation publication No 00/188 project No ARH-IA 29.

Nyoagbe, L. A., Appiah, V., Nketsia- Tabiri J., Larbi, D. and Adjei, I. (2016) Evaluation of African giant snails (Achatina and Archachatina) obtained from markets (wild) and breeding farms. African Journal of Food Science; 10(7): 94-104

Offiong, E. E. A. (2013) Nutritional /Chemical Constituent of Three Local Species of Land Snail, Archachatinamarginata, Achatina achatina and Achatina fulicafound In Uyo-Akwa Ibom. The International Journal of Science and Technology; 1(4): 1-5.

Ojating I, Ogar GE. Effect of feed types on the growth performance of African giant snail Fattened at room temperature.Journal of Sustainable Agriculture and Environment. 2002;4(2):303-308.

Okon B, Ibom LA, Etuk NE, Akpan EW. (2012) Variations in growth patterns and Conformation of snails: Influence of strain and location on isometry of growth inCross River State of Nigeria. Journal of Agriculture, Forestry and the Social Sciences (JOAFSS).;6(2):218-227

Okon B, Ibom LA.Snails breeding and snailery management (2012). Calabar: Freshdew Publication. ;90

Okon B, Ibom LA, Williams ME, and EkongNB.(2008) Effects of breed on reproductive Efficiency of two most popular snails (*A. marginata* (S) and *A. achatina*(L)) in Nigeria. *Journal of Agricultural Science*. 2012a;4(8):236-245...

Omole AJ, Taiwo AA and JA Amusan(2007) Practical Snail Farming – Technical Guide / Bulletin, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria,: 26 pp.

Puurtinen K, Knott E, Suenpaa S, Oik T and Kaitala V (2004). Genetic variability and drift load in populations of an aquatic snail. Evolution 58(4):749-756.

Smith JW and Fowler G (2003). Pathway risk assessment for Achatinidae with emphasis on the Giant African Land Snails, Achatinafulica (Bowdich) and Limicolaria aurora (JAY) from the Caribbean and Brazil, with comments on related taxa Achatinaachatina and Archachatinamarginata (Swainson) intercepted by PPQ, USDA- APHIS, Center for Plant Health Science and Technology (internal concept), Raleigh, NC. pp. 1-7.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30: 2725-2729.

Venette RC, Larson M (2004). Mini risk Assessment Giant African Land Snail, Achatinafulica (BOWDICH) [Gastropoda: Achatinidae]. Department of Entomology, University of Minnesita, ST. PAUL MN 55108. pp. 1-30.

.

Williams, S. T., & Reid, D. G. (2004).Speciation and Diversity on Tropical Rocky Shores: A Global Phylogeny of Snails of the Genus Echinolittorina. Evolution, 58(10), 2227.

Wosu, I.O. (2003). Commercial snail farming in West Africa: a guide. *Ap Express Publishers Ltd, Nsukka*. 172