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Research Article

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Comparative Morphology of the Leaf Epidermis in Six *Citrus* Species and its Biosystematic Importance

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Abstract

Studies on comparative morphology of the leaf epidermis in six *Citrus* species- *C. sinensis, C. limon, C. aurantifolia, C. reticulata, C. paradisi* and *C. maxima* were carried out to ascertain the systematic importance of epidermal features in classification of these taxa. The epidermal carpet shows no appendages or subsidiary cells. The six species studied are hypostomatic with anomocytic type of stomata. Contiguous, mega stomata and special sheath cells are present in some species and could be of taxonomic and ecological potential. There are contiguous stomata in *C. sinensis, C. limon, C. aurantifolia* and *C. maxima* while lacking in *C. reticulata* and *C. paradisi*. The mega stomata are present in *C. sinensis* and *C. limon* but lacking in other species. The sheath cells are present in *C. sinensis, C. limon, C. paradisi* and *C. maxima* in the upper epidermis, and in *C. limon, C. aurantifolia* and *C. reticulata* in the lower epidermis. In the leaf epidermal carpet architecture there is a consistent variation among the species of citrus studied each exhibiting its own character and some with remote characters ranging from rectangular, cuboidal, triangular, pentagonal, hexagonal, pyramidal and trapezial in the upper epidermis while in the lower epidermis *C. reticulata, C. paradisi* and *C. maxima* distinguished themselves from others. The biosystematic implications of these findings have been discussed in the light of current literature.

Keywords: Comparative; Leaf; Epidermis; *Citrus*; Biosystematic; Morphology

Introduction

The six species of the genus *Citrus* namely: *C. sinensis*, *C. limon*, *C. aurantifolia*, *C. paradizi*, *C. reticulate* and *C. maxima* belong to dicotyledonous family Rutaceae. The family has over 150 genera and 1500 species. They are mainly distributed in both tropical and temperate regions; especially in Australia and South Africa. In West Africa, it is represented by 9 genera and about 30 species. Several important genera such as *Citrus*, *Aegle, Murraya, Ruta, Africana, Limnocitrus, Citropsis, Fortunella, Glycosmis, Micromelum, and Fagara* are major representatives of the family. The family comprises of mainly trees and shrubs, rarely herbs. Some shrubs are climbers and xerophytic. The stems are erect, sometimes climbing, thorny branched, woody or rarely herbaceous, cylindrical, solid, green when young while grey when old. Leaves are single or pinnate or palmate. Compound exstipulate. They are usually alternate and rarely opposite. The leaves are dotted with glands which contain volatile oil. This volatile oil gives the leaves its typical smell. Leaves are reduced to spine in early stages of genera such as *Citrus* and *Feronia sp.* The petiole is glabrous to pubescent. Inflorescence may be cymose or racemose. Flowers are solitarity, axillary, bisexual, actinomorphic or occasionally zygomorphic, white or yellow hypogynous. Sometimes, the flowers are unisexual e.g. Evodia, Zanthoxylum, etc.

Calyx four to five sepals, polysepalous, green in colour. The corolla is polypetalous. Petals are coloured white, yellow or red. Androecium contains eight to ten stamens. Filaments are free with bilobed anther. Gynoecium is syncarpous, contains three, four or five carpels. Placentation is axile. The style is short and decidous. Fruits vary in different genera. They may be berry, drupe, capsule, samara or follicle.

Some genus such as *Citrus* is used for medicinal purposes. They contain Vitamin A,B,C and P as well as flavonoids, sugar, organic acids and mineral salts. Orange diminishes blood viscosity and produces a protective effect on blood vessel due to vitamin P, among other substances [1]. In some cultures, genus such as *Citrus* is used for traditional purposes. During China New Year Festivals, Mandarn Orange and Tangerines are considered traditional symbols of abundance and good fortune. They are often decorated and presented as gifts to friends and relations, even business associates [2]. *Citrus* also serve as ornamental trees. Orange flowers have delicious scent and are used in decorations and banquets [3]. The leaves of *Ruta graveolens, Fegara budrunga, Murraya koenigii* and *Toddalia asiatica* are used as condiments and for flavouring curries. The tender shoots of *F. oxyphylla* are used as vegetables. Some are used as fodder (*Aegle marmelos* and *Feronia limonia*). *Citrus* serves as source of raw material to juice industrials. Naringin (a flavour) and neohesperidin dihydroahalcone from grape fruit and pummelo (Shaddock) have application as artificial sweetners. Timbers are yellowish, hand and close-grained, used in cabinet work, turnery and marquetry. The trees are used for afforestation of dry areas since the *Citrus* species have a water conserving system [4].

Plant families known for their wide diversity and complexity constitute many problems to biosystematicists using traditional methods based on gross morphology to characterize the species under the large family. In most recent times, leaf epidermal features as anatomical characters have received very considerable attention by Taxonomists [5-7].

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The application of epidermal characters such as shape of epidermal cells, wall pattern, type and arrangement of stomata, stomatal index, stomatal aperture, size and types of trichomes, row of cell per trichome are some of the important systematic characters used in modern biosystematic studies. The importance of epidermal characters in plant taxonomy can be appreciated from review of works carried out on some plant species by various researchers [8]. Edeoga and Ikem [9] used epidermal morphology of some species of *Boerhavia* to establish interspecific relationships among the different species they investigated. Stace (1965b) was able to review the tribal, generic and specific characters of the family Combretaceae based on their leaf epidermal morphological characters. Mbagwu [10] studied leaf epidermal features of eight *Vigna* species and separated *V. gracellis* and *V. racemosa* from other species based on trichome morphology. Edeoge and Okoli [11] in Dioscoreaceae, Edeoga and Ogbebor [12] in Commelinaceae; Nyananyo and Olowokudejo in Portulaceae; Inyama *et al* [13] in Compositae; Gill and Nyawuama [14] in Labiatae were all workers who have used leaf epidermal features to solve are problem or the other in these families. Inyama *et al.* [15] used the epidermal morphology of three species of *Chinpophyllum* to establish intraspecific relationships among the taxa investigated. According to researchers, epidermal and cuticular traits of plants can serve as useful tools exploitable in the systematic of the present day Angiosperms [5,10,16].

Due to the complex taxonomic and systematic nature of the genus *Citrus*, there is need for revalidational studies on the interspecies relationship among the genus. Till now, only morphological features have been the bases of classifying the genus. There is little or no known work on epidermal, thus the need to use this evidence to revalidate six selected species of the genus *Citrus*. This study shall therefore reassess the taxonomic identities of these taxa using epidermal feature for proper biosystematic data collection. Also, the study shall try to establish inter specie relationship among the taxa investigated.

Materials and Methods

Specimen collections

The studies were made on living plant specimens. The specimen of the six species of the genus Citrus namely: *C. sinensis, C. limon, C. aurantifolia, C. paradisi, C. reticulata* and *C. maxima* were collected from Ministry of Agriculture and Natural Resources Nekede, Agricultural Development Programme (ADP) farms, Plant Garden at Aladinma Housing Estate, Homestead Garden at Amakohia Layout, Songhai Farms, Nekede, Imo ADP Egbeada and Homestead, Obazu Mbieri.

The sample specimen were identified and authenticated by authorities at Imo State University Herbarium were deposited at Imo State University Herbarium with Herbarium No. IMSUH 001-006. The study was conducted at the Plant Science and Biotechnology laboratory in Imo State University, Owerri, Nigeria.

Epidermal features

Fresh leaves from each of the six taxa namely, *C. sinensis, C. limon, C. aurantifolia, C. reticulata, C. paradisi* and *C. maxima* were washed in tap water. The leaves were painted with clear finger nail polish on both surfaces and allowed to dry. After drying, pieces of short clear cellophane tape were firmly pressed over the dried nail polish on the surfaces. The tapes were carefully peeled from the leaves and affixed on clean slides, stained with 1% ethanol safranin for one minute and temporarily mounted in aqueous glycerol solution and observed under. Ten slides were prepared per species and the type of stomata and shape of epidermal cells was determined according to Van Cotthem [17]. Also five out of ten slides were selected per species for the counting of the number of stomata, epidermal cells and other epidermal features. Frequency/Stomatal index per unit area were made from the five slides selected per species. The unit area used was the microscopic field of view at x 10.

Then four different counts were made from different portions of each slide. The thickness of the cell wall, type of trichome and the subsidiary cells were also determined. Data for the counts were organized and presented in Tables 1-2. Photographs illustrating the epidermal features were taken from the slides using Leitz Wetzler Ortholux microscope fitted with Viviter-v-335 camera (Figures 1-3). The stomatal index (S.I.) per specie was calculated as shown below:

Characters	*C. sinensis*	*C. limon*	*C. aurantifolia*	*C. reticulata*	*C. paradisi*	*C. maxima*
Shape of epidermal cell	Rectangular to cuboidal to pentagonal to trapezial	Rectangular to triangular to Haxagonal to Trapezial	Rectangular to Hexagonal to Pyramidal to Trapezial	Rectangular to Pyramidal Trapezial	Hezagonal Cuboidal Trapezial	Rectangular Cuboidal Hexagonal Trapezial
Anticlinal cell wall type	Straight	Straight	Straight	Straight	Straight	Straight
Nature of cell wall	Thick-walled	Thick-walled	Thick-walled	Thick-walled	Thick-walled	Thick-walled
Number of epidermal cells per unit area	320-335 (325.80)	302-309 (305.45)	349-362 (356.05)	490-505 (496.45)	405-419 (413.05)	452-468 (459.45)
Epidermal cell length	15-22.5 μm (18.13)	12.5-27.5 μm (20.94)	13.75-22.5 μm (19.33)	12.5-20 μm (16.58)	15-32 μm (19.70)	12.5-17.5 μm (15.63)
Epidermal cell width	7.5-13.75 μm (11.45)	7.5-17.5 μm (11.63)	7.5-12.5 μm (11.63)	6.25-12.5 μm (8.65)	7.5-13.75 μm (10.25)	3.75-12.25 μm (7.78)
Position of stomata	None	None	None	None	None	None
Stomatal type	None	None	None	None	None	None
Number of subsidiary cells	None	None	None	None	None	None
Length of stomata	None	None	None	None	None	None
Width of stomata	None	None	None	None	None	None
Number of stomata per unit area	None	None	None	None	None	None
Stomatal index	None	None	None	None	None	None
Trichome type	Absent	Absent	Absent	Absent	Absent	Absent
sheath cells	Present cells	Present cells	None	None	Present	Sheath cell

Table 1: Epidermal studies of six *Citrus* species studied (upper epidermis).

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Characters	*C. sinensis*	*C. limon*	*C. aurantifolia*	*C. reticulata*	*C. paradisi*	*C. maxima*
Shape of epidermal cell	Rectangular to Hexagonal to Pyramidal	Rectangular Cuboidal Hexagonal	Rectangular Cuboidal Trapezial	Rectangular Cuboidal Pyramidal Trapezial	Rectangular Cuboidal Pyramidal Trapezial	Rectangular Cuboidal Pyramidal Trapezial
Anticlinal cell wall type	Straight	Straight	Straight	Straight	Straight	Straight
Nature of cell wall	Thick-walled	Thick-walled	Thick-walled	Thick-walled	Thick-walled	Thick-walled
Number of epidermal cells per unit area	258-267 (261.60)	235-246 (241.65)	280-295 (290.40)	389-399 (393.55)	367-378 (371.90)	342-360 (348.85)
Epidermal cell length	12.5-22.5 µm (17.44)	12.5-25 µm (17.10)	12.5-20 µm (16.14)	12.5-22.5 µm (17.21)	12.5-27.5 µm (19.00)	10-17.5 µm (15.19)
Epidermal cell width	5-10 µm (7.94)	2.5-10 µm (7.44)	5-7.5 µm (6.44)	6.25-12.5 µm	5-7.5 µm (5.5)	6.25-10 µm
Position of stomata	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic
Stomatal type	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic
Number of subsidiary cells	None	None	None	None	None	None
Length of stomata	5-9.5 µm (6.91)	5-7 (6.38)	6.25-8.75 µm	7.5-12.5 µm	7.5-12 µm (9.33)	5.25-10 µm
Width of stomata	2.5-7.5 µm	2-5 µm (2.75)	4.5-5 µm (4.85)	5-7.5 µm (6.25)	5-7.5 µm (5.71)	3.75-6 µm (4.68)
Number of stomata per unit area	64-70 (67.80)	60-70 (64.75)	55-66 (61.85)	14-26 (19.55)	40-50 (43.75)	60-70 (64.95)
Stomatal index	20.46 +	+21.13	17.59	4.7	10.53	15.70
Trichome type	Absent	Absent	Absent	Absent	Absent	Absent
Sheath cells	Absent	Present	Present	Present	Absent	Absent
Contiguous Stomata	Present	Present	Present	None	None	Present
Megastomata	Present	Present	Absent	Absent	Absent	Absent

Table 2: Epidermal studies of six *Citrus* species studied (lower epidermis).

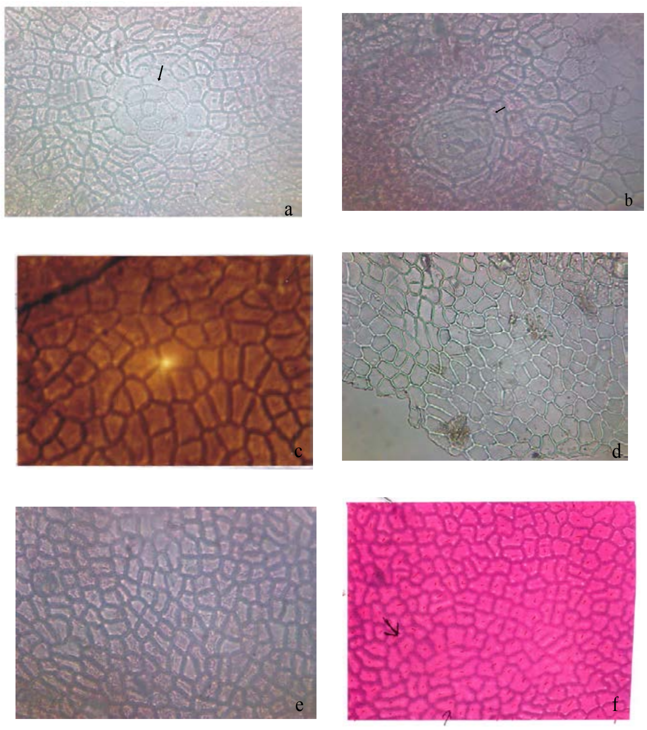

Figure 1: Plate 1: Upper epidermis of (a) *C. sinensis;* (b) *C. limon;* (c) *C. aurantifolia;* (d) *C. reticulate;* (e) *C. paradise and* (f) *C. maxima.* 100×.

$$S.I = \frac{S}{S+E} \times \frac{100}{1}$$

Where S=number of stomata

E=number of epidermal cells

Results

The anticlinal walls of the epidermis are straight and thick-walled at both upper and lower epidermis in all the species of *Citrus* studied. Epidermal cells are flat and variedly shaped in each species studied in the upper epidermis. It is rectangular to cuboidal to pentagonal to trapezial in *C. sinensis;* rectangular to triangular to hexagonal to trapezial in *C. limon*; rectangular to hexagonal to pyramidal to

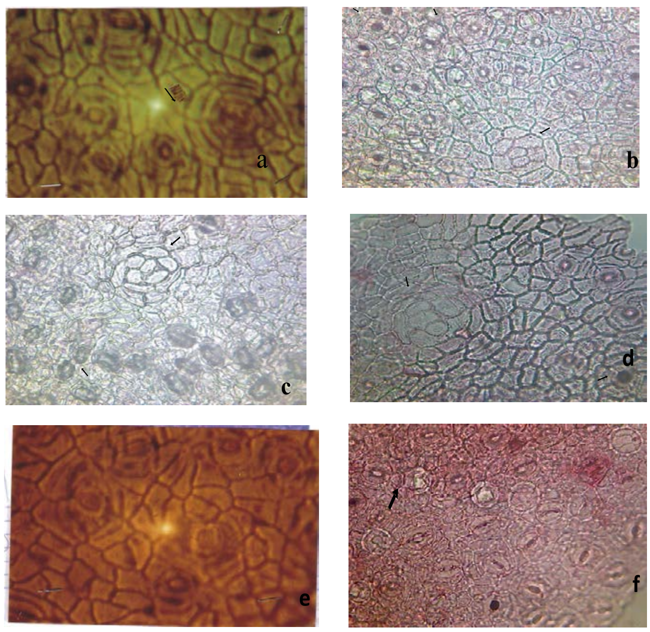

Figure 2: Plate 2: Lower epidermis of (a) *C. sinensis; (b) C. limon; (c) C. aurantifolia; (d) C. reticulate; (e) C. paradise and (f) C. maxima.* 100×.
Plate 2: Lower epidermis of (*d) C. reticulate; (e) C. paradise and (f) C. maxima.* ×.

trapezial in *C. aurantifolia;* rectangular to pyramidal to trapezial in *C. reticulata;* hexagonal to cuboidal to trapezial in *C. paradisi* and rectangular to cuboidal to hexagonal to trapezial in *C. maxima* while in the lower epidermal, the epidermal shape shows variation, being rectangular to hexagonal to pyramidal in *C. sinensis;* rectangular to cuboidal to hexagonal in *C. limon;* rectangular to cuboidal to trapexial but rectangular to cuboidal to pyramidal to trapezial in *C. reticulata, C. paradisi* and *C. maxima*, distinguishing themselves from the rest of the epidermis. In the upper epidermis, the number of epidermis shows consistent variation, ranging from 320-335 in *C. sinensis*; 302-309 in *C. limon;* 349-362 in *C. aurantifolia*; 490-505 in *C. reticulata;* 405-419 in *C. paradisi* and 452-468 in *C. maxima* while in the lower epidermis the consistency persisted ranging from 258-267 in *C. sinensis*, 235-246 in *C. limon;* 280-295 in *C. aurantifolia;* 389-399 in *C. reticulata;* 367-378 in *C. paradisi* and 342-360 in *C. maxima*. The epidermal cell length ranges from 15-22.5 µm in *C. sinensis;* 12.5-27.5 µm in *C. limon;* 13.75-22.5 µm in *C. aurantifolia;* 12.5-20 µm in *C. reticulata*; 15-32 µm in *C. paradisi* and 12.5-17.5 µm in *C. maxima* in the upper epidermis indicating that there is an affinity between *C. sinensis* and *C. paradisi*; and among *C. limon, C. aurantifolia, C. reticulata* and *C. maxima* while in the lower epidermis the epidermal cell length ranges from 12.5-22.5 µm in *C. sinensis;* 12.5-25 µm in *C. limon*; 12.5-20 µm in *C. aurantifolia;* 12.5-22.5 µm in *C. reticulata* 12.5-22.5 µm in *C. reticulata* and 10-17.5 µm in *C. paradisi*, inferring closeness among *C. sinensis, C. aurantifolia* and *C. reticulata* and between *C. limon* and *C. paradisi*; *C. maxima* distinguishing itself from others. The epidermal cell width in the upper epidermis is 7.5-13.75 µm in *C. sinensis*; 7.5-17.5 µm in *C. limon*; 7.5-12.5 µm in *C. aurantifolia*; 6.25-12.5 µm in *C. reticulata*; 7.5-13.75 µm in *C. paradisi* and 3.75-12.25 µm in *C. maxima*. Affinity is indicated among *C. sinensis, C. limon, C. aurantifolia* and *C. paradisi*, showing distinctiveness in *C. reticulata* and *C. maxima* while in the lower epidermis, the epidermal cell width ranges from 5-10 µm in *C. sinensis*; 2.5-10 µm in *C. limon;* 5-7.5 µm in *C. aurantifolia*; 6.25-12.5 µm in *C. reticulata*; 5-7.5 µm in *C. paradisi* and 6.25-10 µm in *C. maxima* showing that *C. sinensis, C. aurantifolia* and *C. paradisi* are related on one hand, while *C. reticulata* and *C. maxima* are related on the other hand, whereas *C. limon* distinguished itself from others. All the six species are hypostomatic with anomocytic type of stomata. The epidermal carpet architecture shows no appendages (trichome) or subsidiary cells in all the species. Sheath cells, modified epidermal cells are found covering the vein areas, prominent in *C. sinensis, C. limon, C. paradisi* and *C. maxima* in the upper epidermis whereas in the lower epidermis, they are prominent in *C. limon, C. aurantifolia* and *C. reticulata*. The number of stomata per unit area ranges from 64-70 in *C. sinensis;* 60-70 in *C. limon;* 55-66 in *C. aurantifolia;* 14-26 in *C. reticulata*; 40-50 in *C. paradisi* and 60-7 in *C. maxima. C. sinensis, C. limon* and *C. maxima* share the same affinity with highest number of stomata per unit area, followed by *C. aurantifolia* and *C. paradisi* but *C. reticulata* having the lowest number of stomata per unit area. This feature followed almost the same trend with the stomatal index, the highest being in *C. limon* with 21.13 and least in *C. reticulata* with 4.7 while it is 20.46 in *C. sinensis;* 17.59 in *C. aurantifolia;* 10.53 in *C. paradisi* and 15.70 in *C. maxima*. The length and width of stomata showed consistent variations being 5-9.5 µm and 2.5-7.5 µm in *C. sinensis*; 5-7 µm and 2 -5 µm in *C. limon;* 6.25-8.75 µm and 4.5-5 µm in *C. aurantifolia;* 7.5-12.5 µm and 5-7.5 µm in *C. paradisi;* 5.25-10 µm and 3.75-6 µm in *C. maxima*, showing relationship between *C. sinensis* and *C. limon; C. aurantifolia* and *C. maxima*; and between *C. reticulata* and *C. paradisi*. Contiguous stomata showed their presence in *C. sinensis, C. limon, C. aurantifolia* and *C. maxima* while absent in *C. reticulata* and *C. paradisi*. The mega stomata are present in *C. sinensis* and *C. limon* but absent in other species, showing affinity between *C. sinensis* and *C. limon*. (Tables 1 and 2; Plates 1-2).

Discussion

Stomata in all the taxa studied are anomocytic and hypostomatic. On the lower epidermis, the anomocytic stomata observed in these taxa agreed with the findings of Mbagwu, *et. al.,* [18] who reported same types of stomata in *C. limon, C. Sinensis; C. auarntifolia* and *C. maxima* which are four of the six species considered. On the contrary, the result disagreed with Obiremi *et al* (2001) who reported paracytic stomata in *C. limon* and *C. auarntifolia;* paracytic to anomocytic in *C. simensis* and *C. reticulate.* The presence of anomocytic stomata have been reported by some authors in different angiosperms. Shah and Gopal [19] in Discoreasceae; Edeoga and Ikem [9], and Mbagwu and Edeoga [20] in *Vigna* species. Presence of contignous stomata distinguished four of the taxa *C. sinensis, C. limon, C. aurantifolia, and C. maxima* from *C. reticulate* and *C. paradise.* This is the first time of reporting contignous stomata in the genus *Citrus*. The absence of sheath cells observed in *C. sinensis* and *C. paradise* is also a differentiating feature to separate *C. rericulata* from *C. paradise.* In the species were they occurred; it was observed that the sheath cells are found mostly above or covering the vein areas. This could be a mechanism to withstand excessive turgor pressure along the vessels during fluid condition as survival strategy. While the presence of mega stomata reported in *C. senensis* and *C. limon* can be used to distinguish *C. sinensis* from *C. paradise.* These mega stomata are usually surrounded by larger epidermal cells. Based on the shape of epidermal cells, rectangular, hexagonal, pyramidal shaped epidermal cells observed in *C. sinensis* definitely distinguished it from the rest of the taxa studied. While *C. reticulate, C. paradise* and *C. maxima* showed strong affinity based on the shape of epidermal cell walls. Rajiagopal, et al. [21] in *Ternera;* Gill and Ngawuame [14] in Bicarpellatea plants, and Patel and Shah [22] in Chilli were some researchers who used shape of epidermal cell as taxonomic tool in differentiating different taxa they studied.

Other features observed such as straight anticlinal and thick celled walls, support the interspecie relationships among the species studied. The taxa studied could be distinguished based on stomatal index as Edeoga et al. [9] on Boerharia. The stomata index showed that *C. sinensis* and *C. limon* are closely related; *C. aurantifolia* and *C. maxima* are closer while *C. reticulate* and *C. paradisi* can be differentiated from each other and the other taxa studied. *C. reticulate* having the lowest stomata index is easily differentiated from all the taxa. It was observed that *C. aurantifolia* has high stomata index than *C. reticulata.* This is contrary to Obirim et al. [4] who reported high stomatal index in *C. reticulate* and lowest stomatal index in *C. aurantifolia.*

On the upper epidermis, the shape of epidermal cells varies in each taxa and presence of rectangular and trapezium shaped cells in all but one specie indicating the close affinity between the taxa studied. The presence of Pentagonal shaped cells separated *C. sinensis* from the rest of the taxa while the absence of rectangular shaped epidermal cell distinguished *C. paradisi* from the other taxa studied. The number of epidermal cells also varies per species and can be used to delimilate the taxa [23-26].

The sheath cells were observed to occur in four of the taxa but absent in two of the taxa: *C. aurantifolia* and *C. reticulata.* Also, the presence of sheath cells on both the upper and lower epidermis of *C. limon* delinate it from the other taxa studied. Therefore, similarities observed on the epidermis of the six *Citrus* taxa studied strongly supported the relationship while the difference established their placement into different species [27,28].

Conclusion

The use of epidermal features of the taxa studied is of great taxanomic importance in supporting both their phylogenetic relationship of the taxa and their treatment as different species, but for a more detailed taxonomic study there is need to extend this type of taxonomic study to other evidences such as cytology, histochemistry, morphology, etc. before taking final taxonomic decision of the six investigated taxa.

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