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PALYNOLOGICAL ANALYSIS OF ANNONA MURICATA, A. SQUOMOSA AND A. SENEGALENSIS

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ABSTRACT
Palynological analysis of *Annona senegalensis* Pers., *Annona squamosa* Linn. and *Annona muricata* Linn. was carried out to compare the pollen morphology of these species and to identify the differences, if any, that might warrant their re-classification. Fresh flowers of these plants were collected, dried and crushed to liberate the pollen grains. The liberated pollen grains were subjected to acetolysis treatment and then examined under the microscope. Photomicrograph of the pollen was taken using trinocular microscope fitted with a digital camera. Results showed that all the plants had tetrad, acalymate pollen that were inaperturate. Pollens of *A. senegalensis* and A. *squamosa* were tetragonal with a globose shape, rugulate exine ornamentation and an intectate exine inner structure. On the contrary, pollen of *A. muricata* was rhomboidal with an ellipsoidal shape, reticulate exine ornamentation and a semitectate exine inner structure. *A. muricata* had the highest pollen size with tetrad and monad diameter of 229 µm and 111 µm, respectively while *A. squamosa* had the least (tetrad diameter = 99 µm; monad diameter = 50 µm). Sexine was only present in *A. muricata* (5.5 µm). The observed differences in the pollen morphology of these three studied species do not warrant their re-classification.

Keywords: *Annona spp.*, acetolysis; aperture; acalymate; exine; pollen sculpture
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INTRODUCTION
Annona belongs to the family Annonaceae. This family is one of the largest tropical and subtropical families of trees, shrubs and lianas with about 135 genera and 2500 species widely distributed in the world (Escribano *et al*., 2007). The genus *Annona*, commonly known as the custard-apple, consists of some 125 species with some widely cultivated for their edible fruits and often becoming naturalised beyond their native range of tropical America and Africa (Wagner *et al.*, 2014). *There are just six* species in the genus *Annona* that produce edible fruits. They are *A. squamosa* (widely cultivated), *A. reticulata, A. cherimola, A. muricata, A. atemoya* (a natural hybrid of *A. squamosa* and *A. cherimola*) and *A. diversifolia.* Majority of the *Annona* species are believed to have originated from South America and the Antilles. However, wild soursop (*A. muricata*) and *A. senegalensis* are believed to be of African origin (Pinto *et al*., 2005).

The *Annonas* spp are hardy and deciduous, quite easy to cultivate and require relatively minimal input with little or no care. However, they have not been fully domesticated *(*Zonneveld *et al., 2012*; Anuragi *et al., 2016*). The genus *Annona* is a multipurpose plant that has found application in numerous aspects of human endeavour: as food, in industries and medicine. Every part of the plant is useful. The fruits are edible and are in high demand by the processing industries, especially those of *A. squamosa* and *A. muricata* (Santos *et al.*, 2011). The pulp is a rich source of minerals and vitamins (Gyamfi *et al.*, 2011). Seeds of *Annona*, especially *A. muricata*, are rich in oil and can be exploited for use in oil and allied industries (Anuragi *et al.,* 2006). All parts of the *Annona* plant including the leaves, roots, barks, fruits and seeds contain bioactive compounds that have been found useful in medicine *(*Pinto *et al., 2005).*

Palynology, the study of spores and pollen grains, is very important in evolutionary and taxonomic research. Several studies have indicated that the various morphological and ultrastructural features found in the spores and pollen grains are of taxonomic values. They are useful for identification of species and in determining the phylogenetic, interspecific and intraspecific relationships of plants (Khaleghi *et al*., 2019; Xu *et al*., 2019). Edeoga and Ikem (2006) and Mbagwu *et al*. (2009) used attributes of the pollen aperture to show probable evidence of relationships among some species in many genera of flowering plants. According to them, pollen morphology can be useful in solving differences in the classification of problematic taxa and in supporting taxonomic evidence.

A number of studies have been carried out on the pollen morphology of the family Annonaceae (Su and Saunders, 2003; Gan *et al*., 2015) with very few centered on the genus *Annona*. Hence, the need for more studies on the pollen features of species of *Annona*. This work was, therefore, undertaken to compare the pollen morphology of three species of *Annona* (*A. senegalensis* Pers., *A. squamosa* L. and *A. muricata* L.) grown in Nigeria and to identify the differences, if any, that might warrant their re-classification.

MATERIALS AND METHODS

Study location

This study was conducted in the University of Nigeria, Nsukka.

Pollen extraction

Fresh flowers of the three *Annona* species used for the study were collected from Umuahia and Uturu in Abia State. They were dried, crushed to liberate the pollen and sieved with a wire gauze. About 5 g of the pollen grain was measured into a well labeled centrifuge tube for acetolysis treatment.

Acetolysis treatment

Acetolysis mixture was prepared by mixing acetic anhydride and concentrated sulphuric acid in the ratio of 9:1, i.e. 9 ml of acetic anhydride and one ml of conc. sulphuric acid in a measuring cylinder. Five (5) ml of the acetolysis mixture was introduced into the centrifuge tube containing the liberated pollen grains and heated in a water bath at a temperature of 100ºC for 10 minutes. The mixture was then allowed to cool and later centrifuged at 2000 rpm for 20 minutes. The filtrate was decanted leaving the polliniferous residue in the tube. Five (5) ml of glacial acetic acid was added to the residue and centrifuged again at 2000 rpm for 20 minutes. The filtrate was decanted and 10 ml of distilled water was added to the residue and centrifuged again. This was done to remove the offensive acids and make the sample neutral. Two drops of glycerol-alcohol were added to the residue as a suspensor and to prevent the sample from drying. The sample was then stored in a vial from where samples were collected for microscopic examination.

Microscopic examination

Two drops of the suspended polliniferous sample were dropped on a standard slide and covered with a cover slip. The cover slip was sealed along the edges with a nail hardener. This preserved the specimen for a few days and prevented desiccation. Morphological examination was carried out at x100 and x1000 magnifications using a light microscope. Photomicrographs of the pollen were also taken at x100 and x1000 magnifications using trinocular compound microscope with digital camera attached (Nikon CoolPix5000).

Assessment of parameters

Parameters assessed were qualitative and quantitative characters of the pollens of the three *Annona* species. The qualitative characters include the pollen type, pollen symmetry, pollen cohesion, pollen aperture, pollen shape, exine ornamentation and exine inner structure. The quantitative characters assessed were the morphological dimensions of the tetrad pollen along both opposite directions as D1 and D2, the dimension of a monad (a unit), the exine, sexine (where applicable) and nexine thickness.

RESULTS

The morphological characters of the pollen grain of the studied *Annona spp*. are summarised in Tables 1 and 2 and the photomicrographs shown in Plates 1-3. All the three species had tetrad pollens that were acalymate and inaperturate. However, *A. senegalensis* and *A. squamosa* had tetragonal pollen symmetry with a globose pollen shape whereas rhomboidal symmetry and ellipsoidal pollens were found in *A. muricata*. Exine ornamentation was rugulate in *A. senegalensis* and *A. squamosa* while *A. muricata* had a reticulate exine sculpture.

In the case of the quantitative characters, it was observed that the size of the pollen grains varied among the studied species. Pollen grain of *A. muricata* was the largest in size with tetrad diameters of 122 µm and 130 µm, exine thickness of 7 µm, nexine thickness of 1.5 µm and sexine thickness of 5.5 µm. The *A. squamosa* had tetrad diameters of 82 µm and 99 µm, exine thickness of 2 µm and nexine thickness of 0.5 µm. Sexine was not found in both *A. senegalensis* and *A. squamosa.*

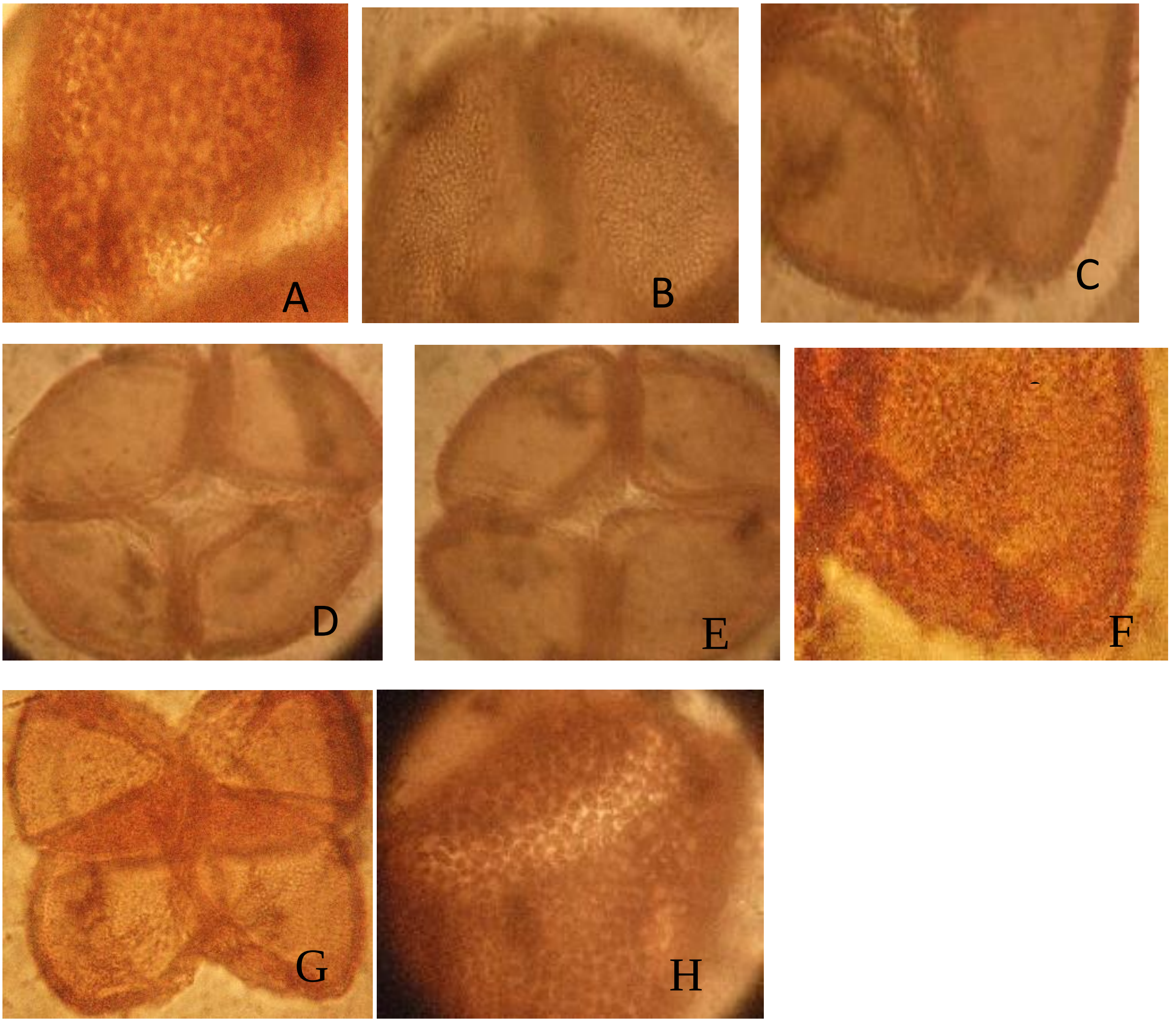

Plate 1(A-H): Photomicrograph of pollen morphology of *Annona senegalensis* A, C, F and H (x1000) show the type of exine sculpture/ornamentation, which is ‘rugulate’’ B, D, E and G (x100) show the tetrad, acalymate, tetragonal pollen.

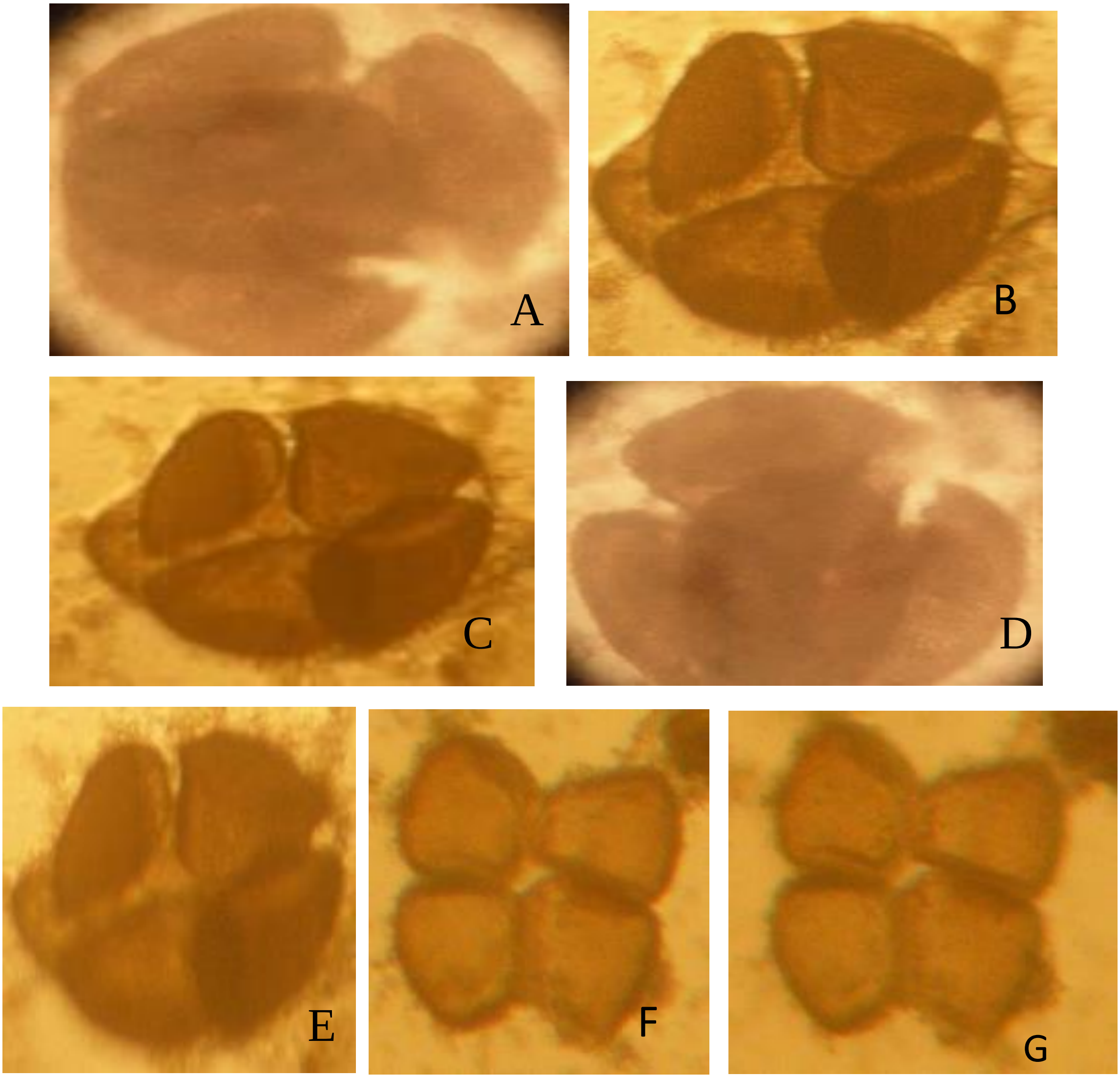

Plate 2 (A-G): Photomicrograph of *Annona squamosa*, A, B, C, D and E (x1000) show the type of exine sculpture/ornamentation, which is rugulate; F and G (x100) show the tetrad acalymate tetragonal pollen and the globose pollen shape.

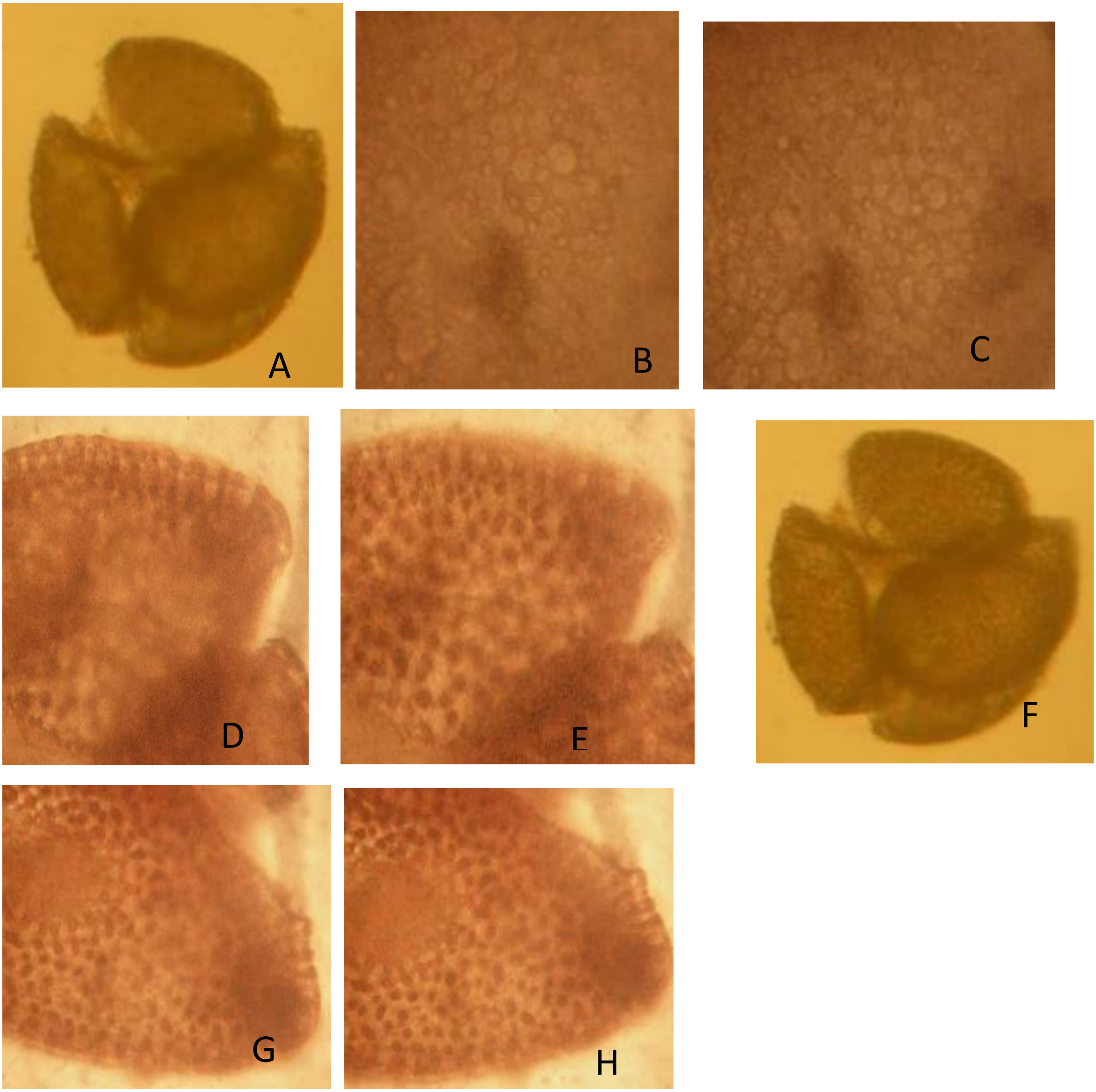

Plate 3 (A-H): Photomicrograph of *Annona muricata.* A and F x100 show the tetrad pollen with rhomboidal tetrahedral symmetry and ellipsoidal pollen shape; B, C, D, G and H (x1000) show the sculptural pattern; B and C show the reticulate (network-like) sculpture of the exine

Table 1: Qualitative characters of the pollen grains of *Annona spp*

	Annona species		
Qualitative character	*A. senegalensis*	*A. squamosa*	*A. muricata*
Pollen type	Tetrad	Tetrad	Tetrad
Pollen symmetry	Tetragonal	Tetragonal	Rhomboidal
Pollen cohesion	Acalymmate	Acalymmate	Acalymmate
Aperture	Inaperturate	Inaperturate	Inaperturate
Pollen shape	Globose	Globose	Globose
Exine sculpture	Rugulate	Rugulate	Reticulate
Exine inner structure	Intectate	Intectate	Semitectate

Table 2: Quantitative characters of the pollen grains of the *Annona spp*

	Annona species		
Quantitative character (µm)	*A. senegalensis*	*A. squamosa*	*A. muricata*
D1	122 (120 - 125)	82 (72 - 87)	200(169- 231)
D2	130 (119 - 150)	99 (91 -106)	229(220- 237)
D3	72 (63 - 80)	50 (45 - 55)	111(110- 112)
Exine thickness	3 (3 - 3.2)	2	7
Nexine thickness	0.4 (0.4 - 0.5)	0.5	1.5
Sexine thickness	NA	NA	5.5

D1 and D2 = tetrad diameters in opposite directions; D3 = monad diameter; NA = not applicable. Values are means of five replicates.

DISCUSSION

Morphological characters of the pollen are a valuable tool in plant taxonomy and systematics. It provides additional features for plant species identification and thus can be employed to solve differences in the classification of problematic taxa (Mbagwu *et al.*, 2009). Different types of pollen aggregation are found in plants namely dyads, triads, tetrads or polyads. Both monad- and tetrad pollen-producing species are reportedly found in the genus *Annona* (Lora *et al*., 2014). However, the three *Annona* species studied in this work were all tetrad, which supports previous pollen morphology reports of these species (Lora *et al*., 2009; Lora *et al*., 2014; Gan *et al.*, 2015; Yangying *et al*. 2015). According to Lora *et al.* (2014), tetrad pollen results from the inability of the microspore to separate during pollen development. This is due to delay in the dissolution of the pollen mother cell wall, which is made of callose and cellulose. It could also be due to failure in the synthesis of the callose layer during microspore separation in the tetrad pollen (Blackmore *et al.,* 2007). Nonetheless, the release of pollen in aggregated forms (dyads, tetrads and polyads) is reported to be of evolutionary and adaptive importance (Blackmore *et al.,* 2007; Harder and Johnson, 2008). It is beneficial for pollen transfer in insect-pollinated species especially where pollinators are infrequent (Harder and Johnson, 2008), where there is short pollen viability and short pollen transport episodes (Lora *et al*., 2009). It can, therefore, increase pollination efficiency in insect-pollinated species. In addition, formation of aggregated pollen helps to protect the pollen grains from dehydration (Harder and Johnson, 2008).

Two types of pollen symmetry were observed viz tetragonal and rhomboidal. Whereas *A. senegalensis* and *A. squamosa* had tetragonal pollen symmetry, *A. muricata* had rhomboidal symmetry. This shows divergence in these species. Lora *et al.* (2014) reported tetragonal pollen in *A. squamosa* and rhomboidal pollen in *A. senegalensis* and other *Annona* species. Pollens were acalymmate in all the three species examined. This implies that the four pollen grains were held together only by partial fusion with simple cohesion as reported by Su and Saunders (2003). This may possibly explain the observed inaperturate condition of the tetrad pollen of the studied *Annona species*. Inaperturate tetrad pollen has also been reported by Su and Saunders (2003) in 42 species of *Pseuduvaria* in the family Annonaceae with the pollen released as acalymmate tetrads. On the contrary, Lora *et al.* (2014) reported punctuated exine cohesion in *Annona* species with tetrad pollen (*A. squamosa*, *A. atemoya* and *A. senegalensis*) and inaperturate pollen in the monad species (*A. emarginata* and *A. neosalicifolia*). According to them, large apertures are reportedly seen in species with permanent tetrad pollen. Pollen aperture formation is linked to delay in dissolution of callose (Lora *et al*., 2009; Albert *et al*., 2011). This is also the reason for the formation of tetrad pollen (Lora *et al.*, 2014). Since the studied *Annona* species released tetrad pollens that were inaperturate, the delay in the digestion of the callose may not explain the reason for the formation of tetrad pollen in these species. The possible explanation for the formation of tetrad pollen in these species could be the failure in the synthesis of the callose layer during microspore separation in the tetrad pollen as suggested by Blackmore *et al.* (2007).

Annona senegalensis and *A. squamosa* had a globose pollen shape, rugulate exine ornamentation and an intectate exine inner structure. On the other hand, ellipsoidal pollen shape, reticulate exine ornamentation and semitectate exine inner structure were found in *A. muricata*. Lora *et al.* (2014) also reported a globose pollen shape in some species of *Annona* including *A. senegalensis* and *A. squamosa*. Xu and de Craene (2012) reported elliptical pollen grains with globose shape and rugulate exine sculpture in some species belonging to seven different genera in the family Annonaceae. The similarities observed in the pollen features of *A. senegalensis* and *A. squamosa* is an indication of monophyllogeny of these two species. Among the pollen characteristics, pollen aperture is said to be the most basic diagnostic attribute of pollen grains followed by exine sculpture (Hayrapetyan, 2008).

Grain size is an important feature in the reproduction of flowering plants. It affects pollen germination, pollen tube growth and fertilization (Giovannini *et al.*, 2017). In this study, variation in the size of the pollen grains of the different species was found. *A. muricata* had the highest pollen diameter (110-112 µm for the monad and 169-237 µm for the tetrad) while *A. squamosa* had the least (45-55 µm for the monad and 72-106 µm for the tetrad). Ertman (1945) as cited by Badamtsetseg *et al.* (2012) categorised pollen into four different sizes: < 10 µm as very small; 10-25 µm as small; 25-50 µm as medium and 50-100 µm as large. Based on this categorisation, *A. senegalensis* (monad diameter of 72 cm) and *A. squamosa* (monad diameter of 50 µm) could be said to have a large pollen while *A. muricata* had very large pollen (unclassified) with a monad diameter of 111 µm. The variation found in the pollen size of the *Annona* species could be attributed to the size of their flowers. Kostryco *et al.* (2020) in their study on pollen grain micromorphology and ultrastructure of six cultivars of *Rubus idaeus* observed variation in the pollen size of the studied cultivars which was attributed to the pollination and habitat conditions. Reports have shown that pollen size are affected by a variety of factors including the mineral content of the soil, shoot defoliation and climate change. Higher concentrations of soil nitrogen and phosphorus have been reported to increase the size, yield and germinability of pollen grains (Lau and Stephenson, 1994). Shoot defoliation decreases the size of the pollen grain resulting in inhibition of the growth of pollen tubes and reduction in the number of seeds (Aizen and Raffaele, 1998). The increased size of the pollen grain has been correlated with reduced water availability, which stems from long-term impact of climate change (Griener and Warny, 2015).

CONCLUSION

The pollen morphology of the studied *Annona* species showed similarities in the form pollen is released, cohesion and aperture of the pollen but variation in the other pollen characteristics. The similarities in pollen features found among the investigated species showed interspecific relationships of the individual species. *A. senegalensis* and *A. squamosa* can be said to be closely related as they share very important pollen features unlike *A. muricata*. However, the differences found are not enough for re-classification of these species.

REFERENCES

Albert, R. A., Ressayre and Nadot, S. (2011). Correlation between pollen aperture and callose deposition in tetrad stage of three species producing atypical pollen grains. *American Journal of Botany*, 98: 189-196.

Aizen, M.A. and Raffaele, E. (1998). Flowering-shoot defoliation affects pollen grain size and postpollination pollen performance in *Alstroemeria aurea*. *Ecology*, 79: 2133-2142.

Anuragi, H., Dhaduk, H. L., Kumar, S., Dhruve, J.J., Parekh, M.J. and Sakure, A.A. (2016). Molecular diversity of *Annona* species and proximate fruit composition of selected genotypes. 3 *Biotechnology*, 6: (204): 1-10.

Badamtsetseg, B., Myoung, L.E. and Yuon, L.H. (2012). Pollen Morphology of the Family Lamiaceae in Mongolia. *Journal of Korean Nature*, 5(2): 169-179.

Blackmore, S., Wortley, A.H., Skvarla, J.J. and Rowley, J.R. (2007). Pollen wall development in flowering plants. *New Phytologist*, 174: 483-498.

Edeoga, H.O. and Ikem, I.C. (2006). Pollen morphology of some Nigerian species. *New Botany*, 23: 223-231.

Escribano, P., Viruel, M.A. and Hormaza, J.I. (2007). Molecular analysis of genetic diversity and geographic origin within an *ex situ* germplasm collection of cherimoya by using SSRs. *Journal of the American Society for Horticultural Science*, 132: 357–367.

Gan, Y., Yang, L. and Fengxia, X. (2015). Pollen morphology of selected species from Annonaceae. *Grana*, 54: 271-281.

Giovannini, A., Macovei, A.. Caser, M., Mansuino, A., Ghione, G.G.. Savona, M., Carbonera, D., Scariot, V. and Balestrazzi, A. (2017). Pollen grain preservation and fertility in valuable commercial rose cultivars. *Plants*, 6: 17.

Griener, K.W. and Warny, S. (2015). *Nothofagus* pollen grain size as a proxy for long-term climate change: An applied study on Eocene, Oligocene and Miocene sediments from Antarctica. *Review in Paleobotany and Palynology*, 221:138-143.

Gyamfi, K., Sarfo, D., Nyarko, B., Akaho, E., Serfor-Armah, Y. and Ampomah-Amaoka, E. (2011). Assessment of elemental content in the fruit of graviola plant, *Annona muricata*, from some selected communities in Ghana by instrumental neutron activation analysis. *Elixir Journal*, 41:5671-5675.

Harder, L. and Johnson, S. (2008). Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Science*, 169: 59-78.

Hayrapetyan, A.M. (2008). Features of the exine ornamentation of pollen grains in the family Solanaceae Juss. I. The simple types of ornamentation. *National Academy of Sciences of RA Electronic Journal of Natural Sciences*, 2(11): 46-50.

Khaleghi, E., Karamnezhad, F. and Moallemi, N. (2019). Study of pollen morphology and salinity effect on the pollen grains of four olive (O*lea europaea*) cultivars. *South African Journal of Botany,* 127: 51-57.

Kostryco, M., Chwil, M. and Matraszek-Gawron, R.S. (2020). Comparison of the micromorphology and ultrastructure of pollen grains of selected *Rubus idaeus* L. cultivars grown in commercial plantation. *Plants*, 9:1194-1224.

Lau, T.C. and Stephenson, A.G. (1994). Effect of soil phosphorus on pollen production, pollen size, pollen phosphorus content and the ability to sire seeds in *Cucurbita pepo* (Cucurbitaceae). *Sexual Plant Reproduction*, 7: 215-220.

Lora, J., Testillano, P.S., Risueño, M.C., Hormaza, J.I. and Herrero, M. (2009). Pollen development in *Annona cherimola* Mill. (Annonaceae): implications for the evolution of aggregated pollen. *BMC Plant Biology*, 9: 129.

Lora, J., Herrero, M. and Hormaza, J.I. (2014). Microspore development in *Annona* (Annonaceae): Differences between monad and tetrad pollen. *American Journal of Botany,* 101(9): 1508–1518.

Mbagwu, F.N., Chime, E.G. and Unamba, C.I.N. (2009). Palynological studies on five species of Asteraceae. *African Journal of Biotechnology*, 8 (7): 1222-1225.

Pinto, A.C.Q., Cordeiro, M.C.R., de Andrade, S.R.M., Ferreira, F.R., Filgueiras, H.A.C., Alves, R.E. and Kinpara, D.J. (2005). *Annona* species. International Centre for Underutilised Crops, University of Southampton, Southampton. 263p.

Santos, M.Q.C., Lemos, E.E.P., Salvador, T.L., Rezende, L.P., Silva, J.W., Barros, P.G. and Campos, R.S. (2011). Rooting cuttings of soft soursop (*Annona muricata*), 'Giant of Alagoas'. *Acta Horticulture*, 923: 241–245.

Su, Y.C.K. and Saunders, R.M.K. (2003). Pollen structure, tetrad cohesion and pollen-connecting threads in *Pseuduvaria* (Annonaceae). *Botanical Journal of the Linnean Society*, 143: 69-78.

Wagner, W.L., Herbst, D.R. and Lorence, D.H. (2014). Flora of the Hawaiian Islands Smithsonian Institution. Washington DC, USA.

Xu, F. and de Craene, L.P. R. (2012). Pollen morphology and ultrastructure of selected species from Annonaceae. *Plant Systematics and Evolution*, doi:10.1007/s00606-012-0698-1.

Xu, C. Y., Hou, X., Li, X., Zhang, X. and Shao, J. (2019). Pollen morphological variation of *Primula merrilliana* and its systematic significance. *Flora*, 253: 43-48.

Yangying, G., Yong, L. and. Fengxia, X. (2015). Pollen morphology of selected species from Annonaceae. *Grana*, 54: 271-281.

Zonneveld, M.V., Scheldeman, X., Escribano, P., Viruel, M.A., Damme, P.V., Garcia, W., Tapia, C., Romero, J., Siguenas, M. and Hormaza, J.I. (2012). Mapping genetic diversity of cherimoya (*Annona cherimola* Mill): application of spatial analysis for conservation and use of plant genetic resources. *PLos One* 7:e29845.