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Determining Accurate Nomenclature of Five Ixoridae Subfamily Members Using DNA Barcoding

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Running Title: Use of DNA barcoding in authenticating voucher specimens in Herbaria

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Abstract

Background and Objective: The enormity of authenticating plant voucher specimens in Herbaria using the usual Expert Recognition Method (ERM) across the scientific, industrial and medical enterprise is disturbingly grave. The study conceived in response to the assignment of different taxonomic names to five different specimens by five Institutional Herbaria in Southern Nigeria was meant to determine the accurate nomenclatural identities of six Ixoridae members with huge indigenous usage in Nigeria.

Materials and Method: Grounded leaf samples of the specimens were subjected to standard DNA protocols of Extraction, Amplification, Sequencing and BLASTING (Basic Local Alignment Tool), for which one specimen (Specimen D) was not successfully amplified.

Results: The results of the other differential nucleic acid purity levels, a sequence length of 550-650 base pairs units and various Amino Acid Residues proved all but two of the specimens as a distinct taxon. When the sequenced products were subjected to Basic Local Alignment Tool, specimen A exhibited a 99.78% homology with *Oxyanthus speciosus* while specimen B, C, E and F exhibited a 100% homology with *Oxyanthus laxiflorus*, *Mussaenda erythrophylla*, *Mussaenda erythrophylla* and *Gardenia jasmonoides* respectively. The result affirmed that two specimens (Specimen C & E) previously considered as two taxa were returned as one (*Mussaenda macrophylla*). The study also established that curators across five Herbaria in Southern Nigeria Universities correctly assigned just one out of six specimens under investigations.

Conclusion: The study concludes by recommending DNA barcoding as an effective tool to replace ERM in voucher specimen authentications in Herbaria.

Keywords: Herbaria, Maturase -k, Rubiaceae, Taxonomy & Universities

Background

DNA barcoding is fast becoming a tool of choice in resolving nomenclatural challenges (Ebigwai et al 2019). In Nigeria as in other Third World Countries, species authentication is often conducted via expert recognition and endless search of voucher specimen in Herbarium that matches a taxon of interest (Ebigwai et al 2019). The challenges associated with this traditional method are grave. First, it is not only fraught with errors but it's disturbingly enshrined in taxa memorization with no documented nor Standard Operating Procedure in arriving at species nomenclature (Ebigwai et al 2019). The practice could best be described as a nonscientific endeavour. Second, the Herbarium may not hold species of interest thus resulting in huge man-hour lost. Third, the high incidence of speciation in the Tropics is further rendering the enterprise obsolete (Ebigwai et al 2017). Fourth and most importantly to this research is the recurring frequency of wrongly assigned nomenclatures to large

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holdings of voucher specimens in most Herbarium. No better evidence exemplifies these challenges than the lack of uniformity in assignment more than one nomenclature per taxon to the species under study by five Herbarium curators in five southern Universities in Nigeria.

Several taxonomic lines of evidence are currently in use, to overcome these challenges including DNA barcoding. Some regions of organismal DNA are capable of encoding and expressing minute variations. Variations, we all know are undoubtedly the bedrock for speciation. These regions called Primers have been used in resolving taxonomic conflicts across all ranks. Reviewed reports where DNA markers were used include (Avisé et al., 1987; Moritz et al., 1987; Harrison, 1989, Templeton, et al., 1992, Segraves K.A. et al. 1999, Joshi et al, 1999, Clement et al., 2000, Posada D & Crandall KA 2001, Mani et al. 2004, Petit & Vendramin 2007, Finkeldey et al., 2010, Wicke et al. 2011, Xiang et al., 2012, Kordrostami, Mojtaba & Rahimi, (2015), Salmataj, et al 2018, Okhale, et al 2018).

The Ixoridae subfamily of Rubiaceae is well represented in Nigeria. They offer provisional ecosystem services to a wide spectrum of the Nigerian economy. (Baliga and Kurian, 2012, Finkeldey et al., 2010, Karou, et al 2011, Salmataj, et al 2018, Queiroz, et al 2019) Despite its members offering such huge services, there exist surfeit of nomenclatural ambiguities as exemplified by repeated endless revisions (Alejandro, 2005; Bremer and Manen, 2000) and lately (Birgitta and Torsten, 2009) within this taxon.

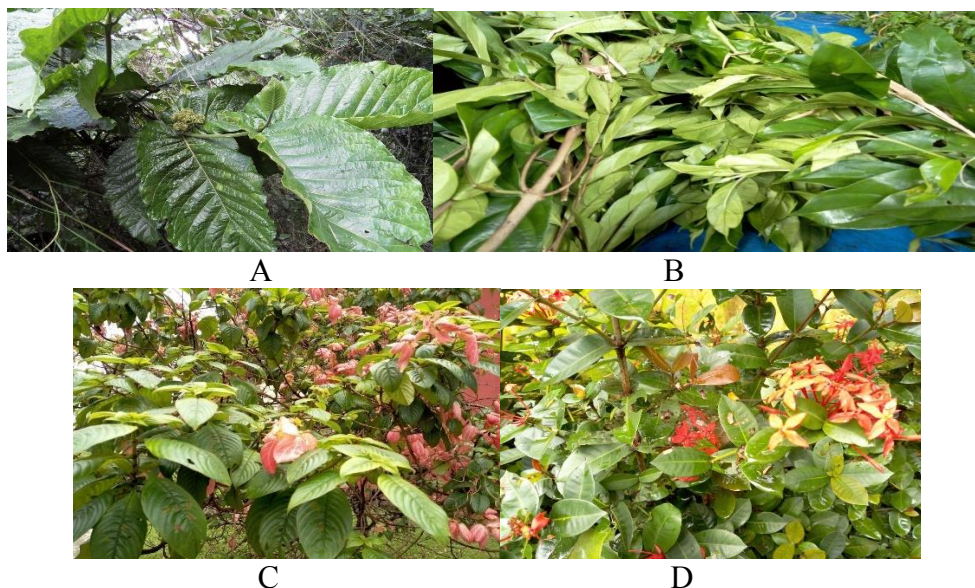
Since authentication in our climes is still heavily dependent on voucher searches in Herbarium, this research aims to commence a process of replacing wrongly assigned taxon with accurate nomenclatures determined by DNA barcoding. It is in light of this, that this study employed the Maturase K gene primer in determining the accurate nomenclatures of six members of Ixoridae.

Materials & Method

Study Area: Samples were collected within Cross River National Park and in August 2019. DNA extraction, quantification was conducted in the Department of Genetics and Biotechnology, University of Calabar while Polymerase Chain Reaction (PCR) and Sequencing were conducted in Inquaba, Pretoria, South Africa. The analyses spanned August to October 2019.

Plant material collection

Fresh plant samples were collected from Mbe Mountain Reserves in Cross River State and subjected to five Herbaria Curators in Nigeria. The result returned all species under consideration as members of Ixoridae though with non-uniformity in names of taxa, hence all specimens under study were coded as A B, C, D, E and F pending nomenclatural determination by a molecular marker.



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Plate 1 a-f: Specimens under investigation

DNA Extraction

Extraction was done using a Zymo R plant DNA miniprep extraction kit supplied by Inqaba South Africa. Heavy growth of the pure culture of the suspected isolates was suspended in 200 microliters of isotonic buffer into a Zymo R Bashing Bead Lysis tubes, 750 microliters of lysis solution were added to the tube. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The Zymo R bashing bead lysis tube was centrifuged at 10,000 rands for 1 minute.

Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7, 000 rands for 1 minute. One thousand two hundred (1, 200) microliters of plant DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1, 600 microliters, 800 microliters was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 rands for 1 minute, the flow-through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 rands for 1 minute followed by the addition of 500 microliters of plant DNA Wash Buffer and centrifuged at 10,000 rands for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microliter centrifuge tube, 100 microliters of DNA elution buffer was added to the column matrix and centrifuged at 10,000 rands microliter for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double-clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microliters of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

1. 1RKIM-forward: 5'- ACCCAGTCCATCTGGAAATCTTGGTTC-3' and
2. 3FKIM-reverse: 5'- CGTACAGTACTTTTGTGTTTACGAG -3',

These primers are universal primers used in plants as used by Udensi et al, 2017?

The primers were introduced on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microliters for 35 cycles. The PCR cocktail mix used for the study included: the X2 Dream Taq Master Mix supplied by Inqaba Biotechnology, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as the template. The PCR conditions were as follows:

- ❖ Initial denaturation, 95°C for 5 minutes
- ❖ Denaturation, 95°C for 30 seconds
- ❖ Annealing, 53°C for 30 seconds
- ❖ Extension, 72°C for 30 seconds for 35 cycles and

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- ❖ Final extension, 72°C for 5 minutes. The product was resolved on a 1 % agarose gel at 120 V for 15 minutes and visualized on a blue light Tran's illuminator.

3.5.4 Sequencing

The Mat K genes in the extracted DNA Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnology, Pretoria South Africa. The sequencing was done at a final volume of 10 ul, the components included 0.25 ul Big Dye terminator v1.1/v3.1, 2.25 ul of 5 x Big Dye sequencing buffer, 10 uM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were as follows 32 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 min.

Phylogenetic and Statistical Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace Bio edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalW W. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Results and Discussion

DNA Quantification

Obtained result showed that Specimen A and E recorded the highest DNA concentration of 72.00 and 34.30 ng/ul respectively as against Specimen D and F with 22.80 and 20.80 ng/ul respectively. This explained in part the sequencing failure of Specimen D (Table 1).

Table 1
DNA extraction and quantification results

S/N	Sample Name	DNA Length (ng/ul)
1	Specimen A	72.000
2	Specimen B	28.700
3	Specimen C	25.800
4	Specimen D	22.800
5	Specimen E	34.300
6	Specimen F	20.800

Key: ng/ul= Nano gam/microliter

DNA amplification

Result revealed that all the samples were successfully amplified as the Amplicons ranged between 650 base pairs for each of specimens A, B, C and E to 550 bp for D and F. (Plate 1). The first well-labelled L represents 1000 bp DNA molecular ladder while wells A to F represents the Amplicons of the various plant samples under study.

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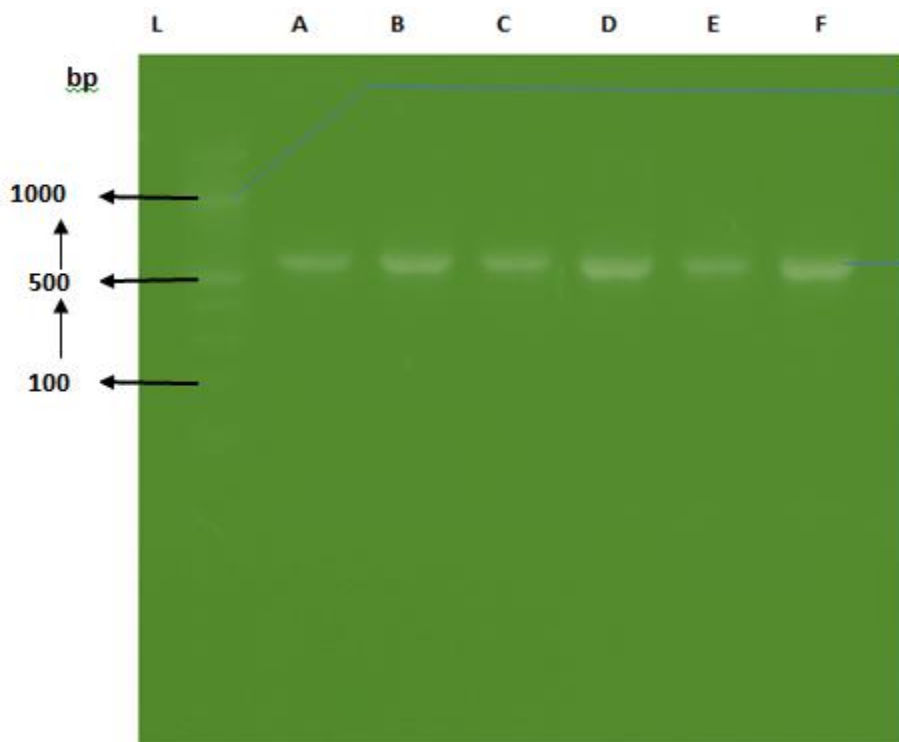


Plate 2: Agarose gel electrophoresis showing the amplified Mat-K gene of samples A to F

Amplicons sequencing and sequence analysis

The *MatK* gene from five of the plant samples was sequenced successfully while just one (Specimen D) failed this test probably because of non-amplification of the *MatK* gene in the sample by the primers during the Polymerase Chain Reaction (PCR). The result showed that samples E recorded a sequence length of 300 base pairs while all others (specimen A, B, C and F) recorded sequence length of 628 base pairs respectively (Fig. 1). The additional result revealed that specimen E, B and A recorded the highest amino acid sequence of 160, 154 and 143 base pairs respectively, while specimen C and F recorded the least, 72 and 25 base pairs respectively. Table 2 reveals the mutation points in the genetic sequence of the specimens while Table 3 shows the nucleotide and amino acid word counts for the samples. However, the decrease in the amino acid sequence was attributable to the increased number of "nonsense codons" within studied species as seen in Tables 2 and 3.

Table 2
Points of mutation in the genetic sequence of samples

Species	First Segment	Middle Segment	Last Segment
Specimen A	1-126	143	573-628
Specimen B	No Mutation	No Mutation	500-628
Specimen C	1-136	143	360-628
Specimen E	1-128	143	No Mutation
Specimen F	1-120	143	200-628

Table 3
Nucleotide and Amino Acid Word Count for samples

Species	Nucleotide Sequence (DNA)	Amino Acid Sequence (RNA)
Specimen A	445	143
Specimen B	499	154
Specimen C	222	72
Specimen E	499	160
Specimen F	78	25

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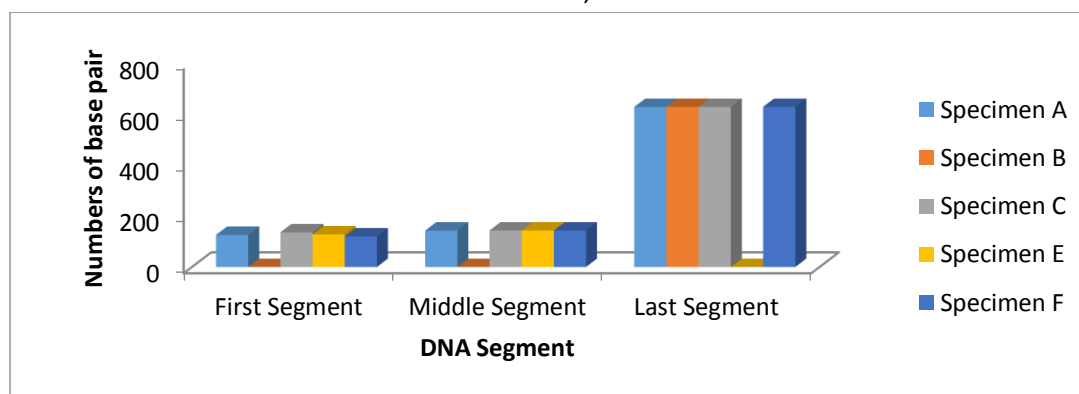


Fig. 1: Characterization of DNA sequence mutation points of the successful species

The result of (Fig. 1) shows that specimen B recorded no mutation at the first and middle sequence segment as well as specimen E at the last segment which may be responsible for some morphological differences observed among these species.

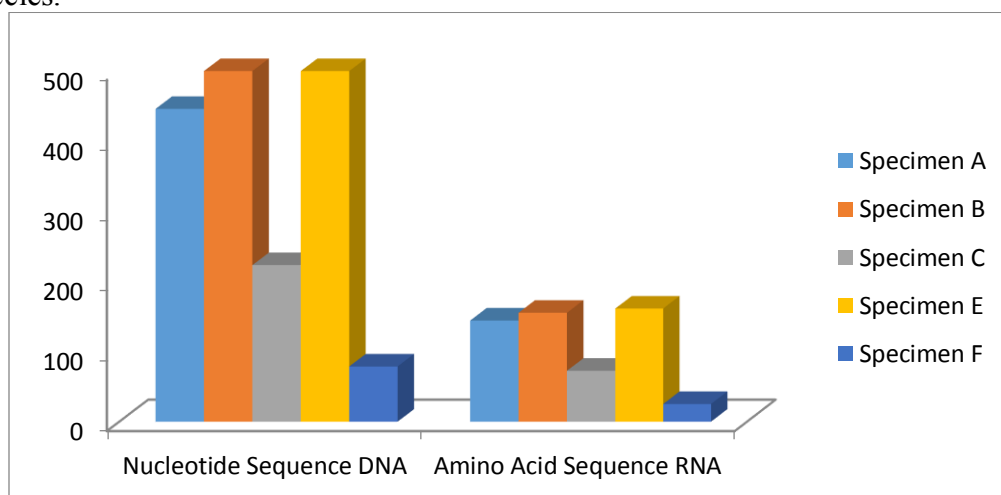


Fig. 2: Characterization of nucleotide and amino acid word count for samples
Sequence identification

The BLAST results showed that the Mat-K sequence of specimen A was *Oxyanthus speciosus*, B was identified as (*Oxyanthus laxiflorus*), specimens C and E (*Mussaenda macrophylla*) and specimen F (*Gardenia jasmonoides*) at 100.00 % similarity while specimen A exhibited 99.78% homology with *Oxyanthus. Speciosus*. All the species under study were identified to specific epithet using the National Center for Biotechnology Information (NCBI) database (Table 4).

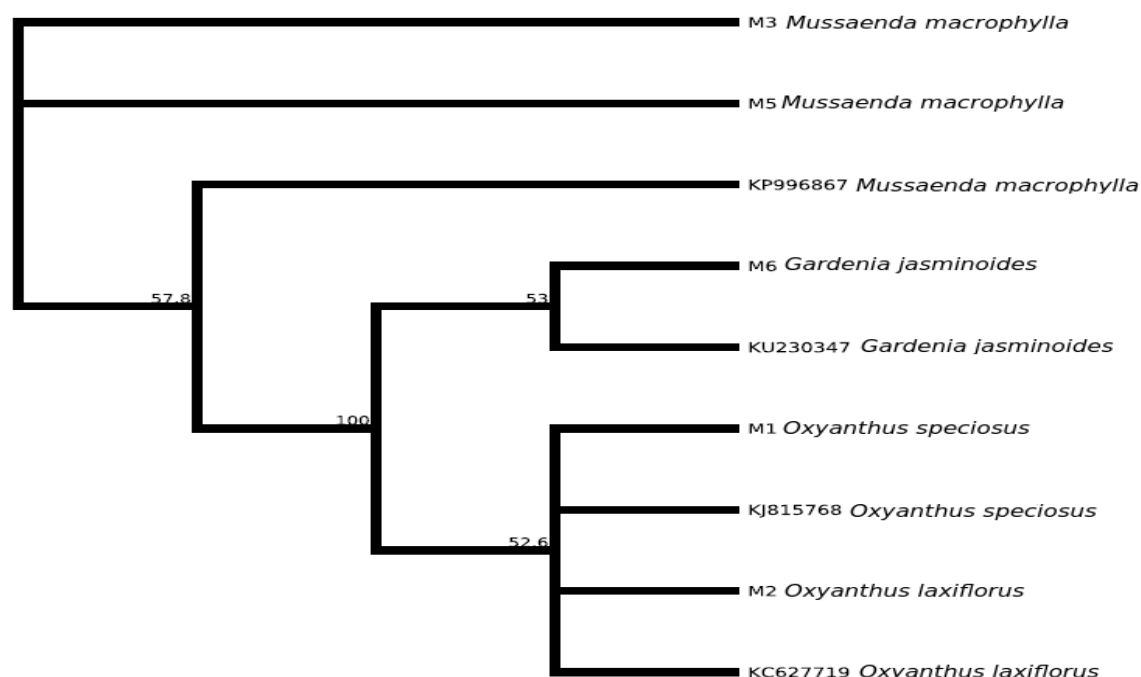
Specimen A (*O. speciosus*) and Specimen B (*O. laxiflorus*) formed a clade at a weak bootstrap value of 52.60 %. While Specimen F (*G. jasmonoides*) showed a close relationship with specimen A (*O. speciosus*) and specimen B (*O. laxiflorus*). The evolutionary distances computed using the Jukes-Cantor method was in agreement with the phylogenetic placement of the Mat-K gene of the plants within similar taxa (Fig. 3).

The evolutionary distance between species computed using Mega 7 software, revealed that sample C (*Mussaenda macrophylla*) and specimen E (*Mussaenda macrophylla*) had 100.00 % similarity implying that these species are the same. Also, the comparison revealed a similarity between specimens A (*O. speciosus*) with all other species at 96.60 % similarity level (Table 5).

Table 4
BLAST authentication of five successfully sequenced species

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Query	NCBI sequence	Accession Number	% homology	Level of success
Specimen A	<i>O. speciosus</i>	KJ815768	99.78	Species
Specimen B	<i>O. laxiflorus</i>	KC627719	100.00	Species
Specimen C	<i>M. macrophylla</i>	KP996867	100.00	Species
Specimen E	<i>M. macrophylla</i>	KP996867	100.00	Species
Specimen F	<i>G. jasmonoides</i>	KU230347	100.00	Species



Key: M1= *O. speciosus*, M2= *O. laxiflorus*, M3= *M. macrophylla*, M5= *M. macrophylla*, M6= *G. jasmonoides*

Fig. 3: Phylogenetic tree showing the evolutionary distance between the various plants under study.

The phylogenetic tree was constructed using the Jukes-Cantor method. The number besides branches corresponds to bootstrap support.

Table 5

Evolutionary/Pairwise Distance between the studied species

	Specimen A	Specimen B	Specimen C	Specimen E	Specimen F
Specimen A					
Specimen B	0.931				
Specimen C	0.069	0.816			
Specimen E	0.069	0.816	0.000		
Specimen F	0.034	0.900	0.069	0.069	

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Key: Specimen A=*O. speciosus*, specimen B=*O. laxiflorus*, specimen C=*M. macrophylla*, specimen E=*M. macrophylla*, specimen F=*Gardenia jasmonoides*.

DISCUSSION

This study assessed the applicability of Mat-K gene sequence segments for discrimination of some Ixoridae subfamily members of Rubiaceae to reduce the problem of misplaced identity and to commence a process of phasing out voucher specimens authenticated by expert recognition in Uncial Herbarium with one authenticated by DNA markers. The results of DNA Extraction, Amplification, Sequencing and Phylogenetic establishes five of the samples that were successfully amplified as belonging to four taxa. Lashermes *et al.* (1996) reported similar DNA concentration among various species of *Coffee*. Lulut *et al.* (2018) reported 615 and 814 bp respectively between two studied species. Similar results with Mat-K were also reported in Liu *et al.* (2015) and Wei *et al.* (2017).

Importantly, this study revealed some salient findings concerning nomenclatural ambiguities. First, except for one institutional Herbarium, the scientific name assigned to *Oxyanthus speciosus* by expert curators in four other Institutional Herbaria were wrong. This species was assigned *Oxyanthus unilocularis*. The implication of this ambiguity is grave. The main active compounds of **oleanolic acid glycosides, triterpene alcohols and flavonoids (quercetin and isorhamnetin)** contained in the latter are responsible for its analgesic, arthritis, pain-relieving, antinociceptive and anti-inflammatory properties as reported by Nkeh-Chungag, et al., (2010). Attempting applying the specimen to soap production (Burkill 1985) would fail due to the absence of Lye which is present in *O. unilocularis*. Same production failures would result also when the specimen is indigenously attempted in the production of tobacco, snuff, fuel and lightening (Hyde et al 2019). Since these active ingredients are not contained in *O. speciosus*, assigning *O. unilocularis* to such specimen would result in designating it with anti-inflammatory functions rather than its anti-tuberculosis functions. There are several implications for this misplacement. First, it would be interpreted that the Herbarium does not hold species with anti-tuberculosis functions. Second, the administration of herbal recipes or inclusion of its active ingredients into drug formulation for anti-inflammatory therapies would not be efficacious and may result in severe side effects instead.

Second, the nomenclatural uniformity expressed by expert curators for specimen B is confirmed by molecular data.

Third, while expert curators returned sample C and E as separate species, results of molecular information returned both as same. Expert curators authenticated the taxon as either *Mussaenda erythrophylla*, *Mussaenda hybrid* or *Mussaenda kwangtungensis* as against *Musaenda macrophylla* that was obtained as the accurate identity using DNA analyses. *Mussaenda erythrophylla* has found usage as anti-diuretic, antichloristic, antipyretic and anti-fertility activities. These functions are as a result of the presence of iridoids, flavonoids and triterpene. *M. macrophylla*, on the other hand, possess a special compound known as mussaeidoside W7 and Triterpene glycosides which has activity against oral pathogens (Astalakshmi and Sundara 2017). the mismatch implies that first, *Musaenda macrophylla* will not be available to serve its purpose and second, the species will be used in administering treatments for the wrong purpose which may lead to the worsening health conditions.

Specimen E on the other hand is recognized by curators in 40% of the Herbaria accurately as against others that assigned *Musaenda pubescens* as the name of the taxon. Nesting of both specimens in the same clade at 99.00 % similarity level is a further testament to them been the same species. This finding is in tandem with Ebigwai et al (2019) who reported that specimens exhibited at least 97% should be treated as the same species.

Specimen F was authenticated by few curators by their generic epithet as against others that assigned *Gardenia resinifera* to it. The main active compounds of *G resinifera* include Carbohydrates, Glycosides, Phytosterol Steroids, Tannins & Phenolic Group and Flavonoids as reported by WenpingXiao, et al., (2017) which is responsible for its wide range of medicinal applications. However, there is no Pharmacognostic work record of

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this traditionally much-valued drug this may be attributed to the miss-match of a wrong taxon. Since these active ingredients are not contained in *G. jasminoides* assigning *Gardenia resinifera* to such specimen once again would result in designating it with other medicinal functions rather than its antioxidant properties, hypoglycemic effect, inhibition of inflammation, antidepressant activity, and improved sleeping quality functions due to the presence of the following compounds iridoids, iridoid glycosides, triterpenoids, organic acids, and volatile compounds (Yang, et al 2009 and WenpingXiao, et al., 2017).

Conclusively, the study affirmed the accurate nomenclatural identities of all but one species that passed Amplification. Second, it affirmed two morphological dissimilar taxa as one, thereby demanding future studies using the deposited vouchers specimens. Thirdly, the study also established that only one species out of five was accurately authenticated by the five Institutional Curators. These drawbacks have made the process of gradual phasing out of voucher specimens authenticated by the Expert Recognition Method by one mediated by DNA barcoding all the more compelling. It is imperative that all specimens so authenticated by DNA analyses be subjected to standard phytochemical protocols that will offer efficient, cheap and on-field authentication process in future by anyone without reversion to the repetition of DNA analyses for the same taxon.

CONCLUSION & RECOMMENDATIONS

The results of present molecular studies have accurately and significantly disputed wrongly assigned specific epithets of three species while providing the accurate botanical names for all of them. It is recommended that molecular markers be deployed as a potent tool for authenticating voucher specimens in Herbaria.

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