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PHYLOGENETIC POSITION OF WEST AFRICAN SPECIES OF THE GENUS *CROTALARIA* L. (CROTALARIEAE, FABACEAE) BASED ON THE CURRENT INFRAGENERIC CLASSIFICATION

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Abstract

Crotalaria L. (Crotalarieae, Fabaceae) is among the largest legume genera consisting of ca. 702 species with the majority of the species (ca. 400) distributed in tropical and sub-tropical Africa. The current existing global sectional classification of *Crotalaria* does not include the majority of the West African species. Nuclear ribosomal internal transcribed spacer (ITS) gene region of seven species of *Crotalaria* from West Africa was sampled with the aim of determining their phylogenetic relationships and position based on the current sectional classification. Dataset was analyzed using parsimony and Bayesian MCMC (MrBayes) approaches. The result showed that *C. macrocalyx*, *C. senegalensis*and *C. pallida*were closely related and belonged to Hediriocarpae clade while *C. retusa* and *C. goreensis*, *C. atrurubens, C. barkae*fell within the Calycinae, Stipulosae, Longirostres and Incanae clades respectively. Our results also showed that *C. retusa*was resolved as sister to southern African *C. papilosa*in Calycinae clade. The result of this is study supports the sectional classification in *Crotalaria* and helps in revealing the phylogenetic position of the poorly studied West African species.

Key words: *ITS*, Phylogenetic positions, Infrageneric classification, *Crotalaria*, West Africa.

Introduction

Crotalaria L. (Fabaceae) is one of the largest genera of Papilionoideae (Fabaceae) consisting of ca. 700 species (Le Roux *et al*., 2013). It is the largest genus of legumes in tropical Africa (Polhill, 1982, Frodin, 2004).

Species within the genus *Crotalaria* differ in their habit ranging shrub - herbs and may be annual or perennial. They are easily recognised by their yellow, whitish to purplish or bluish coloured flowers. The leaves are simple or one to three foliate, alternate, lanceolate to obovate. The genus can also be recognised by a combination of the following five diagnostic characters: rostrate keel, inflated fruit, 5+5 highly dimorphic anther arrangement (five long, basified anthers alternating with five short, dorsified ones), trichomes present on the style and macrocyclicpyrrolizidine alkaloids (Baker, 1914; Polhill, 1968; Van Wyk, 2005, Alexender *et al*., 2017)

Crotalaria is almost cosmopolitan in distribution across the tropical regions of the world (Lewis *et al*., 2005), with its center of diversity in Africa and Madagascar (ca. 543 species) (Polhill, 1968, Polphil, 1982, Le Roux & Van Wyk, 2013) and a secondary center in India (ca. 92 species) (Ansari, 2008; Sibichen & Nampy, 2007). The genus is also widely distributed across the southern hemisphere, extending into Asia and North America. There are ca. 51 species of *Crotalaria* in West Africa (Hutchinson *et al*., 1958).

African and Madagascan members of the genus were revised by (Polhill 1982), following the revision of (Bisby, 1973) and (Bisby & Polhill, 1973). Polhill (1982) published an updated infrageneric classification of the genus where he recognised eight sections and 12 subsections. Later on, Ansari (2008) revised the Polhill classification to accommodate the Indian species. He (Ansari, 2008) maintained the eight sections by Polhill, but modified the subsections. Recently, Le Roux *et al*., (2013) carried out a study on the generic relationship using morphological and molecular evidence. They reported modified sectional classification and recognized eleven sections, after the modification of some sections recognised by Polhill. They raised some subsection to sectional level and abandoned some subsection due to non monophyly.

In this work, we aimed to determine the phylogenetic position of some West African species that are not included in the current infrageneric classification of the genus.

Materials and Method

Taxon sampling: Plant materials were collected through field survey of the genus *Crotalaria* across the study area (Table 2). The plants were identified based on the herbarium specimens and morphological features seen in relevant literatures. *Bolusia* was suggested to be the sister group of *Crotalaria* (Polhill, 1976, 1982), and this has been confirmed in studies of Boatwright *et al*., (2008) with Bootstrap support 92% and PP 1.0. It was also reported that the *Crotalaria* – *Bolusia*clade is sister to monotypic genus *Euchlora* (99% BS; PP 1.0; Boatwright *et al*., 2008). Based on these findings both *Bolusia* and *Euchlora*were used as outgroups in this study. Other sequences of *Crotalaria* species not present in the study area were downloaded from Genbank in the interest of creating more complex phylogeny and to know the phylogenetic position of the species collected for this study in the current infrageneric classification (Table 3).

DNA extraction: Easyprep^{TM}Plant Genomic DNA Kit was used for DNA extraction. The Kit is very efficient in recovering of genomic DNA up to 60 kb in size from plant samples.

DNA was extracted from all the specimens as outlined in the user manual of the kit (the extraction was conducted in DNA Lab Kaduna). Silica gel dried leaf material was crushed to fine powder using a pestle. About 50 mg of powdered dry tissue was put into 2.0 ml tube then 600 µl Buffer PL1 and 10µl of beta mercaptoethanol were added. The tube was vortexed vigorously to mix and disperse all clumps in Deluxe Mixer. Followed by incubating at 65ºC for 10 min in a dry bath. The samples were mixed twice during the incubation by inverting the tubes and vortexing. After vortexing, 200 µl Buffer PL2 was added to the tube and it was mixed by vortexing for 10 s followed by incubation on ice for 5 min, the mixtures was then centrifuged at 11, 000 rpm for 10 min in Centrifuge 5415C, eppendorf. About 400 µl of the supernatant was taken to a sterilized 1.5 ml eppendorf, then 400 µl of Isopropanol was added to the tube. The mixture of the supernant and isopropanol was mixed well by vortexing for 5 s, centrifuged at 12, 000 rpm for 2 min to precipitate the DNA. Supernatant were carefully discarded by using pipette to draw the supernatant to keep away the DNA pellet from dislodge. The tube was inverted on clean towel for 1 min to drain the ethanol (this was also done carefully taking care of the DNA pellet). About 300 µl of pre heated ddH_2O (65ºC) was added to the tube containing the DNA pellet and it was vortex for 10 s to mix the DNA well. It was then briefly incubated at 65ºC to dissolve the DNA, 150 µl of Buffer PL3 and 300 µl of 100% ethanol were added into the tube and the mixture of DNA pellet and pre heated ddH_2O (65ºC), the tube was mixed well by vortexing for 5 s (a precipitation was formed but it did not interfere with the DNA binding to column). The samples were transferred to a clean column and centrifuged at 11, 000 rpm for 60 c. Resulting flow was discarded and the column was put back to the collection tube. This was repeated once. The DNA binding column was centrifuged at 11, 000 rpm for 60 s. The column was transferred into another sterilized 1.5 ml tube, 100 µl of pre warmed (65ºC) elution buffer was added and the tube was centrifuged at 11, 000 rpm for 1 min to elute DNA. This final step was repeated once.

Polymerase chain reaction (PCR): For each specimen, 14 µl of ultra-pure water, 1.5 µl of premix, 1.5 µl of forward and reverse primers, 3 µl DNA sample, totaling 20 µl. PCT was run on an MJ Research PTC – 200 Peltier Thermal Cycler. The primer used are presented in (Table 1).

ITS region was chosen because results from previous studies by Le Roux *et al*., (2013) have shown that it provide robust resolution with the genus under study. Similarly Boatwright *et al*., (2008, 2011) reported that the region provides robust resolution at higher taxonomic levels for the tribe Crotalarieae. Chen *et al*., (2010) in their studies also reported the efficiency of using *ITS* region in species identification within the Fabaceae family.

The PCR starts with initial denaturation of 5 min at 95ºC by 35 cycles of 40 s at 94ºC: 40 s at 50ºC (annealing); 40 s at 72ºC (extension); ending with extension phase of 5 min at 72ºC.

Resulting PCR products were run on agarose gel to confirm successful amplification before purification.

The product was used for the sequencing reactions. The sequencing was done using ABI 3730 capillary DNA sequencer in DNA lab Kaduna.

Phylogenetic analysis: Resulting sequences from the sequencing reactions were assembled with Staden package (Staden*et al*., 1998). ClustalW multiple alignment in the programmeBioedit was used for alignment (Hall, 1999), the sequences were then manually aligned (Bello *et al*., 2015). All positions containing gaps and missing data were eliminated (Shweta *et al*., 2013). The data were analyzed using Maximum parsimony (PAUP version 4.0b10) using heuristic searches with 1000replicates of random taxon addition, tree bisection–reconnection branch swapping, MulTrees on, saving a maximum of 100 trees each replicate. Missing characters were treated as gaps. Support was assessed using 1000 replicates of non-parametric bootstrap analysis (Felsenstein, 1985). Bayesian analysis was carried out using MrBayes version 3.1.2. jModelTest version 2.5.6 was used to select the suitable model.

Table 1. Primers used in PCR reactions.

Region to be amplified	Primer name and direction	Primer sequence
ITS (Nuclear)	ITS5 (forward)	5'-GGA AGT AAA AGTCGT AAC AAG G-3' (White *et al.,*1990
ITS (Nuclear)	ITS4 (reverse)	5'-TCC TCC GCT TAT TGA TAT GC -3' (White *et al.,* 1990)

Table 2. Plant collections used in the study.

Taxon	Collection number	Locality information	Leaf collected
C. macrocalyx	SSY 1	Jibia 13º04′N, 7º15′E	Y
C. senegalensis	SSY2	Mashi 12º58′N, 7º56′E	Y
C. retusa	Bello 395	Kaita 13º05′N, 7º13′E	Y
C. pallida	SSY 28	Dankama13º11′N,7º47′E	Y
C. atrorubens	SSY 12	Mashi 13º06′N, 8º00′E	Y
C. barkae	SSY 15	Jibia 13º04′N, 7º45′E	Y
C. goreensis	SSY 18	Dankama13º17′N,7º47′E	Y

Table 3. Sequences obtained from GenBank and previously used in global infrageneric classification system of *Crotalaria* (Le Roux *et al*., 2013).

Genbank accession no.	Taxa
JQ067143	*Crotalaria virgultaris*
JQ067322	*Crotalaria barnabassii*
JQ067321	*Crotalaria lebeckioides*
JQ067318	*Crotalaria globifera*
JQ067313	*Crotalaria obscura*
JQ067307	*Crotalaria longidens*
JQ067305	*Crotalaria pallida*
JQ067304	*Crotalaria natalitia*
JQ067303	*Crotalaria lanceolata*
JQ067146	*Crotalaria recta*
JQ067145	*Crotalaria lanceolata*
JQ067144	*Crotalaria juncea*
JQ067142	*Crotalaria obscura*
JQ067141	*Crotalaria vasculosa*
JQ067139	*Crotalaria sphaerocarpa*
JQ067138	*Crotalaria humilis*
JQ067137	*Crotalaria excisa*
JQ067136	*Crotalaria meyerana*
JQ067135	*Crotalaria recta*
JQ067134	*Crotalaria griquensis*
JQ067133	*Crotalaria virgulata*
JQ067132	*Crotalaria brachycarpa*
JQ067131	*Crotalaria doidgeae*
JQ067130	*Crotalaria laburnifolia*
JQ067129	*Crotalaria monteiroi*
JQ067128	*Crotalaria dura*
JQ067127	*Crotalaria burkeana*
JQ067126	*Crotalaria macrocarpa*
JQ067125	*Crotalaria globifera*
JQ067124	*Crotalaria distans*
JX120577	*Crotalaria laburnifolia* subsp. *laburnifolia*
JX120576	*Crotalaria agatiflora* subsp. *imperialis*
JX120584	*Crotalaria pilosa*
JX120580	*Crotalaria ulbrichiana*
JX120579	*Crotalaria laburnifolia* subsp. *australis*
JQ067311	*Crotalaria lotoides*
EU347890	*Crotalaria humilis*
EU347889	*Crotalaria humilis*
EU347888	*Crotalaria capensis*
EU347887	*Crotalaria griquensis*
EU347886	*Crotalaria virgultaris*
EU347885	*Crotalaria lanceolata*
EU347884	*Crotalaria capensis*
EU347883	*Crotalaria* sp.
EU347882	*Crotalaria distans*
AM262454	*Crotalaria lebeckioides*
JQ067344	*Bolusia amboensis*
JQ067345	*Euchlora hirsuta*

Results

Phylogenetic relationships were determined using Bayesian inference and maximum parsimony. The data set included 55 accessions (43 species including two outgroups). Out of the 43 species, 36 represent all infrageneric groups across all the continents (Table 3) while the remaining seven (Table 2) are the collections from the present study area. All the analyses (BI and MP) showed that *Crotalaria* is monophyletic (PP= 1.0, BS =99.3%). In the tree some clades are strongly supported (PP =0.8, BS=99%) while other clades are moderately supported (PP value less than 0.8, BS=80%). The results (Figs. 1 & 2) showed that *C. macrocalyx*, *C. senegalensis* and *C. pallida* belonged to Hediriocarpae clade (PP=1.0, BS=61.69%) while *C. retusa* and *C. goreensis*, *C. atrorubens, C. barkae* belonged to Calycinae (PP=1.0, BS=100%), Stipulosae (PP=1.0, BS=94.36%), Longirostres (PP=1.0, BS=97.04%) and Incane (PP=0.91, BS=96.61%) clades respectively. Our results also showed that *C. retusa*is resolved as sister to southern African *C. papilosa*in Calycinae clade (Figs. 1 & 2). The positions of the studied samples across all the sections are strongly supported (PP 1.0), but relationships between the clades and species relationship differs slightly (Figs. 1 & 2). Relationship between Longirostres and Calycinae is strongly supported with (PP=1.0, BS=93.24%), this shows that two of the studied samples (*Crotalaria retusa* and *Crotalaria atrorubens*) are closely related and the relationship between *Crotalaria retusa* and *C. pilosa* (Figs. 1 & 2) is moderately supported (PP= 0.70, BS 52.9%). Incanae and Hedriocarpae relationship is also well supported (PP=0.99, BS 94.36%). Relationships between *Crotalaria barkae* and *Crotalaria burkeana* in the Incanae section is strongly supported (PP=1.00, BS=98.61%) as well as the relationships between *Crotalaria nataliata*and *Crotalaria goreensis* in the Stipulosae section (PP, 0.63, BS=94.36). The resulting tree from maximum parsimony is similar to that of Bayesian; the bootstrap values for the clades are also strongly supported.

Discussion

All the infrageneric classifications before Le Roux *et al*., (2013) were based on morphology. Polhill (1968) used reproductive characters, Bisby (1970, 1973) employed morphometrics in his classification, later on, Polhill and Bisby combined their findings and published a final system in 1973 (Bisby & Polhill, 1973). Polhill in 1982 while working on African and Madagascan species reported eight sections. Ansari while working on Indian species discussed the positions of Indian species where he modified Polhill, (1982) infrageneric classification system (Ansari, 2006, 2008); his work was also based on morphology and accommodated only Indian species.

Le Roux *et al*., (2013) reported the first global infrageneric relationship of the genus for the first time where they combined morphological and molecular data. They reported 15 sections. This system of classification is the one currently in use. Some of the species from West Africa included in this study were not captured in Le Roux *et al* 2013 infrageneric classification. The resulting phylogenetic tree is congruent with finding of (Le Roux *et al*., 2013) because all the taxa shared many morphological features in the clade, as such the findings supported their system of classification as our sampled species were distributed across Longirostres, Calycinae, Incanae, Hidriocarpae and Stipolosae clades respectively with moderately to strong posterior probability value in Bayesian Inference analysis (PP=8.0 to 1.0, BS=90% to 100%). Our results also support the idea that the splitting of the genus into two subgenera is not possible due to uniformity in floral parts. The finding of this study didn't agree with the two sub sections in Hedriocarpae proposed by (Polhill, 1982) because all the species form a monophyletic large clade in the phylogenetic tree.

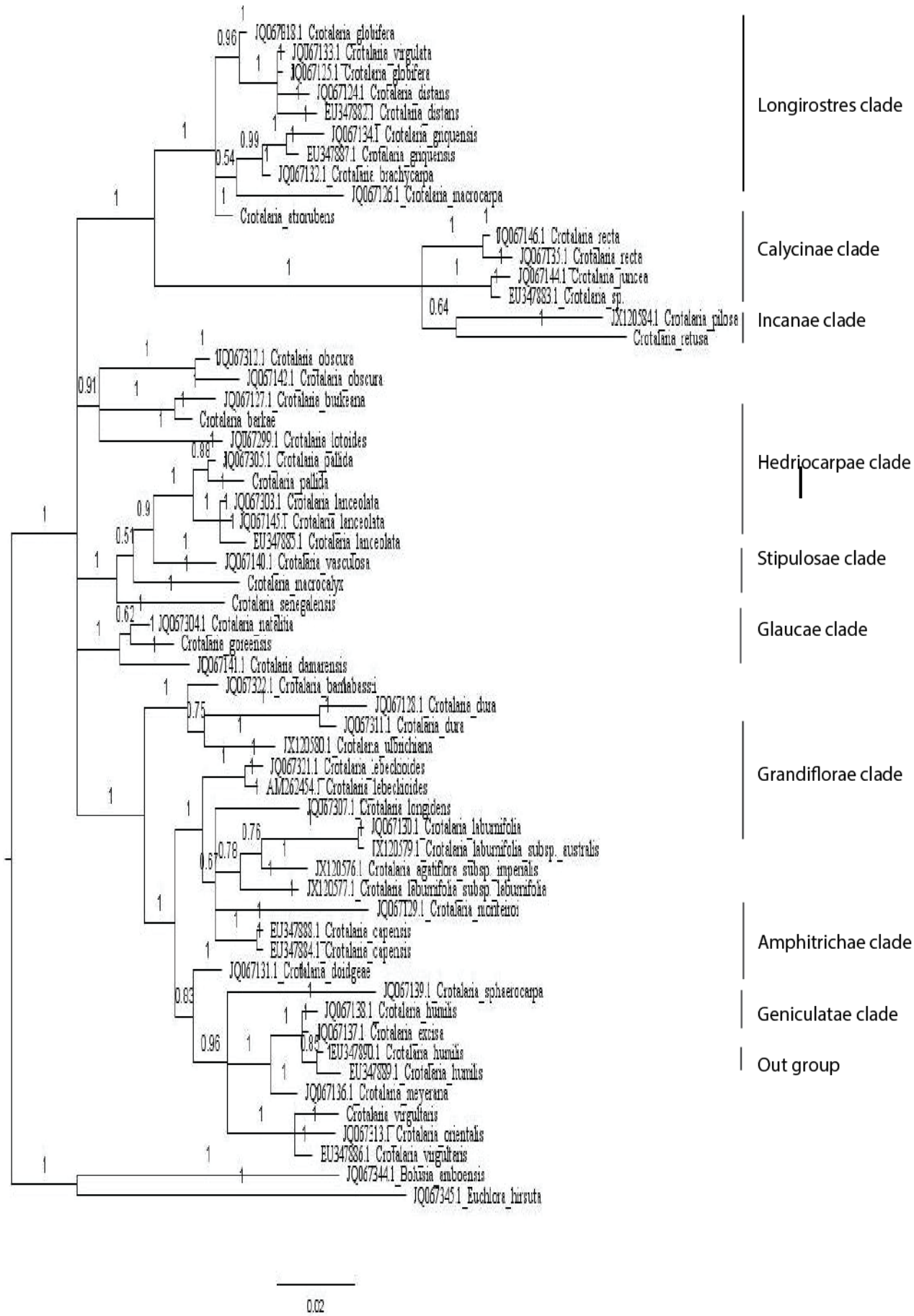

Fig. 1. Phylogenetic tree of *Crotalaria* (43 taxa) based on Bayesian Inference analysis of *ITS* region.

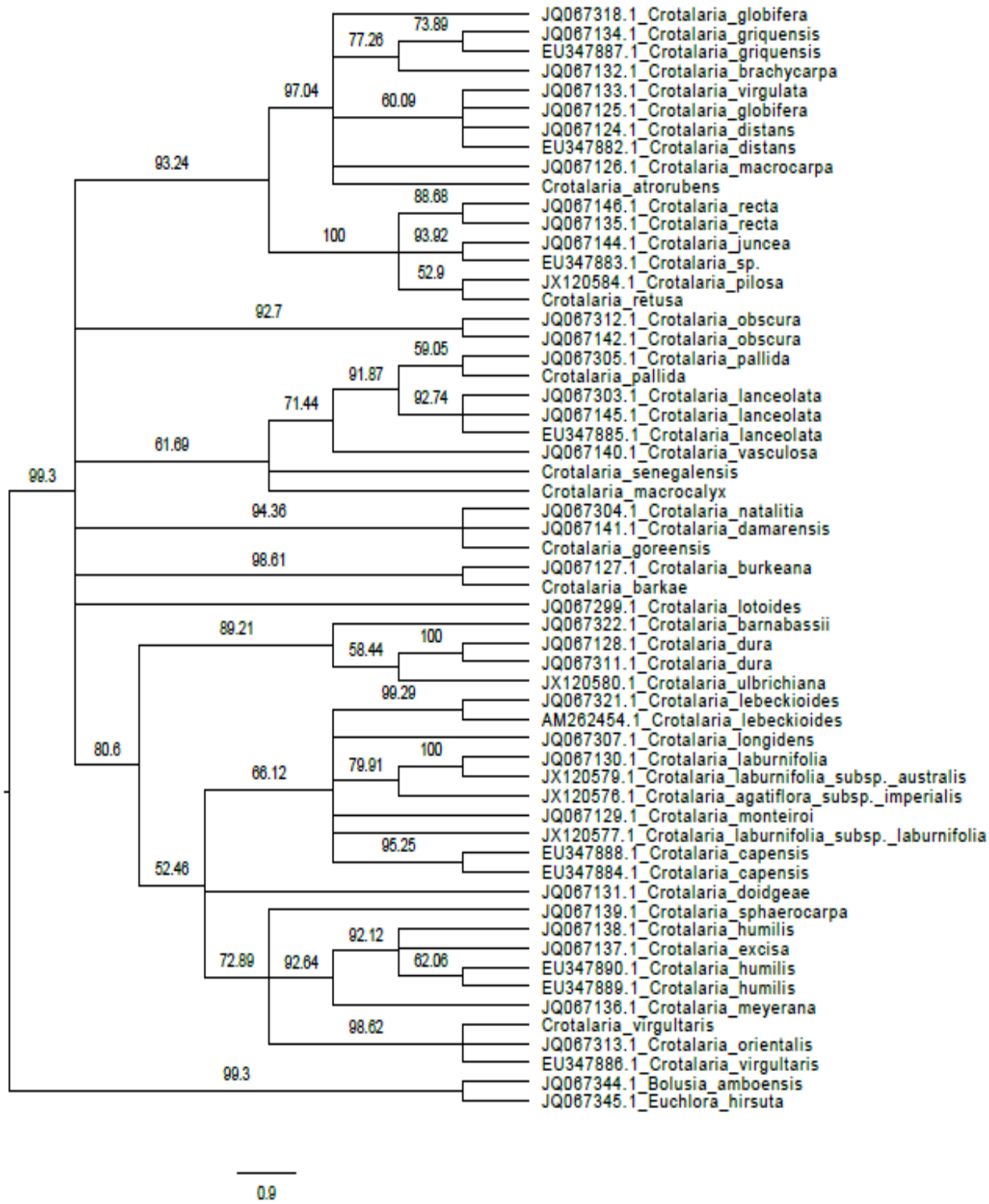

Fig. 2. Phylogenetic relationships of *Crotalaria* (43 taxa) based on Maximum parsimony analysis of *ITS* region.

Description of clades

Longirostres clade: Shrub, annual, leaves 3- foliate; spatulate, leaflet length 30-33 mm, leaflet width 6-8 mm, petiole length 4-6 mm, inflorescence terminal with 7-8 flowers, calyx length 9-11 mm, standard petal length 8-10 mm, standard petal width 8-10 mm, wing petal length 7-9 mm, wing petal width 5mm, fruit length 8-10 mm, pods slightly exceeding calyx, seed number 9-11, seed length 1- 2 mm, pedicel length 2-4 mm, hair present on the stem

Calycinae clade: Shrub, perennial, leaves simple leaf; cuneate, leaflet length 50-52 mm, leaflet width 12-14 mm, length of petiole 3-5 mm, inflorescence terminal with many flowers, calyx length 8-10 mm, standard petal length 12-14 mm, standard petal width s 9-11 mm, wing petal length 12-14 mm, wing petal width 5-7 mm, fruit length 33-35 mm, pods far exceeding calyx, seed number 14-16, seed length 2-4mm, pedicel length 6-8 mm, hair absent.

Incanae clade: Shrub, annual, leaves 3-foliate; Elliptic, leaflet length 30-32 mm, leaflet width 13-15 mm, petiole length 30-32 mm, Inflorescence terminal with 1-3 flower, calyx length 4-6 mm, standard petal length 8-10 mm, standard petal width7-9 mm, wing petal length 6-8 mm, wing petal width 3-5 mm, fruit length16-18 mm, pods size far exceeding than calyx, seed number 10-12, seed length 2-4 mm, pedicel length 3-55 mm, hair present on the stem.

Stipulosae clade: Shrub, annual, leaves 3-foliate; elliptic, leaflet length 40-42 mm, leaflet width 17-19 mm, petiole length 40-42 mm, inflorescence terminal or axial many flower, calyx length 6-8 mm, standard petal length 8-10 mm, standard petal width10-12 mm, wing petal length 12-14 mm, wing petal width 7-9 mm, fruit length 15-17 mm, pods far exceeding calyx, seed number of 10-12, seed length 3-5 mm, pedicel length 3-5 mm, hair present on the stem.

Hedriocarpae clade: Shrub, annual, leaves 3- foliate; Elliptic, leaflet length 40-42 mm, leaflet width 25-27 mm, petiole length 40-42 mm, inflorescence terminal or axial with many flowers, calyx length 4-6 mm, standard petal length 9-11 mm, standard petal width 9-11 mm, wing petal length 9-11 mm, wing petal width 7-11 mm, fruit length 20-22 mm, pods far exceeding calyx, seed number10- 12, seed length 2-4 mm, pedicel length 2-4 mm, hair present on the stem.

Conclusion

This study reported the phylogenetic positions of seven different West African species of *Crotalaria* for the first time. The result supported the monophyly of the genus *Crotalaria* and agreed with the recent infrageneric relationship of the genus. The seven species are distributed in Longirostres clade, Calycinae clade, Incanae clade, Stipulosae clade and Hedriocarpae clade with strong BP and PP support values.

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