



Research article

Comparative epidermal anatomical studies in six taxa of genus *Nephrolepis* Swart in Nigeria

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Abstract: Anatomical studies in six taxa of genus *Nephrolepis*; *N. biserrata*, *N. cordifolia*, *N. exaltata* (i) & (ii), *N. biserrata* var. *furcans* and *N. undulata* were carried out with a view to identify anatomic characters of taxonomic values. Both qualitative and quantitative anatomical studies were carried out. Quantitative data were subjected to descriptive statistical analysis. Anatomical characters studied include venation patterns, trichome types, presence and absence of stomata and values of the stomatal index which are valuable in delimiting the species. The overall results showed overlaps in the quantitative anatomical attributes of the *Nephrolepis* taxa studied suggesting that they belong to the same genus. Qualitative anatomical attributes that separated the genus into distinct taxa are the presence of simple multicellular glandular trichomes in *N. biserrata* and simple multicellular non-glandular trichomes in *N. exaltata* (i) and *N. exalta* (ii) while *N. biserrata* var. *furcans* and *N. undulata* have simple unicellular non-glandular trichomes and absence of trichome in *N. cordifolia*. Presence of anisocytic, diacytic or anomocytic stomata were of diagnostic important in the six taxa.

Keywords: Anatomy - Stomata - Trichomes - *Nephrolepis* - Taxonomy.

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INTRODUCTION

Nephrolepis Schott is a small genus of ferns that form groups of about 12,000 species in the world, with many found in the tropics (Carrington 2003). Alston (1959) reported five species of *Nephrolepis* in both Northern and Southwestern Nigeria, while Oloyede & Odu (2011) reported six taxa of *Nephrolepis* in Southwestern Nigeria. The genus is made up of terrestrial, epiphytic, perennials and sometimes aquatic (growing in the marshy land). They are flowerless, seedless plants that require water at least during sexual reproduction (Sporne 1975, Oloyede & Odu 2011). The leaflets are simple, sessile, small in size and possess a single median vein that fails to reach the apex. These leaves are hairy with serrated margins. The leaves of ferns are called fronds and consist of two main parts: the stipe which is the stalk that connects the leaf blade to the rhizome and the leaf blade or lamina that forms the leaf portion which expands outward from the rachis of the frond.

In ferns, the base of the frond grows faster than the tip which gives the frond a fiddle-head shape (Kenrick & Crane 1997), this is called acropetal maturation. Leaf base in *Nephrolepis* could be cordate in *N. furcans* or truncate in *N. exaltata* (i) and (ii). The leaves are spirally arranged and densely cover the branches as a whorled in *Lycopodium* species (Bhambie 1965). They differ from the primitive thallophytes by having true leaves called megaphylls while they also differ from bryophytes by possessing vascular tissues (Carrington 2003).

The taxonomic values of anatomical features have been stressed by several workers including (Metcalf & Chalk 1950, 1979, Naik & Nirgude 1981, Palmer & Tucker 1981, Oloyede *et al.* 2011). Anatomical features sometimes prove useful in individual identification especially for materials that are not accompanied by flora parts or fruits and can be used to establish the botanical identity of commercial samples of medicinal plants (Metcalf & Chalk 1979). It has a lot of values in forensic Botany. Naik & Nirgude (1981) stressed the value of anatomical characters and reported that anatomical characters provide additional features which along with other characters are of great taxonomic values in the classification and identification of plants. Essiet (2004) reported

that anatomical features are widely used in systematic for identification, and placing anomalous groups in satisfactory position in classification and for indicating patterns of relationship that may have been observed by superficial convergence in morphological features.

Leaf epidermal features have been employed in taxonomy to separate plant genera and species (Scatena *et al.* 2005). The epidermis possesses a number of important diagnostic characters that offer valuable clues for identification like size, shape and orientation of stomata, guard cells and subsidiary cells, structural peculiarities of epidermal cells and stomata frequency (Munir *et al.* 2011). Saheed & Illoh (2010) reported that guard cell area, stomatal index and frequency, presence or absence of trichomes as well as their length on epidermal surfaces and wall types are useful in separating the genera *Senna* and *Chamaecrista* from their initial genus *Cassia*. Oloyede *et al.* (2011) reported that the abaxial surface of *N. biserrata* and *N. undulata* showed that their epidermal cells were irregular in shape, while the stomata were diacytic, anomocytic and elliptic in shape. Crystal sand seen was numerous while non-glandular uniserrate multicellular trichome was seen in *N. biserrata* but absent in *N. undulata*. Watson & Dallwitz (1992) described the important anatomical features of the family Cleomaceae with anomocytic, anisocytic, paracytic, actinocytic or cyclocytic stomata found on the both the abaxial and adaxial surfaces. The presence and types of trichome have long been of considerable importance in comparative systematic investigations of angiosperm Metclfe & Chalk (1979) and ferns (Oloyede *et al.* 2011). Crystals are diagnostic tools in plants for distribution, identification and taxonomy (Illoh & Inyang 1995). In *Nephrolepis biserrata* and *N. undulata*, the common characters like epidermal cell structure, types of stomata, trichomes, crystals, venation patterns and morphological structures can be used to delimit the two species of the genus (Oloyede *et al.* 2011).

From literature, information on anatomical features of *Nephrolepis* genus in Nigeria is scanty. In this study, detail anatomical characters of the six taxa of *Nephrolepis* investigated were examined using Ligth Microscope with a view to (a) elucidate the similarities and differences that exists among the species and (b) fill the knowledge gap observed in the taxonomy of the group in Nigeria.

MATERIALS AND METHODS

Collections of Accessions

Accessions of the six samples of *Nephrolepis* studied were collected from various locations in Ile-Ife and planted at the biological garden of Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria from where macro morphological data were collected. The collections were identified and authenticated at the IFE Herbarium. *N. exaltata* is only species that has two forms of leaf type; unipinnate at young stage [*N. exaltata* (i)] and bipinnate at maturity [*N. exaltata* (ii)] and treated here separately.

Leaf clearing

A sizeable portion of fresh mature leaflets of each taxa was taken from the standard median levels (that is midway between the apex and the base), washed and decolorized by boiling in 100% ethanol for ten minutes to remove chlorophyll. The partially decolorized leaflets were washed carefully with 3–5 changes of water to remove all the traces of alcohol. The leaflets were boiled in sodium hydroxide solution for five minutes. Then soaked in 5% domestic bleach (JIK) in Petri dish until they become completely decolorized. The leaflets were washed in five changes of water and then stored in 50% ethanol. The leaflets were stained with Safranin O for 5 minutes and then mounted on a clean slide in 25% glycerol and the edges of the cover slip were sealed with nail hardener to prevent drying out. Both abaxial and adaxial surfaces of the leaflets were used to study venation patterns.

Epidermal peel

The cleared leaves were used because the stomata and epidermal anatomy were clearly visible under the microscope without peeling. Each taxa, 25 stomata were randomly selected in 20 fields from four prepared slides of the abaxial surface of each taxa. Stomata frequency was determined. The stomata index was estimated for the leaf surfaces using Cutter (1978) formula i.e. by expressing the number of stomata per unit area as a percentage of the total number of epidermal cells.

$$\text{Stomata index (I)} = \frac{S}{E + S} \times 100$$

Where, I = Stomata index

S = number of stomata per unit area

E = number of epidermal cells per unit area.

The guard cell area was calculated by multiplying the length and width of guard cell on the abaxial surface by Franco (1939) constant.

$$\text{Guard cell area} = (\text{length} \times \text{width} \times k) \mu\text{m}^2$$

Where, Franco's constant (k) = 0.78524

Sectioning

Transverse sections (TS) of the leaf of the six taxa were made at 10 μm thickness using the sledge microtome (Reichert, Austria). The sections were preserved in 50% ethanol in vials prior to staining.

The sections were stained with Safranin O for five minutes, rinsed in water thrice and counter stained with Alcian blue for five minutes and rinsed in water thrice. Stained sections were treated in series of grades of ethanol (50%, 70%, 80%, 90% and 100%) to differentiate the sections. The differentiated sections were mounted in 25% glycerol on a clean slide, covered with a cover slip and sealed with nail hardener and properly labeled.

Microscopy

Microscopic observations were done using a light microscope. Tissues, cells and cell inclusions were identified, described and recorded for taxonomic studies.

Photomicrography

Photomicrographs of the slides were made using Accu- scope trinocular microscope (ACCU-Scope 3001 LED Trinocular microscope with 3.2 MP CMOS digital camera).

Statistical Analysis

The results of the quantitative anatomical data generated were subjected to single Linkage Cluster Analysis to show if there exists a significant difference in the six taxa studied.

RESULTS AND OBSERVATION

Anatomical Studies

The summary of the quantitative anatomical features of the six taxa of *Nephrolepis* studied (Table 1) was used to generate dendrogram and cluster analysis (Fig. 1). The Dendrogram produces two main clusters comprising *Nephrolepis biserrata* (Sw.) Schott, and *N. exaltata* (L.) Schott. while the second cluster is made of *N. cordifolia* (L.) C. Presl, *N. biserrata* var. *furcans* hort. ex L.H. Bailey and *N. undulata* (Afzel. ex Sw.) J. Sm. The two main clusters had two sub-clusters each. *N. biserrata* is separated from *N. exaltata* (i) with unipinnate. *N. exaltata* (ii) with bipinnate, which are on the same cluster because they are closely related in having curled and sterile leaflet and some other features e.g. runners. The other sub-cluster gave *N. cordifolia* and *N. furcans* on the same cluster showing the close relationship (leaflet is compound unipinnate), while *N. undulata* is separated for being epiphytic.

Artificial taxonomic key to six *Nephrolepis* taxa generated from the clustered assemblage of *Nephrolepis* using anatomical characters

1a Fertile leaflet

2a parallel vein, vein sheath, veinlet ending in sori, trichome glandular *N. biserrata*

2b vein not parallel, vein not sheathed, veinlet ending in hydathode, trichome not glandular *N. biserrata* var. *furcans*

3a tuber present, mid-rib not thick, trichome present, palisade mesophyll cells cylindrical, root hairs absent *N. undulata*

3b tuber absent, mid-rib thick, trichome absent, palisade mesophyll cells not cylindrical root hairs present *N. cordifolia*

1b Leaflet not fertile

4a reticulate vein, stomata largely anomocytic, uniseriate epidermis, leaflet simple, linear *N. exaltata* (i)

4b non-reticulate vein, stomata not anomocytic epidermis not uniseriate, leaflet coiled *N. exaltata* (ii)

This report displays the dendrogram which visually displays a particular cluster configuration of *Nephrolepis biserrata*, *N. cordifolia*, *N. exaltata* (i), *N. exaltata* (ii), *N. biserrata* var. *furcans* and *N. undulata*. Rows that are close together i.e. *N. cordifolia* and *N. biserrata* var. *furcans*, *N. exaltata* (i) and *N. exaltata* (ii) have small dissimilarities. Therefore, these taxa are closely related and are very similar. Also, *N. undulata* is similar to *N. cordifolia* and *N. biserrata* var. *furcans*. *N. undulata*, *N. cordifolia* and *N. biserrata* var. *furcans* are different from *N. exaltata* (i) and *N. exaltata* (ii). *N. biserrata* is very different from other taxa.

Table 1. Summary of the Quantitative Anatomical features of the six *Nephrolepis* taxa studied.

Characters	<i>N. biserrata</i>		<i>N. cordifolia</i>		<i>N. exaltata</i> (i)		<i>N. exaltata</i> (ii)		<i>N. biserrata</i> var. <i>furcans</i>		<i>N. undulata</i>	
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	adaxial	abaxial	Adaxial	Abaxial	adaxial	Abaxial	adaxial
GCA	10.76	0	8.06	0	2.58	0	2.56	0	10.34	0	10.22	0
GCW	3.05	0	1.15	0	1.64	0	1.52	0	2.59	0	2.52	0
GCL	5.25	0	2.38	0	1.21	0	1.32	0	3.37	0	4.75	0
SFP	8	0	9	0	4	0	4	0	7	0	4	0
STI	13.45	0	18.36	0	29.41	0	29.34	0	8.06	0	13.11	0
LEC	8.96	6.2	2.89	5.8	3.15	4.5	3.12	4.3	8.47	13.84	12.46	13.3
WEC	4.06	3.92	3.15	3.65	3.55	4.08	3.45	4.04	7.24	7.2	5.85	6.68
EPF	37	50	34	46	62	82	66	84	31	48	30	39
NTS	130	142	0	0	66	80	32	12	15	18	3	5
LTF	10	15	0	0	8	8	9	3	10	10	30	30
NVF	48	52	35	28	12	18	14	16	40	42	30	35
LVF	20.5	20.5	10.7	10.7	30.3	30.5	31.2	30.4	19	21	20.3	20.5
TOV	0.05	0.05	0.5	0.5	0.05	0.03	0.04	0.03	0.2	0.3	0.05	0.02
DME	1.5	1.5	1.5	1.5	1.5	0.9	1.5	1.1	1.8	1.6	1.3	1.2
TOR	2.5	2.5	4	4	1	2	1.1	1.1	0.7	0.6	0.4	0.2

Note: GCA- Guard cell area (μm^2) \pm S.E. N=25; GCW- Guard cell width (μm); GCL- Guard cell length (μm); SFP- stomata frequency per field; STI- stomata Index %; LEC- length of epidermal cell (μm); WEC- width of epidermal cell (μm); EPF- epidermal cell per field (μm); NTS- number of trichome per leaflet surface; LTF- length of trichome per field; NVF- number of veins per field; LVF- length of veins per field; TOV- thickness of veins; DME- distance between leaflets margin; TOR- thickness of costa (mid-rib); and veinlet endings.

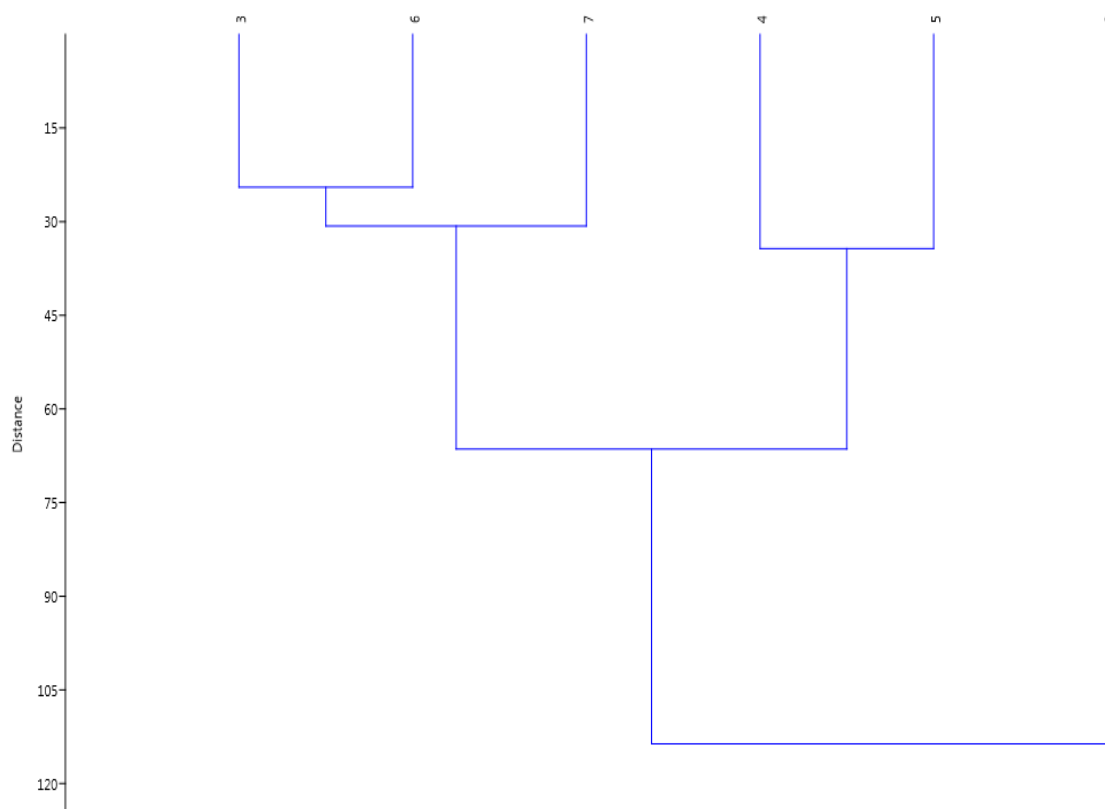


Figure 1. Dendrogram of the six taxa of *Nephrolepis* studied based on quantitative data from anatomical features. [2- *Nephrolepis biserrata*, 3- *N. cordifolia*, 4- *N. exaltata* (i), 5- *N. exaltata* (ii), 6- *N. biserrata* var. *furcans* and 7- *N. undulata*]

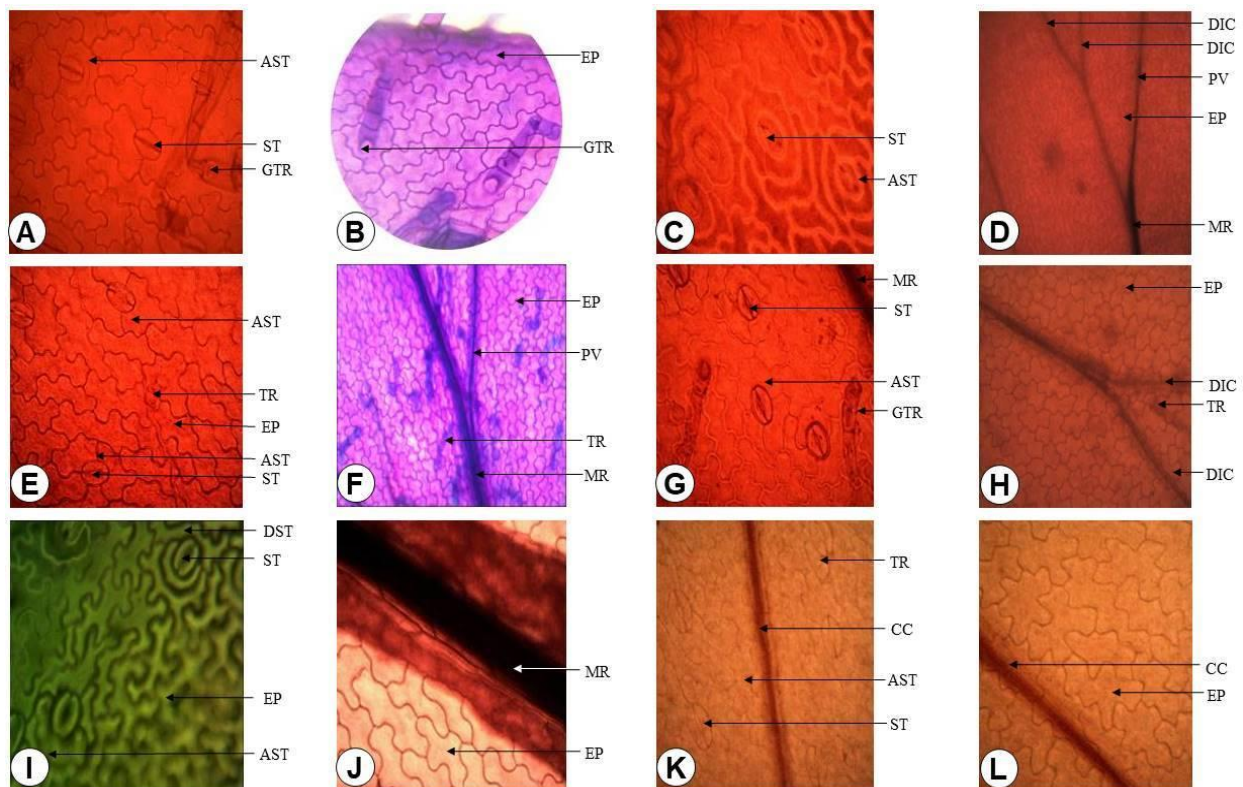


Figure 2. Leaflet anatomical study of *Nephrolepis* taxa. (AST- anisocytic stomata; EP- epidermal cell; GTR- trichome; ST- stomata; DIC- dichotomously branched vein; PV- primary vein; MR- mid-rib; VE- vein ending in hydathode/sori; VI- diacytic stomata; DG- deep groove at the margin; CC- costa cell; all in 400x)

Leaflet anatomical study of *Nephrolepis biserrata*

(Fig. 2A & B)

Abaxial Surface:

The epidermal cell is irregular and undulating. The anticlinal wall is thin and undulating. Stomata are anisocytic, diacytic and anomocytic. Stomata frequency was 7–9. Long, glandular multicellular trichomes which are numerous are seen on the abaxial surface.

Adaxial Surface:

Epidermal cells irregular, thin and undulating. No stomata. Long, glandular multicellular trichomes are seen.

Leaflet anatomical study of *Nephrolepis cordifolia*

(Fig. 2C & D)

Abaxial Surface:

The epidermal cell is irregular in shape, thick and undulating. The anticlinal wall is thick and undulating. Some depressions or grooves at the margin. Stomata are anisocytic. Stomata frequency was 8–10. No trichome. Few starch grains are present. There is groove at the margin.

Adaxial Surface:

The epidermal cell is thick, irregular in shape and undulating, no stomata and no trichome. Few starch grains were seen. There is no groove at the margin.

Leaflet anatomical study of *Nephrolepis exaltata* (i)

(Fig. 2E & F)

Abaxial Surface:

The epidermal cell is irregular in shape and anticlinal wall were thin and undulating. Stomata are present and are diacytic, anisocytic and anomocytic. Stomata frequency was 3–5. Trichome was simple, uniseriate, multicellular non-glandular and numerous in the lamina. Few starch grains are present. Depression or groove was seen at the margin.

Adaxial Surface:

Epidermal cells were irregular in shape, thin and undulating. Stomata are absent. Trichome was simple, uniseriate, multicellular non-glandular and numerous at the lamina. Depression or grooves were seen at the proximal to the median of the margin.

Leaflet anatomical study of *Nephrolepis exaltata* (ii)**(Fig. 2G & H)***Abaxial surface:*

Epidermal cells were polygonal to irregular in shape. Anticlinal walls were thin and undulating. Anisocytic, diacytic and anomocytic stomata type were seen. Stomata frequency was 2–4. Non-glandular uniseriate multicellular trichomes present. Depression or grooves were seen at the margin.

Adaxial surface:

The epidermal cells were polygonal to irregular in shape. Anticlinal walls seen were thin and undulating. No stomata seen. Non-glandular, uniseriate, multicellular trichomes were seen. Starch grains present and numerous.

Leaflet anatomical study of *Nephrolepis biserrata* var. *furcans***(Fig. 2I & J)***Abaxial surface:*

Epidermal cells were irregular, thin and undulating. Diacytic and anisocytic stomata were seen while stomata frequency was 4–9. Few trichomes present. Leaflets form deep depressions or grooves at the tip to form emarginate apex. Numerous starch grains are present.

Adaxial surface:

Epidermal cell was irregular in shape, thin and undulating. Stomata are absent. No trichome. Depression or groove at the leaflet surface.

Leaflet anatomical study of *Nephrolepis undulata***(Fig. 2K & L)***Abaxial surface:*

Epidermal cells were irregular in shape, thin and undulating. Anisocytic stomata were seen. Stomata frequency was 3–5. Few unicellular non-glandular trichomes were seen at the lamina. Few starch grains are seen.

Adaxial surface:

Epidermal cells are irregular in shape. Anticlinal wall was thin and undulating. Stomata are absent. Few unicellular non-glandular trichomes were seen.

DISCUSSION

The anatomy of the leaflets of *Nephrolepis* showed various degrees of differences and similarities that can be used for taxonomical studies. These anatomical features such as the absence of trichome in *N. cordifolia* which can be used to separate this species while the presence of anisocytic stomata shows that they are related and have a common evolutionary origin are a lot of taxonomic values in the six taxa of *Nephrolepis* studied in this work. The result of this study is similar to other workers who reported similar results. They include Carlquist (1961) who stated that the leaf provides a variety of anatomical features that can be of taxonomic importance. Illoh (1995) reported on *Celosia* species that the presence and absence of crystals were used in identification, Adedeji (2004) on the anatomy of *Emilia* species reported the shapes of the mid-ribs are relatively the same in the two species, Ogundipe (2004) on Sapindaceae observed anomocytic stomata in the three species of *Blighia* studied and Oloyede *et al.* (2011) on *N. biserrata* and *N. undulata* reported non-glandular uniseriate multicellular trichome in *N. biserrata* but was absent in *N. undulata*. In this study, the leaflets of the six taxa showed remarkable variations on both abaxial and adaxial surfaces. There is variation in the number of veins and length of veins per field as *N. biserrata* recorded the highest of 52 veins on the adaxial surface and 48 veins on the abaxial surface while the lowest of 17 veins on the adaxial and 15 on the abaxial surface in *N. exaltata* (i) and (ii). The length of veins per field as in *N. exaltata* (i) and (ii) having 30.5 and 30.4 μm at the adaxial surface and 30.3 and 31.2 μm at the abaxial surface respectively as the highest while *N. cordifolia* recorded the lowest value of 10.7 μm at both the adaxial and abaxial surfaces respectively. Epidermal cells in this genus are irregular in shape, with thin undulating anticlinal walls. However anticlinal wall of *N. biserrata*, *N. cordifolia* and *N. biserrata* var. *furcans* on both abaxial and adaxial surfaces are thick thus delimiting the taxa. From the study the length of the epidermal cells of members of this genus shows that *N. biserrata* var. *furcans* has the longest 8.74–18.93 μm long and 6.92–8.94 μm wide on the adaxial surface while the least was observed in *N. exaltata* (i) & (ii) as 2.28–5.75 μm long and 2.65–4.12 μm wide. But the abaxial surface of *N. biserrata* var. *furcans* as the highest with 10.92–16.02 μm long and 6.92–7.56 μm wide while *N. exaltata* (i) had the lowest with 2.75–3.55 μm long and 2.85–4.25 μm wide. Based on the epidermal cell lengths *Nephrolepis* species can be delimited.

Solerender (1908) and Metcalfe & Chalk (1950) reported that stomata type is of taxonomic value. All the

six taxa studied are hypostomatic and they are largely anisocytic. In addition, *N. biserrata*, *N. exaltata* (i), *N. exaltata* (ii) and *N. biserrata* var. *furcans* have diacytic as well as anomocytic. The presence of anisocytic stomata in all the taxa shows that they are related and have a common evolutionary origin and are generic characters for the genus *Nephrolepis*.

A marked difference in the stomata frequency was reported in the study. For instance, stomata frequency in *N. cordifolia* was 8–10 which is the highest while *N. undulata* had 3–5 as the least. *N. biserrata* had 6–8, *N. exaltata* (i) & (ii) 2–4, while *N. biserrata* var. *furcans* (a variety of *biserrata*) had 1–3 respectively. These differences in the stomata frequency could be used as taxonomic value to delimit the taxa (Table 1; Fig. 1).

Stomata indices in the members of this genus are different. For example, stomata index in *N. exaltata* (i) was highest with 24.41% while *N. biserrata* var. *furcans* had the least with 8.06%. The variation in the stomata index in this study can be reasonably employed in delimiting the *Nephrolepis* species. Adedeji & Jewoola (2008) reported that the stomata index is constant for any given species and the value is more uniform on the abaxial surface than the adaxial surface except in an isobilateral leaf.

Essiett *et al.* (2010) reported that stomatal index and the guard cell area provide values that will serve as parameters for comparison among taxa, which can be useful for identification of the studied taxa. Essiett & Etukudo (2012) on their study on three species of *Acalypha* occurring in Nigeria also reported that variation in stomata index and guard cell areas are useful diagnostic tools. This study reported differences in guard cell areas as. *N. biserrata* had the highest guard cell area of 4.70–5.80 μm square long and 2.5–3.6 μm square wide while *N. exaltata* (i) had the least with 1.17–1.25 μm square long and 1.52–1.75 μm square wide.

The uses of trichomes in delimiting taxa are reported in literature for example Saheed & Illoh (2010) used the presence and absence of trichomes to separate the genera *Senna* and *Chamaecrista* from their initial genus *Cassia*. *N. biserrata* recorded the highest number of trichome per leaflet surface with 142 on the adaxial and 130 on the abaxial surface followed with *N. exaltata* (i) having 80 on the adaxial and 66 on the abaxial while *N. biserrata* var. *furcans* has 18 on the adaxial and 15 on the abaxial and *N. undulata* had the least with 5 on the adaxial and 3 at the abaxial respectively (Table 1). In addition, *N. biserrata* have simple multicellular glandular trichome which is specific to the taxon while *N. exaltata* (i), *N. exaltata* (ii) and *N. biserrata* var. *furcans* and *N. undulata* have simple unicellular non-glandular trichome. *N. cordifolia* have no trichome. Trichomes of different types, sizes and numbers are therefore good diagnostic feature for taxonomic studies in this genus (Table 1).

CONCLUSION

The results from anatomical studies revealed the affinities, similarities and differences among members of the genus *Nephrolepis* studied. Anatomically, *Nephrolepis* taxa studied showed generic features common to all the selected taxa. In all the six taxa, vein arrangement was dichotomously branched but the veinlet ending can be grouped into two; the fertile leaflets of *N. biserrata*, *N. cordifolia* and *N. undulata* have their veinlets terminated with sori while the sterile leaflets of *N. exaltata* (i), *N. exaltata* (ii), and *N. biserrata* var. *furcans* do not have sori. Stomata type, stomatal index and stomata area are useful in delimiting the taxa. Anisocytic stomata are common to all. However, the presence of diacytic in *N. biserrata*, *N. exaltata* (i), *N. exaltata* (ii) and *N. biserrata* var. *furcans* and the presence of anomocytic in *N. biserrata*, *N. exaltata* (i) and *N. exaltata* (ii) clearly demarcate them from the other taxa. The presence of simple multicellular glandular trichomes in *N. biserrata*, simple multicellular non-glandular trichomes in *N. exaltata*, and simple unicellular non-glandular in *N. biserrata* var. *furcans* and *N. undulata* is diagnostic while total absence of trichomes in *N. cordifolia* is taxon specific and delimits it from the remaining.

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