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MORPHOMETRIC AND MOLECULAR CHARACTERIZATION OF IROKO GALL BUG, *Phytolyma species* (Hemiptera: Psyllidae) FROM EASTERN AND WESTERN NIGERIA

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ABSTRACT

Iroko gall bug, Phytolyma species is a major pest of Milicia excelsa which has hindered the establishment of Milicia plantations in many West African countries. To date, the genus Phytolyma consists of Phytolyma lata (Scot), Phytolyma fusca (Walker) Phytolyma minuta (Hollis) and Phytolyma tuberculata (Alibert). In Nigeria, there is no clear record of the Phytolyma spp found in the country. This has become a major concern due to irregularities in researchers' report of species present in Nigeria. This further hampers proper deployment of management strategies of the pest in affected regions. Hence, this study used morphometry and DNA barcoding to delineate Phytolyma species in three regions of Nigeria. The Principal component analysis yielded 19 components which correspond to the 19 morphometric measurements. The first two principal components resulted to 54.6% and 68.8% of the total variation in males and in females, respectively. However, the first two canonical variates showed partial separation for males and full separation of females. Phylogenetic analyses inferred through Maximum Likelihood method yielded a monophyletic clade with two distinct branches. Due to limited taxonomic information on the pest in Nigeria coupled with lack of genetic information at the GenBank on Phytolyma spp, this study reports on the creation of a first DNA barcode reference library of Phytolyma fusca

Keywords: *Phytolyma*, Species identification, morphometry, DNA barcoding,

INTRODUCTION

The Iroko gall bug of the genus *Phytolyma*, Psyllidae is a major insect pest of *Milicia excelsa*, a forest tree that is found naturally in Sub-Saharan tropical Africa. *Milicia excelsa* is known as Iroko or African Teak in the commercial market. Establishment of *Milicia* spp. in plantations in many parts of Africa including Nigeria has been hindered by attacks by *Phytolyma* spp pests the most prominent being *Phytolyma lata* (White, 1966; Cobbinah, 1986 Nichols *et al.*, 1999; Wagner *et al*., 2000 and Bosu *et al.*, 2006;Ugwu and Omoloye,2014). *Phytolyma* spp attack the buds and young leaves of *Milicia* spp, mainly the seedlings resulting in the formation of galls on the shoot and leaves (Ofori and Cobinnah 2007; Ugwu and Omoloye 2014). The adult female lay eggs on the young stems, leaves or shoots in high numbers, which is followed by subsequent hatching to first instar nymph (after 8 days) that punctures the plant tissues and eventually induces gall formation. These galls later develop to enclose the nymph (Ugwu and Omoloye, 2014). The nymph stages feed within the gall tissue, in the process breaking down the epidermal cells which eventually cause fermentation of the leaf parenchyma (Wagner *et*

al.,2000). The galls become turgid and later burst to release the adults, after 17 – 18 days. The galls rupture is followed by fungal decay accompanied by terminal dieback which causes growth reduction and seedling mortality in most cases (Cobbinah, 1986; Ugwu and Omoloye, 2014).

According to Hollis, (1973) the genus *Phytolyma* consists of four species: *Phytolyma lata* (Scot), *Phytolyma fusca* (Walker), *Phytolyma minuta* (Hollis) and *Phytolyma tuberculata* (Alibert). In Ghana, where two *Milicia* species overlap, three *Phytolyma* species have been collected; *P. lata* (Walker) *P. fusca* (Alibert) and *P. tuberculata* (Alibert) (Wagner, *et al*, 2000). In Cameroon, a new species of *Phytolyma* described as *Phytolyma tchuentei* has been reported (Tamesse *et al*., 2011). *Phytolyma tchuentei* develops on *Morus mesozygia* (Moraceae) and does not cause galls on leaves and buds of its host like the other *Phytolyma* species (Tamesse *et al*., 2011). In Nigeria, there is no clear record of the pest species as of date that attack *Milicia*. As such, this study aimed to combine both morphometrics analyses and DNA barcoding to delineate the *Phytolyma* species, make a comparison with what has been reported or characterized and create a DNA barcode reference library for the species to ease pest identification and potentially hasten better deployment of management measures of the species.

DNA Barcoding is the current molecular tool of choice, a system that employs sequence diversity in short, standardized cytochrome oxidase subunit I (COI) gene regions aiding in identification of species (Ratnasingham and Hebert 2007). Hebert *et al*. (2003) clearly elaborated that DNA barcoding assigns unknown specimens by comparing sequences to reference sequences of known species and thus, its success depends on the availability of a comprehensive database of DNA sequences for species identification, delineation, genetic diversity and variability studies (Morrow *et al.* 2000; Barr *et al*. 2006; Khamis *et al*, 2012). In retrospect, there have been major irregularities in researchers' reports of the *Phytolyma* spp present in Nigeria. Hence, this study aimed to characterize the genus *Phytolyma* using morphological and molecular characteristics to establish the *Phytolyma* species found in the country. Morphometry will be used to confirm the identity of the pest using features such as the wing veins, tibia length etc. (Khamis *et al*., 2012, Willig *et al* 1986; McNamee and Dytham 1993; Adsavakulchai *et al* 1999).

MATERIALS AND METHODS

Sample Collection

Phytolyma species were collected from three locations in Nigeria, namely; Olido Enugu –Ezike in Enugu state, Nigeria (South East Nigeria), Ido local government area of Oyo state in (South West Nigeria) and Idishin – Ibadan North west of Oyo State (South West Nigeria). The adult *Phytolyma* were collected from mature galls on *Milicia excelsa* plants and preserved in 95% ethanol until use. Thirty (30) samples from each location were selected for morphometric analyses and molecular characterization at the Centre of Insect Physiology and Ecology (icipe).

Morphometric study

The body, thorax width and antenna length measurements of the preserved insect specimens were recorded using the program Image-Pro H Plus version 4.1 for Windows TM and the data exported directly to an Excel data sheet. Measurements of all insect parts were taken in triplicate (to an accuracy of 0.001 mm). Further to this, the insects' right forewing and right hind leg were removed under a stereo microscope for morphometric analyses. The remaining parts were preserved in 75% ethanol in 1.5 mL Eppendorf tube for DNA extraction. The insects' right forewing and right hind tibia were prepared and mounted on slides as described by Billah *et al*., (2005), after which images were captured using a Leica MZ 125 Microscope, fitted with Toshiba 3CCD camera using the Auto Montage software. Fourteen wing distances between 15 selected landmarks on the wing were computed to characterize the shape and size of the wings for differentiation. These distances were; the Humeral break – Sub costal break, Sub costal break – vein R1, r– m, Upper length of dm-cell, Basal height of dm-cell, Lower length of dm-cell, Apical height of dm-cell, Vein R1 – Vein R2+3, R4+5, M, C, the wing length and width, and tibia length (Figure 1, 2 and 3). Slides were deposited at the Biosystematics Unit of the *icipe*.

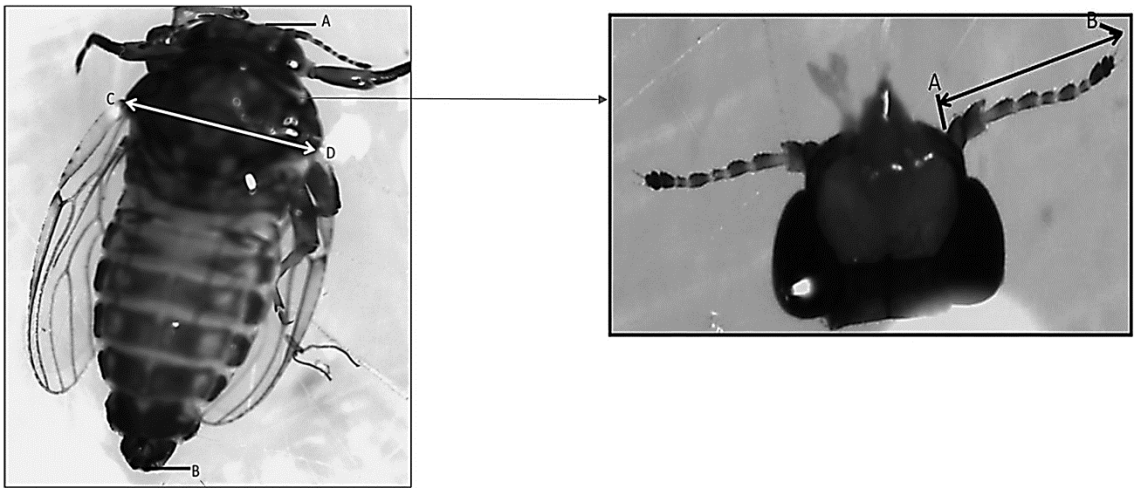

Figure 1: Adult *Phytolyma* and head part showing points of readings taking for morphometric analysis

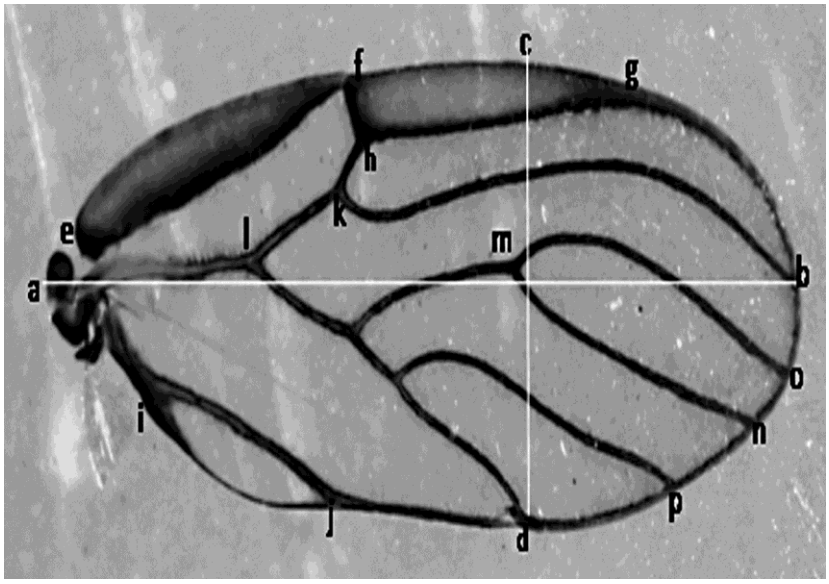

Figure 2: Wing of adult *Phytolyma* showing points of reading taken for morphometric analysis

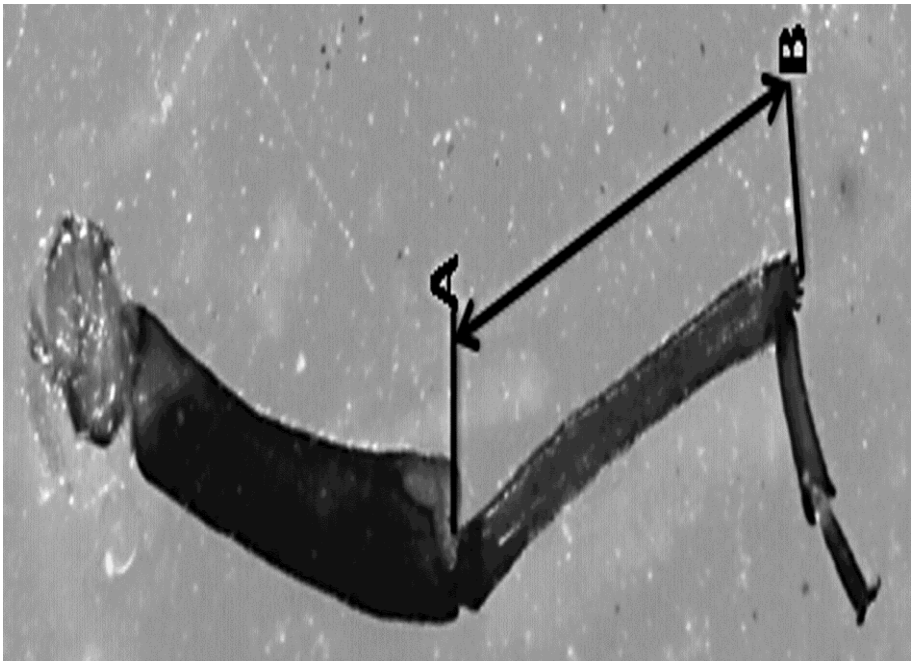

Figure 3: Tibia points of measurement for adult *Phytolyma* (measurement taken from point A to point B).

DNA Extraction

Each individual insect sample was surface-sterilized using 3% NaOCl and rinsed three times with distilled water. Genomic DNA was extracted using the Isolate II genomic DNA Kit (Bioline, London, UK), following the manufacturer's instructions. The purity and concentration of the resultant extracted DNA was determined using Nanodrop 2000/2000c Spectrophotometer (Thermo Fischer Scientific, Wilmington, USA).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was done to amplify the mitochondrial region and 2 sets of markers were used (Table 1). The PCR was carried out in a total reaction volume of 20 µL containing 5X My *Taq* reaction buffer (Bioline, London, UK) (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers), 0.5 pmol μl^{-1} of each primer, 0.5 mM $MgCl_2$, 0.0625 U μl^{-1} My *Taq* DNA polymerase (Bioline, London, UK) and 15 ng μl^{-1} of DNA template. This reaction was set up in the Nexus Mastercycler thermal cycler (Eppendorf, Germany). The cycling conditions involved an initial denaturation for 2 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 45 sec annealing and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. The amplified PCR products were resolved through a 1.2% agarose gel. DNA bands on the gel were analyzed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp, Meadowvale Way Sparks, Nevada, USA). Successively amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline, London, UK) following the manufacturer's instructions. The purified samples were shipped to Macrogen Inc Europe Laboratory, the Netherlands, for bi-directional sequencing.

Table 1: Primer information used in this assay

Name	Sequence5' - 3'	Target	Source	Range	Annealing Temp (°C)
LepF1	ATTCAACCAATCATAAAGATATTGG	COI	Hajibabaei *et al*., 2007	Insects	52
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	COI			
LCO1490	GGTCAACAAATCATAAAGATATTGG	COI	Folmer *et al*., 1994	Insects	50.6
HCO2198	TAAACTTCAGGGTGACCAAAAAATA	COI			

Morphometric Analysis

The nineteen morphometric measurements were log-transformed (log_{10}) prior to multivariate analysis, to stabilize the variance. Principal Component Analysis (PCA), a multivariate technique that assumes all samples are from the same population was used to reduce the dimensionality of the data to visualize it in two-dimension space using principal component scores. A projection of the morphometric measurements using principal components scores is expected to reveal clustering of the population samples. The transformed data were further subjected to Canonical Variate Analysis (CVA), a multivariate procedure where groups are defined prior to analyzing the data. Canonical variates produced from the CVA are then plotted to visualize the population groups. The analyses were implemented in R version 3.2.1 (R Core Team, 2015).

Sequence Analyses

The successful sequences were assembled and edited using Chromas Lite Version 2.1.1 (Thompson *et al*., 1997) and Geneious Version 8 (http://www.geneious.com) (Kearse *et al*., 2012). For conclusive identification of the species, both similarity and phylogenetic analyses were done. Similarity searches were conducted by querying the consensus sequences via BLAST at the Gene Bank database hosted by National Centre of Biotechnology Information (NCBI). BLAST (Basic Local Alignment Search Tool) algorithm finds regions of local similarity between sequences, in which consensus sequences were compared to reference sequences in the Gene Bank database. The sequences were also compared to reference sequences in the Barcode of Life Data systems (BOLD) using the species identification engine tool http://www.boldsystems.org/index.php/IDS_OpenId Engine (Ratnasingham and Hebert 2007).

Multiple alignments of the cleaned sequences were then done using Clustal X software (version 2.1) (Thompson *et al*., 1997) and MUSCLE v3.8.31 (Edgar 2004), with the ends trimmed and gaps removed using Jalview v2.8.2 The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumaret al., 2016). Pair-wise and multiple alignment of the sequences was done using Clustal W in MEGA. Neighbour Joining method was applied as the tree-building algorithm to visualize the patterns of divergence among the *Phytolyma* species (Saitou and Nei, 1987). The reliability of the clustering pattern in the tree was evaluated using a bootstrap analysis with 1000 replicates

RESULTS

Principal Component Analysis showed that the first two principal components demonstrated 54.6% (PC1 = 37.3% and PC2 = 17.3%) of the total variation in the morphometric measurements for the males while for the females, the first two principal components showed 68.8% (PC1 = 57.2% and PC2 = 12.6%) of the total variation. The measurement loadings of the first two principal components (PC1 and PC2) for the transformed female wings of the *Phytolyma* species were all positive (Table 2) while in the males the coefficients were negative (Table 3). Projection of the data on the first two principal components showed no separation of samples for males but partial separation for the females (Fig. 4). However, projection of the data on the first two canonical variates (CV) showed partial separation for males and full separation of females (Fig. 5). For both the male (CV1 = 68.8% and CV2 = 32.2% of the total variation) and female (CV1 = 69.8% and CV2 = 31.0% of the total variation) samples measurements, the first two canonical variates contributed a total of 100%. Mahalanobis distances for the females were twofold higher than those of male samples. For the DNA barcoding, a partial sequence of 658 bp of mitochondrial COI gene was obtained from the successfully sequenced specimens representing one of the localities sampled. When BLAST query was done, it was observed that there are no *Phytolyma* spp sequences in the database. The percentage identities obtained from GenBank from the closest linking relative was 83% to *Mycopsylla gardenensis* voucher DP1.idba (NC_036944.1) with query coverage of ≥98%. This

posed a challenge in conclusive identification because there were no reference voucher sequences in the resource databases. Hence, the samples were taxonomically confirmed as *Phytolyma fusca* (Alibert) 1947. There were no stop codons, insertions or deletions in the sequences. Phylogenetic analyses involved 31 nucleotide sequences. The tree generated clustered all samples from the study in one cluster separately from the closest *Psyllidae* spp; *Mycopsylla gardenensis* and *Craspedolepta canadensis* that were used as out groups in the phylogenetic analyses. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1703.9769) is shown in Fig 6. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 626 positions in the final dataset.

Table 2: Eigen values and coefficient (loadings of first two principal components (PC1 and PC2) for the transformed female wings measurement data of *Phytolyma* species populations.

Features	**PC1**	**PC2**
	37.3%	17.3%
Body length	0.150	0.095
Thorax	0.192	0.039
Antennae	0.065	0.256
V1	0.205	0.015
V2	0.200	0.042
V3	0.217	0.090
V4	0.222	0.038
V5	0.241	0.142
V6	0.475	0.686
V7	0.279	0.071
V8	0.189	0.016
V9	0.230	0.597
V10	0.249	0.017
V11	0.187	0.074
V12	0.221	0.112
V13	0.145	0.194
V14	0.183	0.008
V15	0.212	0.052
HTL	0.252	-0.027

Table 3: Eigen values and coefficient (loadings of first two principal components (PC1 and PC2) for the transformed male wings measurement data of *Phytolyma* species populations

Features	**PC1**	**PC2**
	57.2%	12.6%
Body length	-0.150	0.176
Thorax	-0.232	0.101
Antennae	-0.217	0..315
V1	-0.218	-0.011
V2	-0.221	-.034
V3	-0.243	0.005
V4	-0.304	-0.282
V5	-0.170	0.229
V6	-0.256	-0.099
V7	-0.299	0.163
V8	-0.243	-0.625
V9	-0.224	0.497
V10	-0.212	-0.040
V11	-0.201	-0.115
V12	-0.234	-0.089
V13	-0.214	-0.113
V14	-0.223	-0.010
V15	-0.218	0.127
HTL	-0.228	0.025

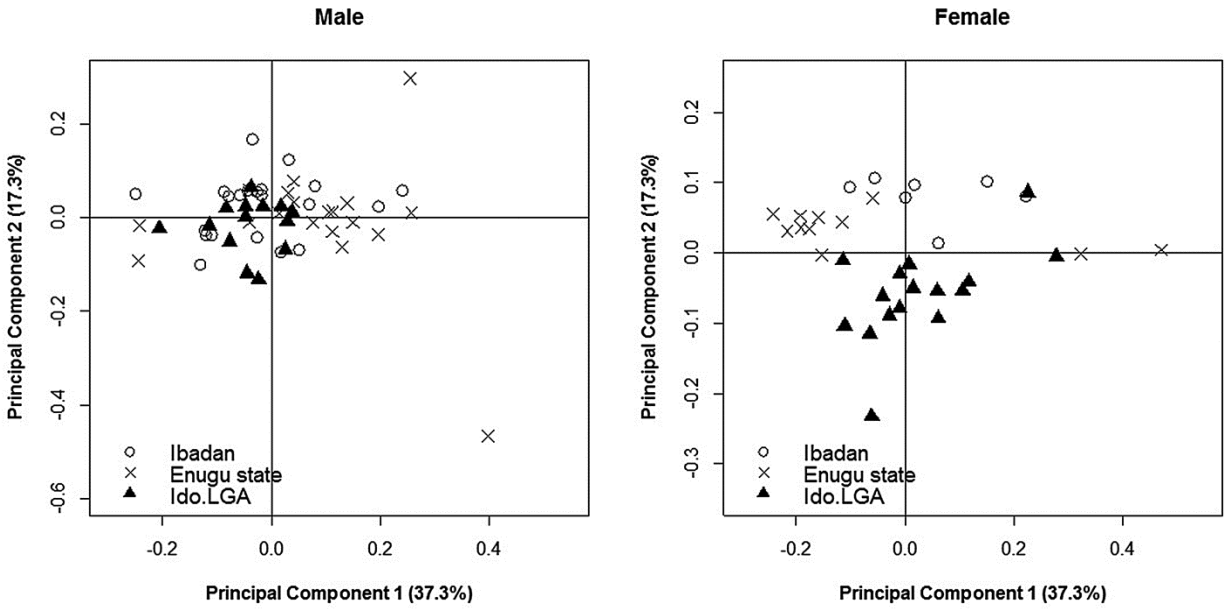

Figure 4: Projection of the wing and tibia data of male and female *Phytolyma* species compared with female from three regions on two principal components

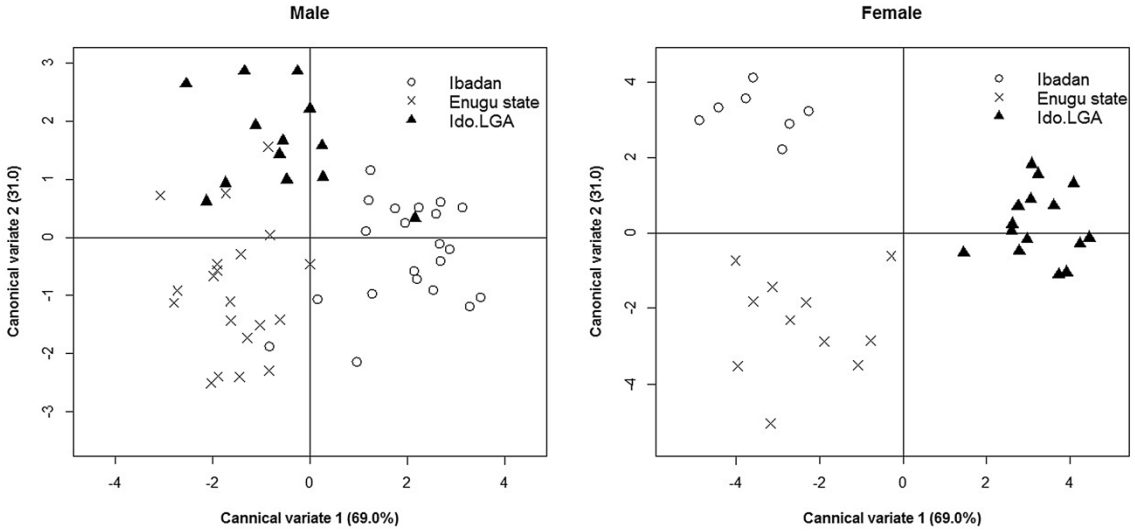

Figure 5: Projection of the wing and tibia data of male and female *Phytolyma species* compared with species from other regions of the first two Canonical variates

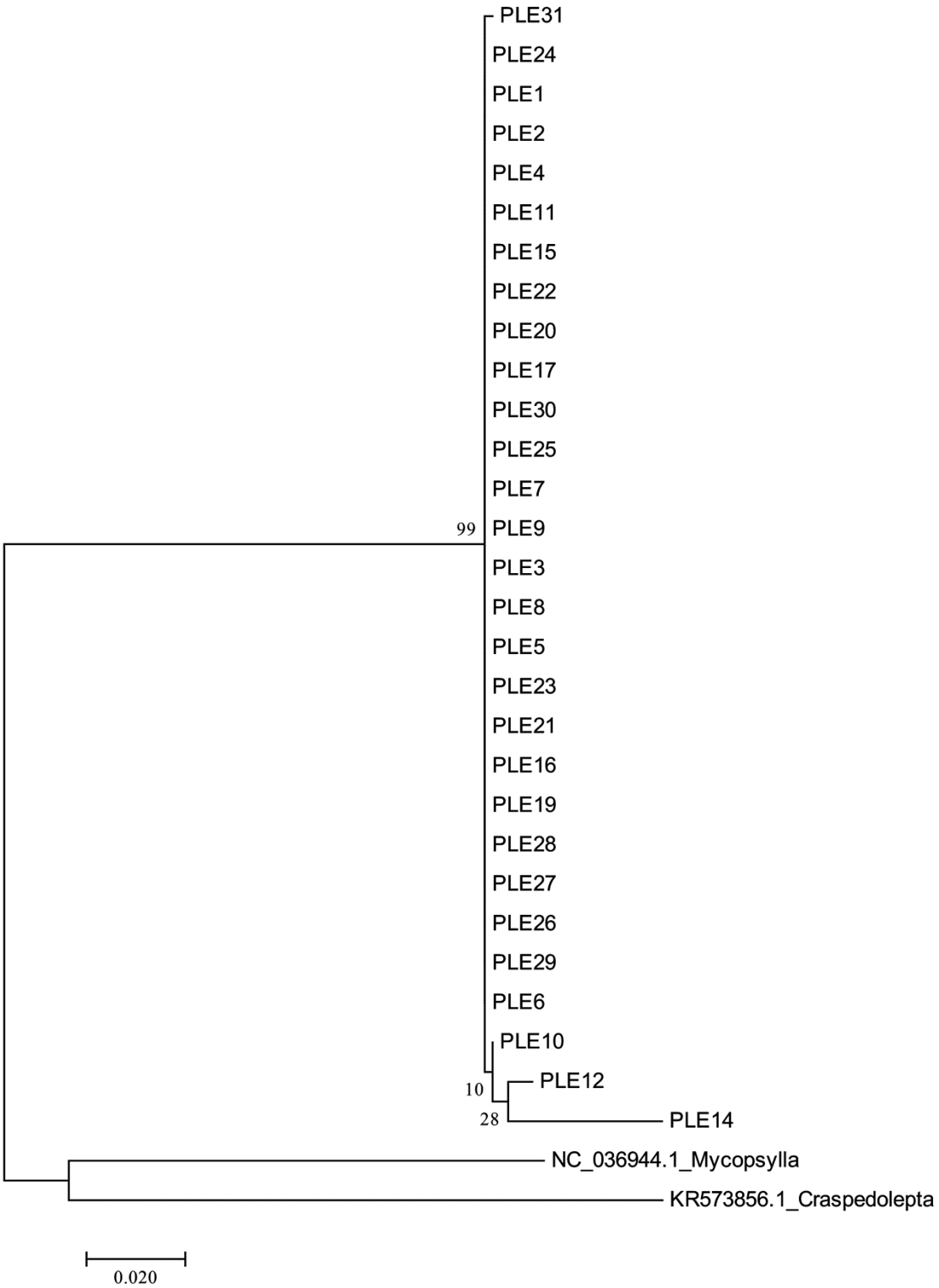

Figure 6: Molecular Phylogenetic analysis by Maximum Likelihood method showing evolutionary relationships between *Phytolyma fusca* samples from the study inferred by MEGA 7 (Kumar, Stecher, and Tamura, 2016). **NC036944.1**: *Mycopsylla gardenensis*; **KR573856.1**: *Craspedolepta canadensis*

DISCUSSION

Psyllids are phytophagous, phloem feeding insects that are typically monophagous or oligophagous. Together with aphids, coccids and whiteflies, they form the monophyletic group, Sternorrhyncha (Homoptera), which is considered basal within the true bugs (Hemiptera). Psyllids are probably the most benign of these four insect groups and therefore the least well studied. Co-evolution and co-speciation are usually defined by species-host interactions, and in this case, it can be hypothesized as insect-plant interaction. To understand this interaction, thorough characterization is necessary and hence this study aimed at delineating the *Phytolyma* species found in Nigeria. In many cases, herbivorous insect speciation is sequential in relation to the host plant which can bring about differences that taxonomically can be used to classify, name or identify a species. The principal components analyses (PCA) could clearly differentiate the species from the 3 locations at 54.6% of the total variation in males while for the females, it was 68.8%. Furthermore, projection of the data on the first two canonical variate(CV) showed partial separation for males and full separation of females. Considering the morphometric analysis alone, females could be considered as appropriate for morphometric measurement for the identification of the specimen from the three localities. The principal component analysis and canonical variate analysis results imply that without prior knowledge about the specimen's source, the morphometrics analysis (thus PCA) could not distinguish the specimens from the three localities.

A major challenge observed during this study was the lack of reference sequences of *Phytolyma* species in the GenBank. To date, this is the first report on work done on *Phytolyma* species using cytochrome oxidase subunit I (COI) gene and hence the 1st reference library for this species. Due to this, the samples could only link up to *Craspedolepta canadensis*, which is psyllid found in North America, at 82% relative positive identity. This is the closest relative that the Nigerian samples could match up with those in the database. Hebert *et al.*, (2003) correctly asserted that, the success of DNA barcoding largely depends on the availability of a comprehensive database of DNA sequences for comparison of species and analysis. It is in this premise that thorough taxonomic review of the samples used in this study was classified as *Phytolyma fusca* (Alibert), 1947. The species is widely distributed throughout the Afrotropical regions and has been reported from Nigeria. The sequences were uploaded to GenBank (accessions no. MK682895-MK682922) and hence their first DNA barcode reference library created to ease in identification.

With the creation of the first *P. fusca* DNA barcode reference library, this study has paved way for robust identification of this pest in Nigeria, Africa and globally for proper management strategies to be put in place.

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