



SYSTEMATIC STUDY OF THE GENUS “*Combretum*” Loefl. IN NORTHERN NIGERIAN SAHELIAN SAVANNAH: A CASE STUDY OF KATSINA STATE

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ABSTRACT

A systematic investigation of the genus *Combretum* in the Northern Nigerian Sahelian savannah was conducted using Katsina State as a case study, with the aim of assessing phylogenetic relationships and revising the taxonomy of species present in the region. Field surveys resulted in the collection of four *Combretum* species, which were analyzed using morphometric and molecular approaches. Cluster analysis based on morphometric characters revealed that all species were distinct at a Euclidean distance of 6. The species were identified as *Combretum ghasalense*, *C. molle*, *C. micranthum*, and *C. glutinosum*. A high cophenetic correlation coefficient ($r = 0.98$) indicated strong similarity among the taxa. Ordination analysis supported the cluster results by separating the specimens into four distinct groups. Principal Component Analysis (PCA) showed that the first two components accounted for 73.3% and 25.4% of the total morphological variation, respectively. For phylogenetic inference, genomic DNA was extracted from all specimens, and the chloroplast *rbcL* gene region was amplified, sequenced, and analyzed using Bayesian inference on the CIPRES portal. The results demonstrated that *Combretum* is monophyletic with strong posterior probability support (PP = 1.0). Several clades were strongly supported, while others showed moderate support. The phylogenetic tree revealed that *C. ghasalense* and *C. micranthum* are sister species within the same clade, whereas *C. molle* and *C. glutinosum* formed a closely related clade, indicating shared evolutionary history.

Keywords: Genus *Combretum*, Katsina state, Northern Nigerian, Systematic study, Sahelian savannah

INTRODUCTION

Combretum Loefl., is the largest genus of the family Combretaceae, belong to subfamily Combretoideae, tribe Combretae DC, subtribe Combretinae (Maurin, O. (2009).), and comprising about 255 species with a center of diversity in Africa, but also extending to Tropical Asia, and Northern Australia (Pattharahirantricin & Poopathn (2022)). The genus was divided in to three subgenera by Exell and Stace (1966), namely *Combretum*, *Cacoucia* (Aubl.) Exell and Stace, and *Apetalanthum* Exell and Stace, and each of these subgenera are now regarded as independent genus. In West Africa, the family Combretaceae is represent by 9 genera with about 72 species and the genus *Combretum* is the largest genus with about 48 species and 8 imperfectly known species (Gill, 1998). In Nigeria, the genus *Combretum* is represented by 25 species, which are mainly straggling shrubs and lianas (Ekeke, *et al*, (2014)). *Combretum* occurs on tropical and subtropical regions (America, Africa, Madagascar, Australia, India, Malaysia), and is rarely present in the pacific island (Stace, 2007). *Combretum* species are mainly shrubs, lianas, trees, shrublets, or woody climbers, very rarely sub herbaceous. The genus *combretum* is characterized by the presence of scales on the body of the plant, whereas the sub genus *Cacoucia* has stalked glands present on the leaves. The genus *Combretum* was recently classified in to 11 sections based on the floral, scale, and fruit anatomy (Fhyrquist, 2007). Members of *Combretum* have trifoliolate leaves which are opposite, verticillate or rarely alternate.

The existing information on these species in Nigeria is strictly base on the morphological description by Hutchinson and Dalziel (1954). This information were however, source from herbarium specimens. Literature search revealed that neither recent description nor taxonomic treatment of Nigerian members of the genus. Hutchinson and Dalziel (1954), reported indeterminate species and noted that much gap need to filled with identified species in tropical west Africa. This

underscores the need for a study to confirm the identity of the species in northern Nigerian sahelian savannah and other part of tropical West Africa. In the savannah vegetation of katsina state in northern Nigeria where the species occurs, there is conflicting identities with morphological signs of introgressions. Thus, this study aimed to identified the potential members of the genus *Combretum* in the study area and evaluate their phylogenetic relationships. Most of the species of *Combretum* have economic value, members of the genus *Combretum* are highly concentrated with antioxidant compounds, which possess anti-inflammatory and anti-viral activities (Masoko, 2007). Anti-cancer compounds that could be utilized for anti-cancer drug development were produce successfully from *Combretum* species (Fhyrquist, 2007).

However, many of these *Combretum* taxa with unique bioactive properties still having taxonomic delimitation issues due to close relatedness, It is therefore necessary to combine traditional taxonomy, cladistics, DNA barcoding and molecular phylogeny to authenticate the species and resolved nomenclatural ambiguities in the genus and also to determine the phylogenetic position of the species present in Katsina State. Some of the Nigerian species are missing in the current infrageneric classification of the genus, which is due to lack of sampling from the country and most of the taxonomic studies of the genus in Nigeria were based on morphological features. Over the years phylogenetic analysis using *rbcL* gene has not only been effectively utilized as a core plant barcode for amelioration of taxonomic problems and problems related to the used of wrongly identified plant for medicinal purposes among the taxa of angiosperm but has also addressed some critical issues relating to ecology of higher plants.

MATERIALS AND METHODS

Taxon Sampling

Fresh Specimens were collected from the field to study their vegetative morphology. Specimens were pressed carefully, to not affect their vegetative characters. Morphological measurements of the characters were made immediately. Specimens from the herbarium of biological science, Umaru Musa Yar'adua University, Katsina were also used as guides for identifying the plants. Plant were collected following the

procedure for collection of plants specimen for standard herbarium (Natalie, 2009). The collected specimens were authenticated and compared with the already deposited specimen in the herbarium of the department biological sciences Umaru Musa yar'adua university Katsina to ensure the correct identification of the species. A total number of four species were used for morphological studies. Detail of the collection are presented in table 1, and the map of Katsina state showing the collection spot is also presented in figure 1.

Table 1: Species Inventory within the Genus *Combretum* Collected for the Study

Taxon	Collection Number	Locality Information	Date of Collection
<i>C. ghasalense</i>	MSI1	Jibia 13°11'N, 7°22'E	August 12, 2024
<i>C. molle</i>	MSI2	Danmusa 12°19'N, 7°47'E	September 6, 2024
<i>C. micranthum</i>	MSI3	Batagarawa 12°89'N, 7°58'E	September 8, 2024
<i>C. glutinosum</i>	MSI4	Batagarawa 12°89'N, 7°58'E	September 8, 2024

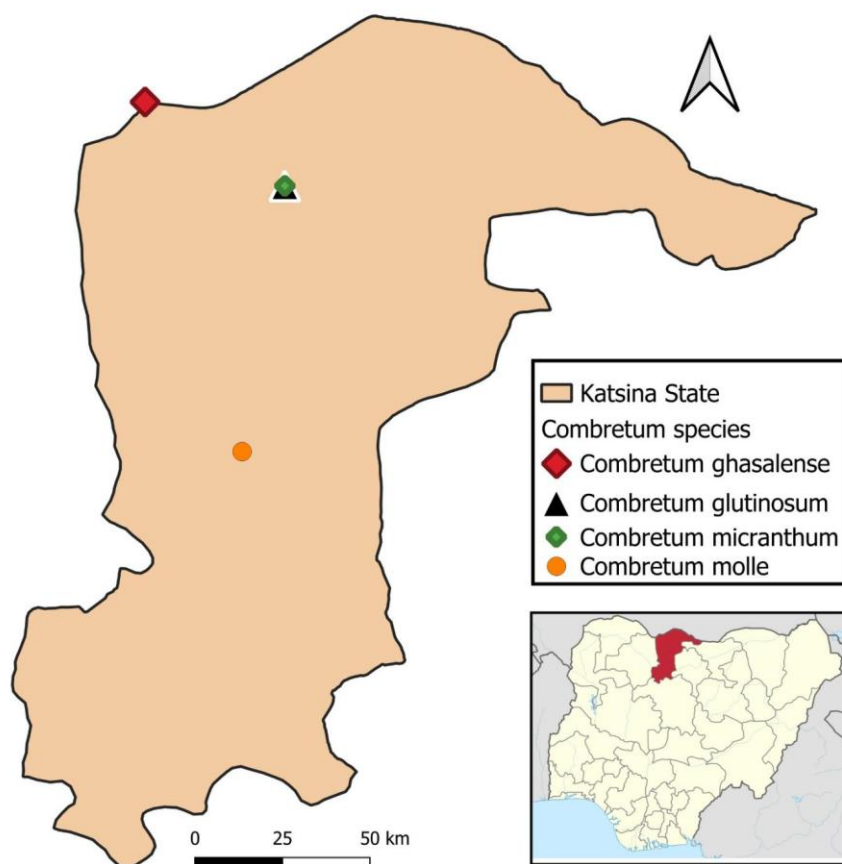


Figure 1: Map of Katsina showing the Collection Spot of *Combretum* Species

Character Observation

Species used in this study were considered as operational taxonomic unit, and each species was represented by one individual in morphometric analysis. A character sets consisting of 19 characters (6 quantitative and 13 qualitative characters which include number of leaflet, length of petiole, length of leaflet, width of leaflet length between nodes, length of seed, leaflet tips, leaflet arrangement, leaflet color, leaflet shape, habit, compound leaf, leaflet venation, number of venation, stem color, fruit shape, fruit color, flower color, and inflorescence were used for morphometric analysis. Table 2 list of quantitative and qualitative characters were used to construct a dendrogram.

Multivariate Analysis

Multivariate analysis was carried out using cluster analysis (CA) and principal component analysis (PCA) and Jaccard's similarity coefficient using PAST 3 program (version 3.05) to determine the morphological similarity among the species and the characters that contribute to the variation of the taxa.

Cluster Analysis (CA)

Cluster analysis is a multivariate data mining technique, whose goal is to group objects base on a set of user selected characteristics. The cluster analysis groups individuals that are very similar in one cluster and show similarities and differences among and within the cluster by building similarity matrix between the studied individuals. Individuals in the same cluster will be recognized as belonging to the same species. The cophenetic correlation coefficient between

the distance matrix and the tree matrix was calculated to examine how well the cluster analysis fits the distance matrix (Sokal and Rohlf, 1997; Rohlf 1998).

Principal Component Analysis (PCA)

PCA is an accepted method to study multivariate character correlation. Its purpose is to study linearly correlated variables (Hotelling, 1936). PCA enables the distinguishing of groups as well as identification of the relative contribution of size-dependent and size-independent variation to species discrimination (Humphries *et al.* 1981). Therefore, it shows

those characters that are useful in showing delimitation among the species. The Jaccard's similarity coefficient was calculated to find the morphological similarity among the species.

All the 19 morphological characters including both quantitative and qualitative traits, for all the 4 species were used for the analysis and each individual specimen is considered as an operational taxonomic unit (OTU). In all the analysis, data were first log₁₀ transformed for the standardization of the matrix.

Table 2: List of the Quantitative and Qualitative Characters and Character State used In Morphometric Analysis

S/No.	Character	Character State
Quantitative Characters		
1	Number of leaflet (NL)	
2	Length of petiole (LP)	mm
3	Length of leaflet (LL)	mm
4	Width of leaflet (WL)	mm
5	Length between nodes (LN)	mm
6	Length of seed (LS)	mm
Qualitative characters		
7	Leaflet tips (LT)	Obtuse (1), Acute (2)
8	leaflet arrangement (LA)	Opposite decussate (1), Whorled (2)
9	leaflet color (LC)	Green (1), Dark green (2), Light green (3)
10	leaflet shape (LS)	Elliptic (1), ovate (2), elliptic oblong (3)
11	Habit (H)	Herb (1), shrub (2), small tree (3)
12	Compound leaf (CL)	Even pinnate (1), Trifoliolate (2)
13	Leaflet venation (LV)	Reticulate venation
14	Number of venation (NV)	7 to 10 pairs (1), 7 to 12 pairs (2)
15	Stem color (SC)	Grey bark reddish (1), Grey bark black (2)
16	Fruit shape (FS)	Four winged
17	Fruit color (FC)	Yellow brown (1), light green (2), reddish (3)
18	Flower color (FC)	Greenish color
19	Inflorescence (IF)	Axillary spike (1), drooping spike (2)

mm= millimeter

Molecular Characterization

DNA Extraction

Accuprep™ Plant Genomic DNA Kit (K-3031) was used for DNA extraction. The Kit was designed for efficient recovery of genomic DNA up to 60kb in size from fresh and dried plant tissue.

DNA was extracted from all the specimens as outlined in the user manual of the kit (the extraction was conducted at DNA Lab Kaduna). Silica gel dried leaf material was ground to powder using a pestle. 100 mg of powder dried tissue was put in 2.0 ml tube. 300µl Buffer PL1 and 10µl of beta mercaptoethanol were added and the tube was vortexed to mix vigorously to disperse all clumps in Deluxe Mixer, followed by incubation at 60°C for 10 minutes in a dry bath. The samples were mixed twice during the incubation by inverting the tubes and also vortexing it. 200 µl Buffer PL2 was added to the tube and it was mixed by vortexing for 10 seconds and it was incubated on ice for 5 minutes, and centrifuged at 11,000 rpm for 10 minutes in centrifuging machine (Centrifuge 5415C, Eppendorf). 400 µl of the supernatant was removed to a clean 1.5 ml Eppendorf tube. 400 µl of Isopropanol was added to the tube and the tube was mixed well by vortexing for 5 seconds, centrifuged at 12,000 rpm for 2 minutes to precipitate the DNA (this removed polysaccharides and improved DNA binding ability to the spin column). The supernatant was carefully discarded by using a pipette to draw

the supernatant to avoid dislodging the DNA pellet. The tube was inverted on absorbance paper towel for 1 minute to drain any residual ethanol (this is also done carefully, taking care of the DNA pellet). 300 µl of pre-heated ddH₂O (65°C) was added to the tube containing the DNA pellet and it was vortexed for 10 seconds to mix the DNA well, it was then briefly incubated at 65°C to help dissolve the DNA. 150 µl of Buffer PL3 and 300 µl of 100% ethanol were added to the tube containing the mixture of DNA pellet and pre-heated ddH₂O (65°C), the tube was mixed well by vortexing for 5 seconds (a precipitation was formed but it doesn't interfere with the DNA binding to column). The sample was transferred to a clean column and centrifuged at 11,000 rpm for 1 minute, the flow was discarded through and the column was put back to the collection tube, it was repeated once. The column was centrifuged at 11,000 rpm for 1 minute (this step is critical for removing residual ethanol that may otherwise interfere with downstream treatment application). The column was transferred into a clean 1.5 ml tube. 100 µl of pre-warmed (65°C) elution buffer was added and the tube was centrifuged at 11,000 rpm for 1 minute to elute DNA. This final step was repeated once.

Polymerase Chain Reaction (PCR)

The mixture used in the PCR was the same for all the specimens. For each specimen, 14 µl of ultra-pure water, 1.5

μ l of both the forward and reverse primers, 3 μ l of DNA sample, giving a total of 20 μ l per reaction. Reactions were run on an MJ Research PTC – 200 Peltier Thermal Cycler. The primer used to amplify the *rbcL* region is given in table 3, together with their sequences.

The PCR process involved an initial denaturation phase of 5 minutes at 94°C by 35 cycles of 1 minute at 72°C: 30 seconds

at 54°C (annealing); 30 seconds at 94°C (extension); and a final extension phase of 5 minutes at 72°C.

Resulting PCR product were run on agarose gel to confirm successful reactions, before being purified using 2xEasyTaq* PCR Super mix kits, according to the protocols supplied by the manufacturer. The product was used for the sequencing reactions. The sequencing was done using ABI 3730 capillary DNA Sequencer in DNA lab Kaduna.

Table 3: Primers used in PCR Reactions

Region Amplified	Primer Name and Direction	Primer Sequence
<i>rbcL</i> (Chloroplast)	<i>rbcL</i> (forward)	ATGTCACCACAAACAGAGACTAAAGC
<i>rbcL</i> (Chloroplast)	<i>rbcL</i> (reverse)	GTAATAATCAAGTCCACCRGC

rbcL region was chosen according to the result from previous studies by (Lahaye *et al.* 2008., Maurin *et al.* 2010, Nithaniyal and Parani, 2016.), there study have shown that it has provided robust resolution with the genus under studies. Similarly Maurin *et al.* (2010) reported that the region provides robust resolution at higher taxonomic levels for the tribe Combretinae. Uzoehina (1978), the studies also reported the efficiency of using *rbcL* region in species identification within Combretaceae family.

Phylogenetic Analysis

Resulting sequences from the sequencing reactions were assembled and edited using Staden package (Staden *et al.*, 1998. Bioedit was used to align the sequences (Hall, 1999),

using ClustalW multiple alignment followed by manual adjustments (Bello *et al.*, 2015). All positions containing gaps and missing data were eliminated (Shweta *et al.*, 2013). Phylogenetic analyses were done using Bayesian Inference Analysis. Bayesian Inference Analysis was run using MrBayes.

Sequences derived from the encountered taxa during fieldwork were combined with sequences downloaded from Genbank in the interest of creating more complex phylogeny and to know the phylogenetic position of the collected species in the current global infrageneric classification of the genus. The four species collected in this study and the genbank accession numbers used in this study are presented in table 4.

Table 4: List of the Genbank Accession Number of the Closest Matches of 4 *Combretum* Species

Query ID	Top hit species name	GenBank accession	% identity	Alignment length (bp)	E-values
<i>C. ghasalense</i>	<i>C. platypetalum</i>	EU338124.1	99.81	1433	0.0
<i>C. glutinosum</i>	<i>C. glutinosum</i>	FJ381789.1	99.25	1399	0.0
<i>C. micranthum</i>	<i>C. micranthum</i>	FJ381793.1	99.82	1295	0.0
<i>C. molle</i>	<i>C. molle</i>	EU338147.1	99.62	1407	0.0

RESULTS AND DISCUSSION

Cluster Analysis

The result of the cluster analysis separated the data set in to 4 clusters (at a Euclidean distance of 6. Figure 1) and a cophenetic correlation coefficient value of $r = 0.98$ indicate a very perfect match between triangular matrix and the phenogram (Sneath and Sokal 1973, Rohlf, 1998). The groups were recognized as distinct taxa at different taxonomic hierarchies if all their OTUs did not mix between clusters. *C. molle* and *C. ghasalense* are the most similar and formed a cluster at a low distance of (4), indicating high similarity between them, *Combretum glutinosum* join the cluster of *C.*

molle and *C. ghasalense* at higher distance of (-10) indicating it is similar, but less so than the previous pair of *C. molle* and *C. ghasalense*, and *Combretum micranthum* is the more distinct and joins the cluster at a distance near (18) indicating it is the most dissimilar species among all four taxa. Therefore *C. molle* and *C. ghasalense* are mostly close related. *C. micranthum* is the most genetically or phenotypically distinct from the all other three species. This hierarchy suggests evolutionary divergence within the genus *Combretum*.

The result of the Jaccard's similarity coefficient varied between 0.73 and 0.83, indicating closer relationships among the species (Table 5).

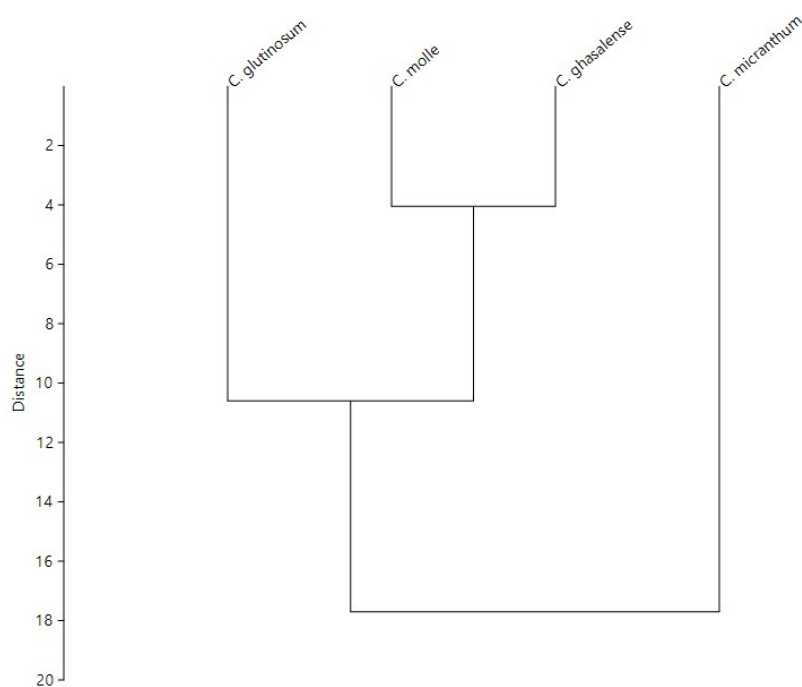


Figure 1: Unweighted Pair Group Method with Arithmetic mean (UPGMA) phenogram resulting from cluster analysis. Cophenetic correlation (r) = 0.987

Table 5: Similarity Matrix Base on Jaccard’s Coefficient

	<i>Combretum ghasalense</i>	<i>Combretum molle</i>	<i>Combretum micranthum</i>	<i>Combretum glutinosum</i>
<i>Combretum ghasalense</i>	0.73	0.73	0.83	0.83
<i>Combretum molle</i>	0.73	0.73	0.83	0.83
<i>Combretum micranthum</i>	0.83	0.73	0.83	0.83
<i>Combretum glutinosum</i>	0.83	0.73	0.83	0.83

The result of the Jaccard’s similarity coefficient varied between 0.73 and 0.83, indicating closer relationships between the species.

Ordination

The ordination result base on the result of PCA also separated the 4 specimens in to four (4) distinct groups that largely correspond to those obtained in the cluster analysis (Figure 1). Principal component 1 accounted for 73.3% of the variation while principal component 2 accounted for 25.4% of the

variation among the taxa. The loading of the PC 1 and PC 2 are presented in table 6. The character mostly correlated with the first PCA axis with value ($r>50$) is: Number of leaflets (Figure 2 & 3). The result of the PCA showed that majority of the clusters in the ordination plot, correspond largely to those obtain by the cluster analysis. The number of leaflet, leaflet length, number of venation and plant height contributed more to showing delimitation among the species. The scree plot indicating the contribution of variation of PC 1 and 2 is also presented in (Figure 2).

Table 6: Loading of the First and Second Components of the Principal Component Analysis

PC	Eigen Value	%Variance
1	72.4218	73.323
2	25.1729	25.486
3	1.17613	1.1908

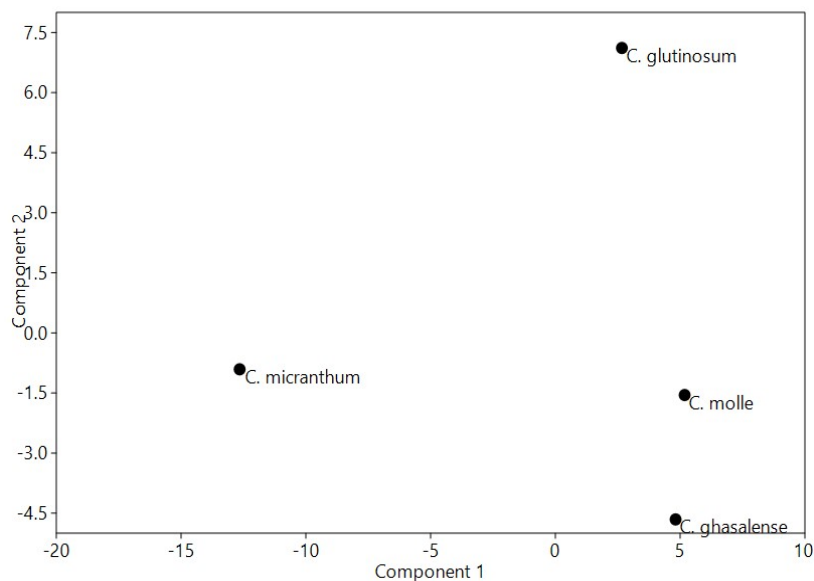


Figure 2: Plot of the first two principal component analyses (PCA) of morphological variation among the species obtain from analyses of morphological data set of the species within the genus *combretum*

The result of the PCA showed that majority of the cluster in ordination plot, correspond largely to those obtain by cluster analysis.

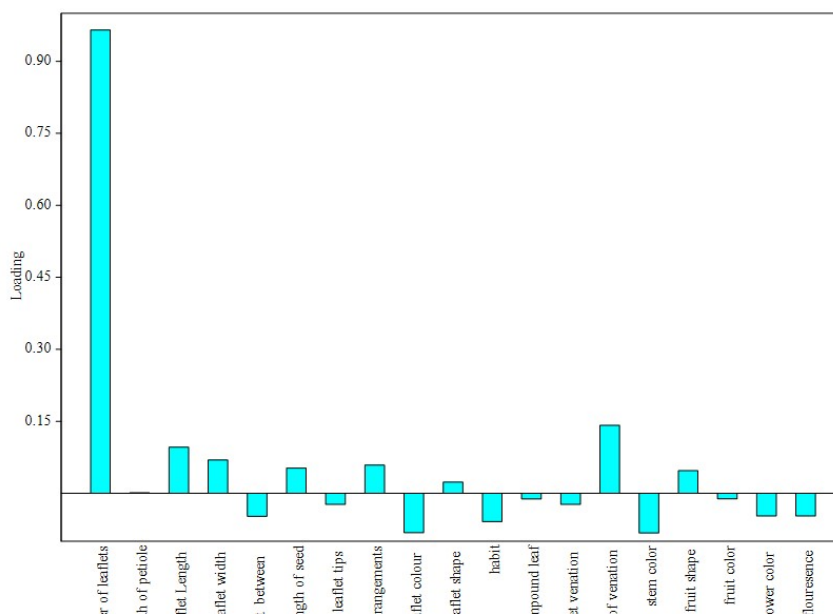


Figure 3: PCA Loading's indicating morphological characters that has the highest and lowest contribution in the variation among the species. Number of leaflets, plant height, and number of venation have higher loading in the result

Phylogenetic Analysis

Phylogenetic relationships were determined using Bayesian inference on MRBAYES run on CIPRES portal. The data set included 11 accessions (10 species of *Combretum* and *Gueira senegalensis* as groups). Out of the 11 species, 7 were downloaded from GenBank and the remaining 4 are the collections from the present study area. The analysis showed that *Combretum* is monophyletic (PP 1.0). In the tree some clades are strongly supported (PP 9= 1.0) while other clades are moderately supported (PP value less than 8.0). The results showed that results showed that *C. ghasalense* and *C. micranthum* are sister species and closely related belonging to the same clade. *C. glutinosum* and *C. molle* belong to the same clade, indicating closely relationship between the species. The

C. micranthum sequence, sequenced from this study is sister to *C. micranthum* sequence in another study, confirming the identification of *C. micranthum* collected in this study. Similarly, *C. molle* sequenced in this study is also sister to *C. molle* downloaded from GenBank, confirming the identification of the *C. molle* collected in this study. The clade of *C. molle* includes other species of *Combretum*, *C. apiculatum* and *C. albopunctatum* indicating a close relationship with *C. molle*. The clade containing *C. micranthum* and *C. ghasalense* contained *C. fragrans*, indicating close relationship between the species. Among the species included in the phylogenetic tree, *C. fragrans* is the earliest diverged species and *C. molle* is the recent diverged species.

the NCBI database. Result from this study could however be sufficiently applied to the scientific classification of the Nigerian *Combretum*, especially where morphological and electrophoretic base molecular studies have failed. It can also be used as barcode for monitoring appropriate use of this genus for medicinal application and drug development. This method which was employed in this study has become important in systematic studies in the past few years in authenticating morphological findings.

CONCLUSION

This study identified four *Combretum* species in Katsina State namely, *C. glutinosum*, *C. molle*, *C. ghasalense* and *C. micranthum*. The morphometric analyses revealed that *C. molle* and *C. ghasalense* show similar morphological features and these two species shared morphological features with *C. glutinosum*. The *C. micranthum* is the species with different morphological features compared with the other three species. *C. ghasalense* and *C. micranthum* are sister species and phylogenetically related among the four species studied. *C. glutinosum* and *C. molle* are sister species found on the same clade. There is genetic diversity among *C. molle* species globally. The morphological data set of the species of the genus *Combretum* studied, the character with the highest value of ($r > 50$) is number of leaflet and the character with lowest value of ($r < 50$) is fruit color.

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