

Original Research Paper

Karyological studies of *Achatina fulica* and *Achatina achatina* (Mollusca: Gastropoda) in Nsukka, Enugu, Nigeria

Running Head: Karyological studies of Achatina fulica and Achatina achatina

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Abstract

Karyological studies were carried out on two *Achatina* species (*Achatina fulica* and *Achatina achatina*) that inhabit Nsukka Local Government Area of Enugu State, Nigeria. This study was undertaken to determine their chromosome morphology and the level of relatedness. Chromosomes were prepared from the ovotestes of the snails which were injected with 0.4% colchicine solution. Chromosomes in well-spread cells were counted and measured directly from the slide using a calibrated eyepiece graticule. Arm ratios and centromeric indices were determined from these measurements. The chromosomes were classified based on the centromeric indices obtained and idiograms were constructed from the measurements. The fundamental number (FN) of arms was determined for each species. Photomicrographs of well-spread representative mitotic metaphase chromosomes were made and used to construct a standard karyotype for each species. Photomicrographs of well-spread meiotic cells were also made. After the examination of over 150 well-spread metaphase cells, the diploid chromosome number of $2n = 60$ was recorded for both *Achatina fulica*, and *Achatina achatina*. However, there are observable differences in their karyotypes which support their classification into different species. The similarity in their chromosome numbers indicates a closer evolutionary relationship when compared to other species in the same family. ancestral lineage.

Keywords: Chromosome, Diploid, Karyotype, Metaphase, Snail

Introduction

The knowledge of chromosomal number (ploidy) including inter- and intra-species alterations is essential to understanding the evolutionary dynamics of most living species in terms of natural selection, hybridization, and the implications that may have arisen from breeding processes (Justin and Tara, 2014). Molluscs including snails are good models for genetic and cytogenetic studies because they are abundant and highly diverse in most afro-tropical regions such as Nigeria. Studies aimed at highlighting the karyological relationship between groups or conspecific species of snails found in Nigeria could contribute to other existing studies in the field of genetics and evolution. Besides, highlighting karyological relationships in snails could reveal important new information about their genetic, systematic, and even evolutionary relationships. Previous studies have established that there are successive cycles of polyploidization, hybridization, and differentiation in certain genera of molluscs, however, during the evolutionary process, sometimes the chromosome morphology could be altered by their environment (Guo *et al.*, 2008).

The relationships between mollusc species (particularly snails) and their taxonomic interpretations are not well understood. This is due to the lack of knowledge about their morphological diversification across different lineages, as it has occurred in different cryptic species or species showing overlapping variability (Elejalde *et al.*, 2008). Some conventional banding techniques used in previous studies (e.g., Guan *et al.*, 2008; Lee *et al.*, 2008; Leitao *et al.*, 2009; Puslednik *et al.*, 2009; Stunzenus *et al.*, 2011) to highlight the karyological and evolutionary relationships across groups of molluscs are commendable; however, most records are from snails found in the temperate regions. The current study aimed to ascertain the diploid chromosome number of two species of *Achatina* (*Achatina fulica* and *Achatina achatina*) within Nsukka, Enugu in Nigeria; construct an appropriate karyotype for the species and determine their level of relatedness. These two species of snails are abundant and visibly distinguished by their unique morphological features (Sogbesan and Ugwumba, 2012; Chukwuka and Ejere, 2019).

Materials and Methods

Study Area

This study was conducted in Nsukka Metropolis in Enugu Nigeria. This area, situated at longitude 7°23'45"E and latitude 7°24'N has a land area of 40,750 km² and a mean elevation of 551.68 m (Onyenucheya and Nnamchi, 2018). Owing to the nature of Nsukkas' climatic condition favoring different species of snails, four (4) communities within the metropolis were visited for sampling (i.e., Obukpa, Ede, Oballa, and Eha-Alumona). Snails were randomly handpicked during their active period in the morning (6:00 to 7:00 am) and evening (6:00 to 7:00 pm). Identification was through Herbert and Kilburn (2004) guide emphasizing the use of morphology (Chukwuka and Ejere, 2019). Afterward, the snails were preserved in aerated plastic containers and transported to the Department of Zoology and Environmental Biology Laboratory, University of Nigeria, Nsukka. The snails were placed in a well-marked improvised ventilated wooden box (0.9 m x 0.6 m x 0.3m) and fed appropriately.

Cell extraction, fixing, spreading, and staining

Snails were injected with 0.1 ml of 0.04% colchicine to arrest the cells at the metaphase stage of cell division. Two hours later, the shells of the snails were broken through a puncture of the apex whorl to reach the ovotestis within the digestive gland. The ovotestis collected in specimen bottles were homogenized using freshly prepared 0.5 ml of 0.075M KCl hypotonic solution (to swell the cells and enhance chromosomal visibility for observation). Afterward, small pieces of the ovotestis were cut through using a sharp razor blade and placed in a mortar containing 2ml of a hypotonic solution, minced to obtain finer cell suspension which was left to stand for 45-60 minutes under room temperature before centrifuging the sample in a test tube (at 2000 rpm for 5 minutes). After discarding the supernatant liquid, cells were re-suspended, followed by the addition of freshly prepared fixative (3:1 methanol: acetic acid) that was flushed with a Pasteur pipette, and centrifuged (at 2000 rpm for 5 minutes) before allowing the mixture to cool in a refrigerator for 24 hours. Afterward, separated cell suspensions in the test tube were shaken to re-suspend the cells and centrifuged at 2000 rpm for 5 minutes to obtain a pellet that was dissolved and fixed. The mixture (fixed cells) was allowed to stand for 10 minutes, re-centrifuged (2 times), and decanted from the test tube until a clear suspension was obtained at 0.5-1.0ml mark into which a few drops of fixative was added to make 5ml. Using a Pasteur pipette, 3-4 drops of the suspension were dropped over a pre-refrigerated clean slide at an inclined angle, before placing on a BIOBASE® slide warmer maintained at 60°C for 24 hours. The dried slides were placed in a BIOBASE® coupling jar containing 6% stock Giemsa stain solution for 30 minutes, then rinsed in distilled water before drying.

Chromosome analysis test

Slides were first viewed for dividing cells (at 10x objective) before viewing under oil immersion to reveal the metaphase stages and to aid eye karyotyping of the chromosomes complement under the Olympus® BX51 light microscope. Karyogamy of the chromosome length, long arm length (q), short arm length (p), and arm ratio (q/p) were done afterward. The photographic documentation of the chromosomes was assessed under the microscope attached with a multi-scan camera before classification into various morphological categories which include: the metacentric (Ms), sub-metacentric (SMs), and telocentric. The chromosomes were further classified into uni-and-biarmed which was used to calculate the fundamental arm number (FN).

Construction of Idiogram and Histogram

Idiograms of chromosome pairs were constructed to represent the shape and size of the chromosomes. These were prepared according to the centromeric position and length of chromosomes in order of decreasing length by using the haploid formula as the basic parameter. Histograms were prepared by taking the chromosome pair number on the X-axis and the corresponding relative length percentage on the Y-axis. Chromosome pairs were arranged in decreasing order of their length.

Results

The Chromosome Morphology of *Achatina fulica*

Well-spread mitotic metaphase chromosomes were examined from the ovotestis of *Achatina fulica* and the diploid chromosome number was found to be $2n=60$ with a fundamental number (FN) of 108. Plate 1 shows the mitotic metaphase chromosome spreads of *Achatina fulica*. The karyotype indicating the paired chromosomes according to size and centromere position is shown in Plate 2. The chromosomes were divided into three main groups according to their size. Group one consists of large chromosome sizes from 1 to 10 which are relatively visible and can be identified clearly in all the metaphase spreads. Ten pairs of these chromosomes (1 – 10) were categorized into sub-metacentric. Group two consists of the medium size chromosomes from 11 to 20 which are visible and identifiable. Three pairs of these chromosomes (11 – 13) were categorized into sub-metacentric, two pairs (14 – 15) were sub-telocentric and five pairs (16 – 20) were telocentric. Group three consists of small size chromosomes from 21 to 30 and they are categorized into metacentric (21 – 27 and 29), telocentric (28), and sub-metacentric (30). The chromosomal formula for the complement was calculated as $2n=8m+13sm+3st+6t$ and the corresponding fundamental arm number was calculated as $NF= 108$. The chromosome measurements were accurately used to construct the idiogram and histogram as shown in Figures 1 and 2. Figure 1 was constructed based on the chromosome number and position of the centromere and it was observed that there were variations in the centromeric location, resulting in variations in the lengths of the long (q) arms and short (p) arms. Plates 3a-d show the different stages of meiotic division observed in *Achatina fulica*. The first meiotic division begins with a long prophase, which is subdivided into four stages, leptotene, zygotene, pachytene, and diplotene. During the leptotene, the chromatin of the chromatids was stretched out very thinly, and it was not possible to identify individual chromosomes i.e., the chromosomes were entangled, slender thread-like structures with densely stained granules (Plate 3a).

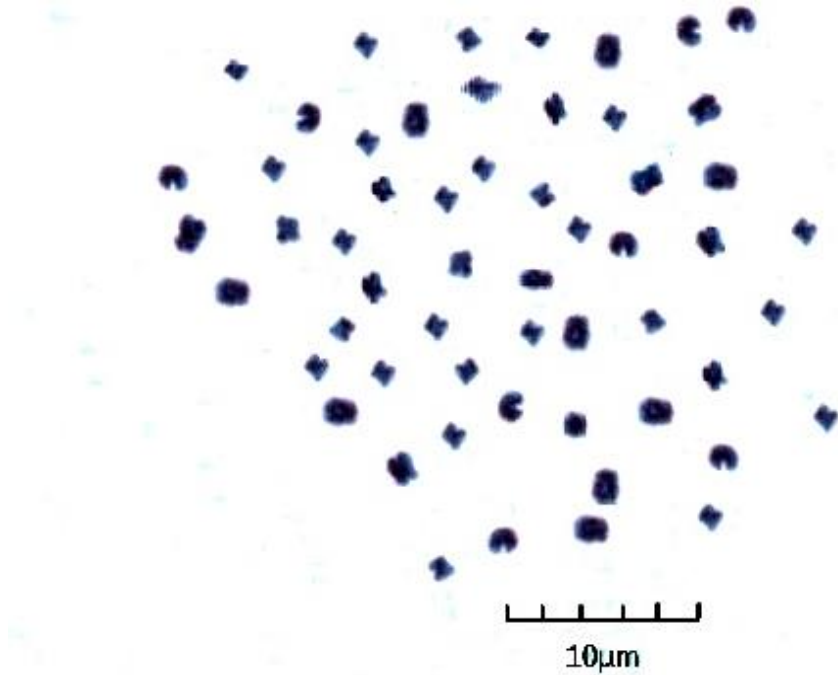


Plate 1: The mitotic metaphase chromosomes obtained from ovotestis of *Achatina fulica* showing diploid chromosome number ($2n=60$)

At the pachytene stage, chromosomes were present in paired form and synapse (Plate 3b). At the zygotene, the chromosomes were much shorter, in the form of long and fine threads that cross and intercross each other, more contracted and quite clearly visible (Plate 3c). During the diplotene, the chromosomes were much shorter, more contracted, and clearly visible (Plate 3d). Sex chromosomes and polyploid cells were not observed.

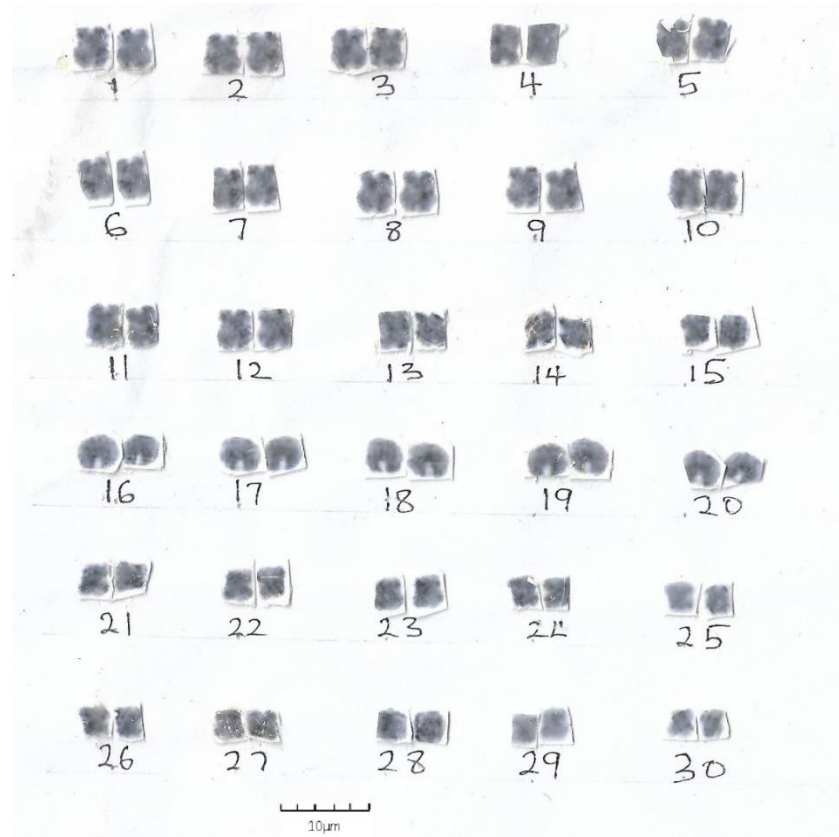


Plate 2: The karyotype of *Achatina fulica* (2n=60)

The centromeric index (i) for each chromosome was derived from the formula, $i = 100p/t$

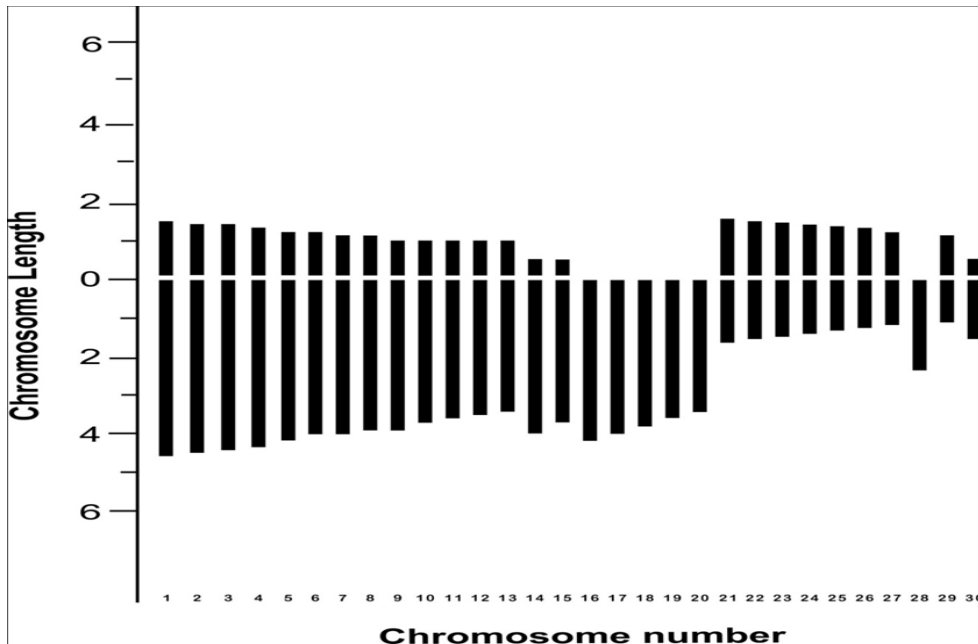
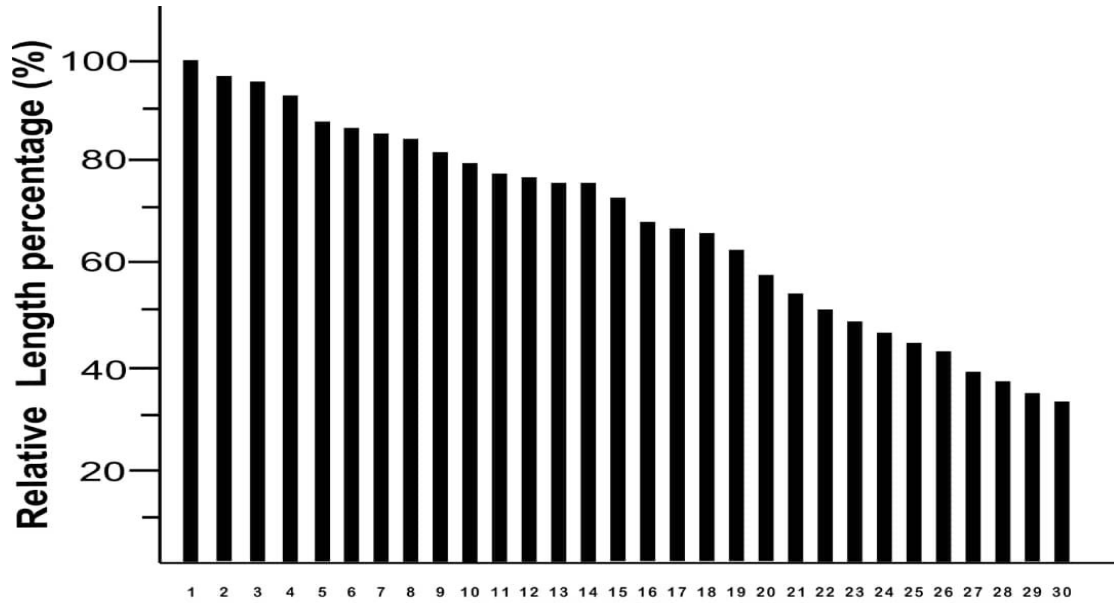


Figure 1: The idiogram of the karyotype of *Achatina fulica* constructed based on chromosome numbers and the position of the centromere.



Chromosome number

Figure 2: The histogram of the karyotype of *Achatina fulica* showing the relative Length Percentage of chromosomes.

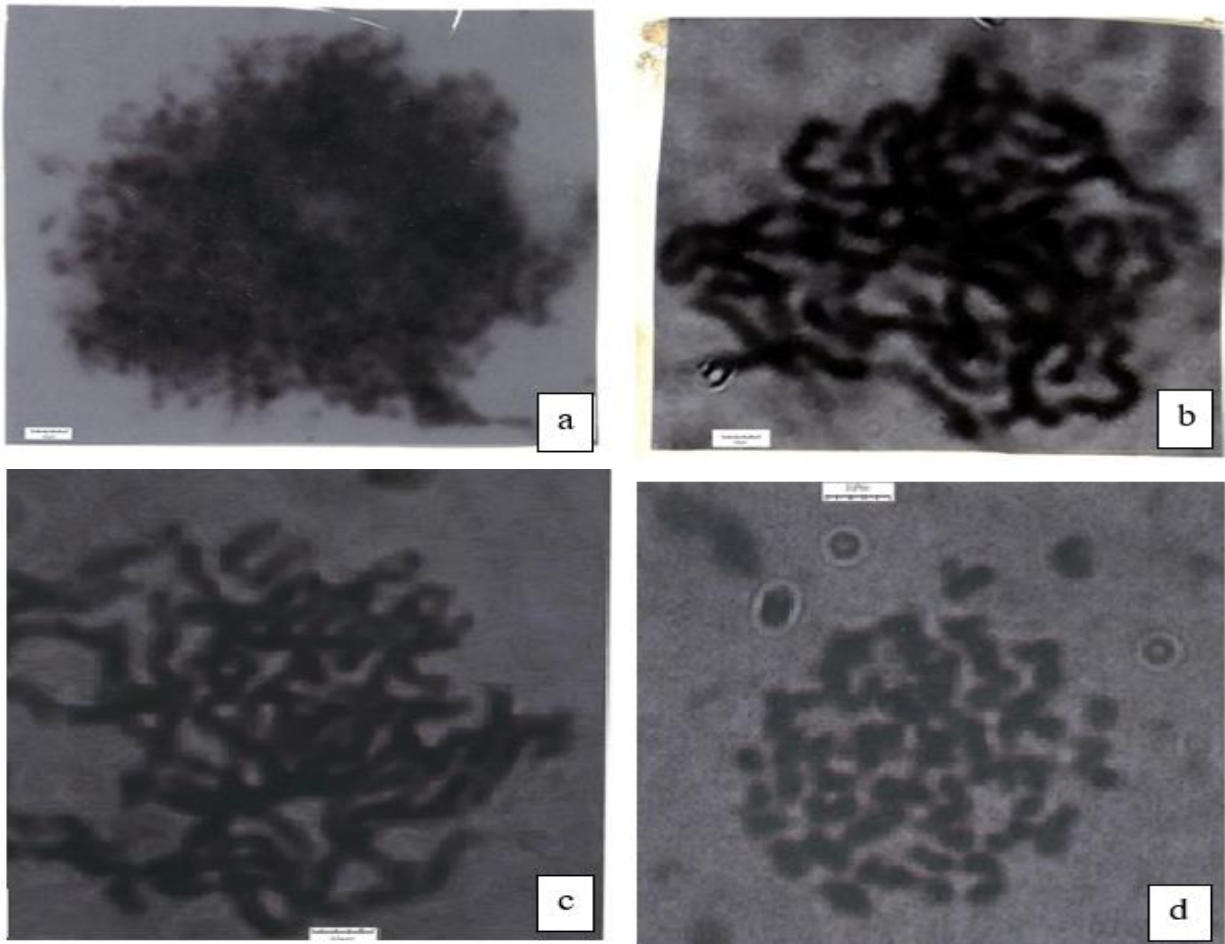


Plate 3: the meiotic chromosome stages observed in ovotestis cells of *Achatina fulica* (a) leptotene stage; (b) Pachytene stage; (c) Zygotene stage; and (d) Diplotene stage.

The Chromosome Morphology of Achatina achatina

Well-spread mitotic metaphase chromosome was examined from the ovotestis of *Achatina achatina* and the diploid chromosome number was also found to be $2n=60$ with a fundamental number (FN) of 100. Plate 4 shows the mitotic metaphase chromosome spreads of *Achatina achatina*. The karyotype is shown in Plate 5. From the karyotype, it was observed that the chromosomes were divided into three main groups according to their size. Group one consists of large chromosome sizes from 1 to 10 and they are relatively visible and can be identified clearly in all the metaphase spreads. Chromosomes 1, 2, and 3 were categorized into metacentric, two pairs (3 and 9) were telocentric, and five pairs (5, 6, 7, 9, and 10) were sub-telocentric. Group two consists of medium-sized chromosomes from 11 to 20 which are visible and identifiable. Eight pairs of these chromosomes (11, 12, 14, 16, 17, 18, 19, and 20) were categorized into metacentric, and two pairs (13 and 15) were telocentric. Group three consists of small-sized chromosomes from 21 to 30 and four pairs were categorized into metacentric (21, 22, 23, and 25), and six pairs were telocentric (24, 26, 27, 28, 29, and 30). The chromosomal formula for the complement was calculated as $2n=15m+5st + 10t$ and the corresponding fundamental arm number was calculated as $NF= 100$. The chromosome measurements were accurately used to construct the idiogram and histogram as shown in Figures 3 and 4. Figure 3 was constructed based on the chromosome number and position of the centromere and it was observed that there were variations in the centromeric location, resulting in variations in the lengths of the long (q) arms and short (p) arms. Figure 4 shows the size variation of the chromosomes and it was observed that there is a decrease in the size of the chromosomes, beginning from chromosomes 2 and 3 which extended to the rest of the chromosomes. It was observed also that chromosomes 13 and 14 have approximately the same length. Plates 6a-6d show the different stages of meiotic division observed in *Achatina achatina*.

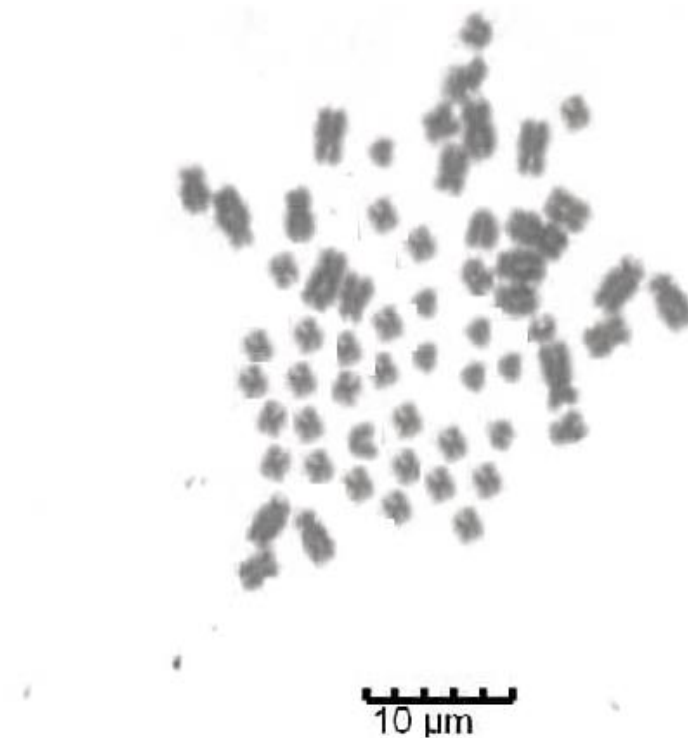


Plate 7: The mitotic metaphase chromosomes obtained from ovotestis of *Achatina achatina* showing diploid chromosome number ($2n=60$).

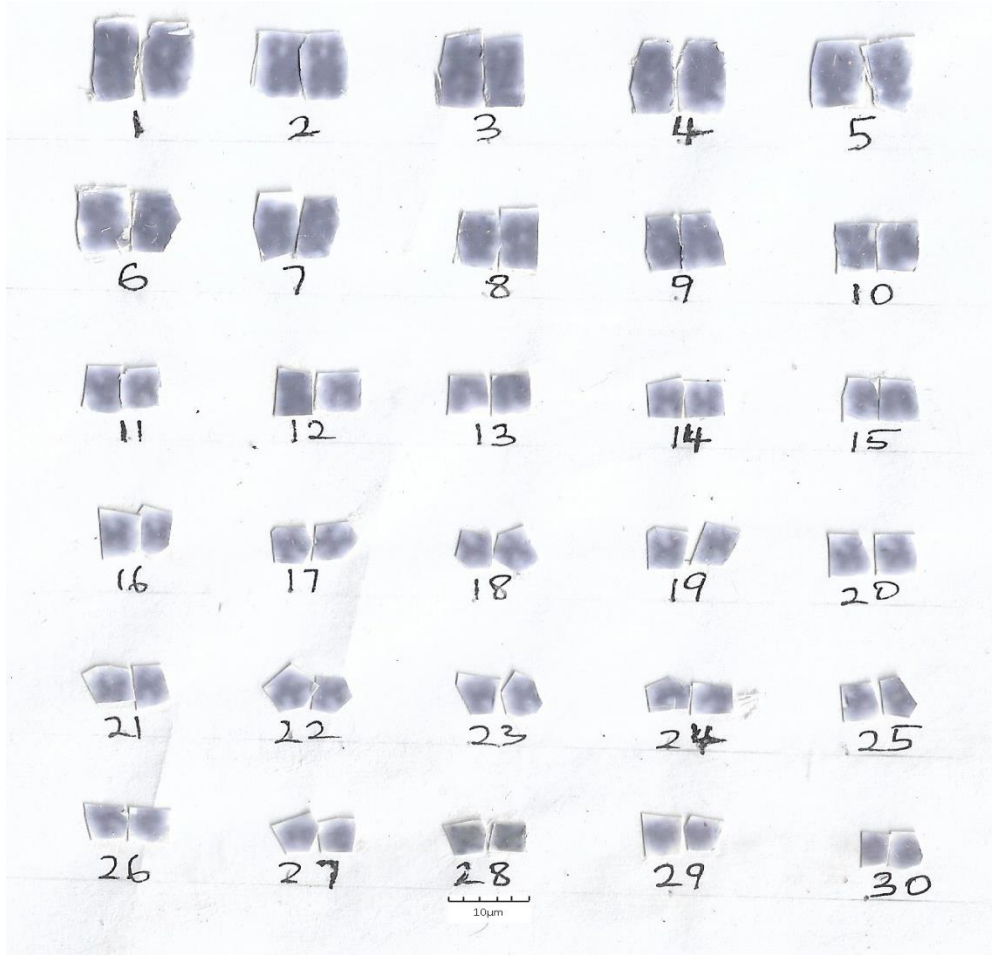


Plate 8: The karyotype of *Achatina achatina*

The centrometric index (i) = 100 p/t.

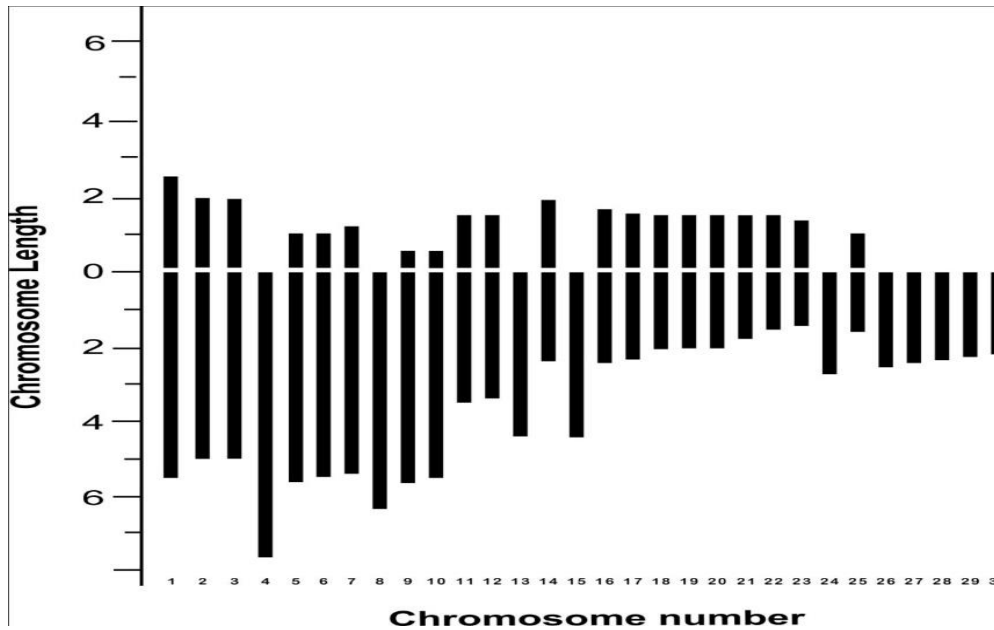


Figure 6: The idiogram of the karyotype of *Achatina achatina* constructed on the basis of chromosome numbers and position of the centromere.

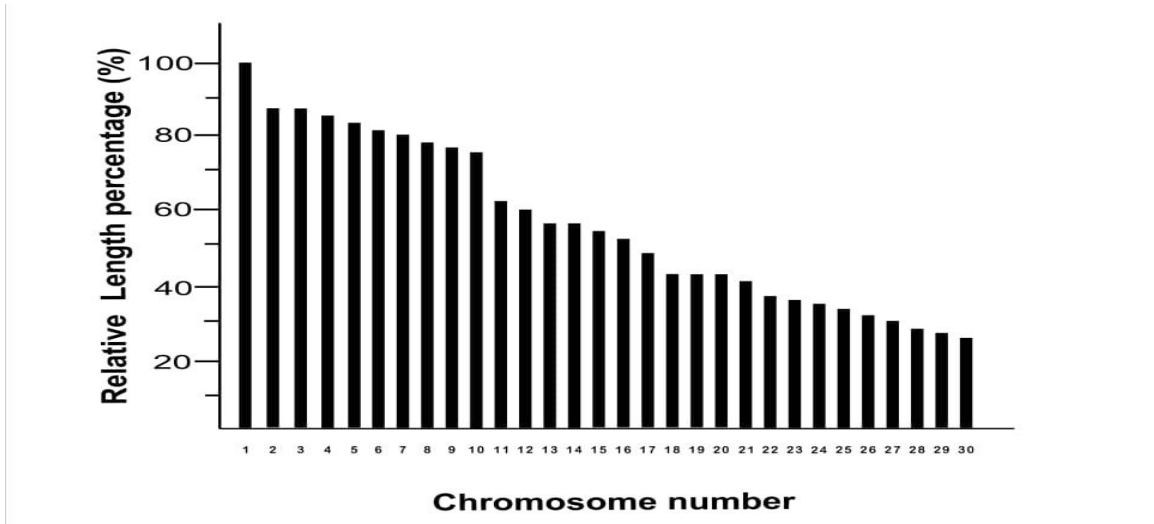


Figure 7: The histogram of the karyotype of *Achatina achatina* showing the relative length Percentage of chromosomes

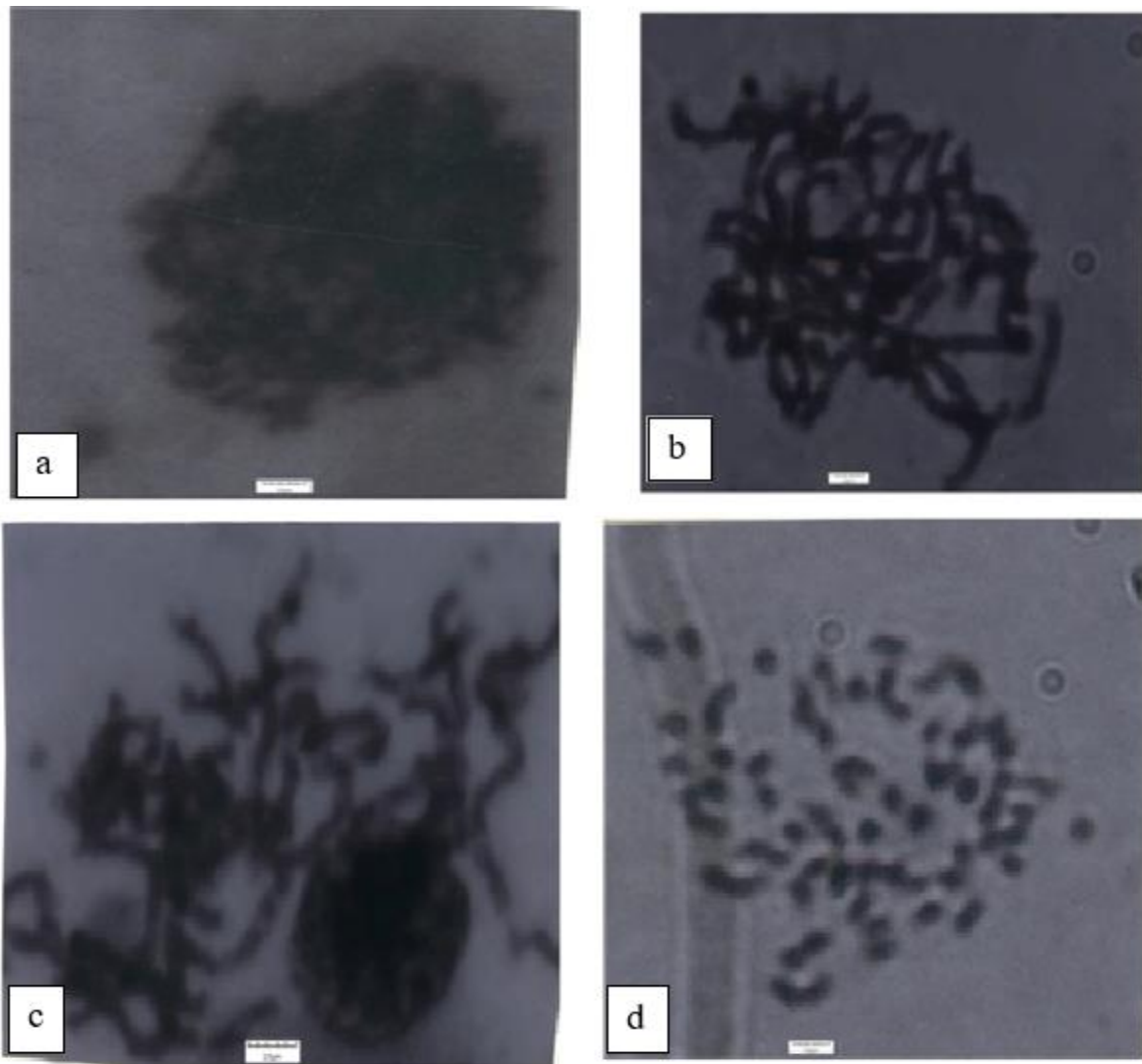


Plate 9: Meiotic chromosome stages observed in ovotestis cells of *Achatina achatina* (a) leptotene stage; (b) Pachytene stage; (c) Zygotene stage; and (d) Diplotene stage.

Discussion

Generally, in molluscs, the diploid chromosome numbers vary from five (land snail, *Catinella rotundata* of Hawaii, United States of America) to 60 (the European, *Ancylus fluviatilis*) and 72 (the Ethiopian freshwater planorbis, *Bulinus octoploidus*) (Burch, 1964). Reported studies on karyotype analysis of molluscs are not abundant. This may be due to difficulties in obtaining mitotic fields with adequate quality to characterize the chromosomes (Park *et al.*, 1999). There are very few reports on cytological studies of the species of land snails available in Nigeria (Fagbuaro *et al.*, 2002, Idowu and Akinnusi, 2006; Awodiran *et al.*, 2012, Okon *et al.*, 2017) and most studies from earlier works report only the chromosome number without the karyotype analysis. This may be due to the old technique of slide preparation such as the squash technique, used by earlier workers. The squash technique involves placing tissue samples between two glass slides and gently pressing them down to spread the cells into a thin layer. The slides are then moved apart in a quick, horizontal motion, creating a "squashed" layer of cells. The preparation is then stained and examined under a microscope. The chromosome number reported in this study is similar to earlier reports. The diploid chromosome number of $2n=60$ obtained for *Achatina fulica* in this study is consistent with the report of Natarajan (1960); Choudhury and Mohapatra (1991), and Jayashankar *et al.* (2014). Meanwhile, this diploid chromosome number ($2n=60$) reported for *Achatina fulica* is quite higher than the $2n=54$ reported by Idowu and Akinnusi (2006) and $2n=44$ by Fagbuaro *et al.* (2004). The disparity observed in the chromosome number might be attributed to variations in mitotic index and the presence of a large number of very small chromosomes (Choudhury, 1992), as well as the types and sizes of chromosomes observed. The haploid chromosome number, $n=30$ of *A. fulica* has also been reported previously in India (Patterson, 1969; Choudhary and Mohapatra, 1991); whereas the haploid number of $n=31$ was reported for the same species in China (Sun, 1995). Although the karyotype of these two populations was found to be different, the population from India has the majority of metacentric chromosomes while that of China was found to possess more telocentric chromosomes. (Choudhary and Mohapatra, 1991).

Similarly, the diploid chromosome number of $2n=60$ was obtained for *A. achatina* in this study which is consistent with Awodiran *et al.* (2012) and Okon *et al.* (2017).

Although the two species reported in this study have the same diploid chromosome number ($2n=60$); their chromosome morphology differs. Their karyotypes vary in size, form, and shape, which could serve as the basis for grouping them into different species. Generally, most molluscan groups tend to maintain stable chromosome numbers across various taxonomic levels. Higher taxa typically have distinctive chromosome numbers, with only a few species showing significant deviations from the basic haploid set (Patterson, 1969). However, karyotypic evolution in these organisms cannot be inferred solely from chromosome numbers. Extensive data on the detailed morphology of mitotic and meiotic chromosomes, as well as their banding patterns, are essential for such studies. The occurrence of sex chromosomes in some gastropod species has been reported by Patterson (1969) and Nakamura (1986) while natural polyploid species of gastropods have also been reported (Patterson and Burch, 1978). In this present study, sex chromosomes and polyploids were not observed.

Conclusion

The chromosome number and morphology of the two different species of snails reported in this study have contributed to the understanding of the cytogenetics of members of the family *Achachatiniidae*. This information would be useful in solving taxonomic problems as well as providing insight into their phylogenetic relationships. Further studies involving DNA sequences that will help in the investigation of the karyo-systematical evolution of members of the family *Achachatiniidae* are hereby recommended.

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Authors Contributions

Hinmikaiye Funmilayo Faith conceived the study, conducted laboratory work, and wrote the first draft of the manuscript. Ejere Vincent Chikwendu supervised the study and edited the manuscript. Awodiran

Michael Olufemi provided laboratory resources and also edited the manuscript. Oladimeji Tofunmi Elizabeth critically reviewed and edited the manuscript.

Ethics

Standard ethical considerations were followed for the snails used for this study.

Conflict of interests

The authors declare no competing interests.

References

- Awodiran, MO., Awopetu, JI. and Akintoye, MA. 2012.** Cytogenetic study of four species of land snails of the family Achatinidae in South-Western Nigeria. *Ife Journal of Science*, 14(2): 233 – 235.
- Burch, J. B. 1964.** Chromosomes of the succineid snail *Catinella rotundata*. *Occasional Paper of the Museum of Zoology, University of Michigan*, 638: 1 - 8.
- Choudhary, RC. and Mohapatra, I. 1991.** Chromosomes of a pestiferous land snail, *Achatina* (*Lissachatina*) *fulica fulica* (Bowdich) (Achatinidae: Pulmonata: Gastropoda). *Caryologia*, 44:201 – 208.
- Chukwuka, CO. and Ejere, VC. 2019.** Physiological adjustment of the genus *Limicolaria* (Gastropoda: Pulmonata) during aestivation and activity in tropical agro-ecosystem. *Journal of Molluscan Research*, 39: 89 – 97.
- Elejalde, MA., Madeira, MJ., Arrebola, JR., Munoz B. and Gomez-Moliner, BJ. (2008).** Molecular Phylogeny, taxonomy and evolution of the land snail genus *Iberus* (Pulmonata: Helicidae). *Journal of Zoology and Systematic Evolution Research*: 46(3): 193–202.
- Fagbuaro, O., Mosuro, AA., Bakare, AA. and Odaibo, AB. 2002.** Cytological studies of *Archachatina marginata* and *Achatina* species from South Western Nigeria. *Tropical Veterinary* 20(2): 74 – 79.
- Guan, F., Niu, A.O., Attwood, S.W., Li, YL., Zhang, B. and Zhu, YN. (2008).** Molecular phylogenetics of Triculine snails (Gastropoda: Pomatiopsidae) from Southern China. *Molecular Phylogenetics and Evolution*, 48: 702 – 707.
- Herbert, D. and Kilburn, D. 2004.** *Field Guide to the Land Snail and Slugs of Eastern South Africa*. Natal Museum, Pietermaritzburg, South Africa.
- Idowu, AB. and Akinnusi, O. 2006.** The structure of the ovotestis of the common African land snails found in Abeokuta, South/ West Nigeria. *Journal of Animal Production*, 33(2): 286 – 293.
- Jayashankar, M., Arun, KR., Reddy, MS., Venu, G. and Ramakrishna, S. 2014.** Shell banding pattern and chromosomal studies on the giant African snail, *Achatina fulica* (Bowdich) (Achatinidae: Gastropoda). *Indian Journal of Applied Research*, 4: 2249 – 555.
- Justin, R. and Tara, SR. 2014.** Ecological studies of polyploidy in the 100 years following its discovery.
- Lee, R., Lovatelli, A. and Ababouch, L. 2008.** Bivalve depuration: fundamental and practical aspects. *FAO Fisheries Technical Paper Rome, Italy*, 511: 139 – 142
- Leitao, A., Vasconcelos, P., Ben-Hamadou, R., Gaspar, MB., Barroso, CM. and Ruano, F. 2009.** Cytogenetics of *Bolinus brandaris* and phylogenetic inferences within the Muricidae (Mollusca: Gastropoda). *Biological Journal of the Linnean Society*, 96: 185 – 193.
- Nakamura, H. K. (1986).** Chromosomes of Archeogastropoda (Mollusca: Prosobranchia) with some remarks of their cytotaxonomy and phylogeny. *Publications of the Seto Marine Biological Laboratory*, 31: 191 – 268.
- Natarajan R., (1960).** - Further cytological studies in Pulmonata (Mollusca-Gastropoda). *J. Zoo. Soc. India.*, 12: 69-79

- Okon, B., Ibom, LA., Dauda, A., Bassey, AE., Awodiran, MO., and Etukudo, MO. 2017.** Chromosome numbers, evolutionary relationships and divergence among three breeds of Giant African land snails in Nigeria. *Nigerian Journal of Animal Production*, 44(4): 1 - 10.
- Onyenucheya, CO. and Nnamchi, HC. 2018.** diurnal and annual mean weather cycles over Nsukka, Nigeria during 2010/2011. *Nigerian Journal of Technology.*, 37(2):519 – 524
- Patterson, C. M. (1969).** Chromosomes of molluscs. Proceedings of the 2nd Symposium of Mollusca, Ernakulam, Cochin, Marine Biological Association of India.
- Patterson, C. M. and Burch, J. B. (1978).** Chromosomes of pulmonate molluscs. In: Fretter, V. and Peake, J. (Editors). Pulmonates: Systematics, Evolution and Ecology. Academic Press, New York.
- Park, G., Kim Jae-Jin, Chung, P., Wang, Y. and Min Duk, Y. 1999.** Karyotypes on three Species of Chinese mesogastropod snails, *Semisulcospira libertine*, *S. dolichostoma* and *Viviparus rivularis*. *The Korean Journal of Parasitology*, 37(1): 5 – 11.
- Puslednik, L., Ponder, WF., Downton, M. and Davis, AR. 2009.** Examining the phylogeny of the Australasian Lymnaeidae (Heterobranchia: Pulmonata: Gastropoda) using mitochondrial, nuclear and morphological markers. *Molecular Phylogenetics and Evolution*, 52: 643 – 659.
- Sogbesan, AO. and Ugwumba, AAA. 2012.** Culture and nutrient values of *Limicolaria aurora* (Jay, 1989) (Mollusca: Achatinidae) raised in two different substrates *Revista Cientifica UDo Agricola* 12 (2): 479-485
- Sun T. 1995.** Chromosomal studies in three land snails. *Sinozoologia*, 12: 154 – 162.
- Stunzenas, V., Petkeviciute, R. and Staneviciute, G. 2011.** Phylogeny of *Sphaerium solidum* (Bivalvia) based on karyotype and sequences of 16S and ITS1 rDNA. *Central European Journal of Biology*, 6(1): 105 – 117.