Phylogenetic patterns in the tribe Acacieae (Caesalpinioideae: Fabaceae) based on *rbcL*, *matK*, *trnL-F* and ITS sequence data

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Abstract. The tribe Acacieae is one of the three tribes of the distinct mimosoid clade nested within the re-circumscribed sub-family Caesalpinioideae. Many uncertainties exist with the taxonomic status of tribe Acacieae in relation to tribe Ingeae and genus *Acacia*. To unravel the phylogenetic patterns within Acacieae, nine members of the tribe were phylogenetically analysed employing both parsimony and Bayesian methods. Six data matrices (ITS, *rbcL*, *matK*, *trnL-F*, *rbcL+matK+trnL-F* and ITS+*rbcL+matK+trnL-F*) representing 46 sequences, and 2 outgroup taxa were used for the analysis. Our results are in support to some previous studies on the phylogeny of the Acacieae. It supports the polyphyly of tribe Acacieae. The monophyly of *Vachellia, Senegalia* and *Faidherbia* taxa were strongly supported at >70% bootstrap support values and >0.90 bayesian inference. An unresolved basal paraphyletic clade of *Acacia auriculiformis* with the outgroup taxa was shown in all the datasets, at mostly low support values. *Faidherbia albida* was nested within the *Senegalia* grade while *A. auriculiformis* (*Acacia* s.s.) was the closest taxon to the outgroup taxa. A key finding of this study is the polyphyly of *Albizia* and its close association with *A. auriculiformis*.

Keywords: African Acacias, molecular phylogeny, Ingeae, cp DNA, nr DNA

INTRODUCTION

The tribe Acacieae is one of the three tribes of the distinct mimosoid clade (Mimosoideae) nested re-circumscribed within the sub-family Caesalpinioideae (LPWG 2017). Tribe Acacieae was formally grouped with many genera that are today classified in tribe Ingeae Benth (Bentham 1842). Initially, Bentham (1875) restricted the genus Acacia Mill. to the Acacieae as the only member of the tribe. Today, tribe Acacieae is now regarded to comprise just two genera, the very large cosmopolitan genus Acacia s.l. and the monotypic genus Faidherbia A. Chev. (Chappill and Maslin 1995; Miller and Bayer 2000; Miller et al., 2003; Murphy et al., 2010). However, many uncertainties still exist, not only with the status of tribe Acacieae, especially in relation to tribe Ingeae and to a lesser extent tribe Mimoseae, but

also with the definition, classification, and phylogeny of genus *Acacia* (Luckow *et al.*, 2000; Maslin *et al.*, 2003; Miller *et al.*, 2003; Murphy *et al.*, 2010). More recently, Miller and Seigler (2012) noted that the tribal relationships within the mimosoid clade are now in disarray, and new phylogenetic data is imperative for establishing an acceptable tribal and generic system of classification of the Mimosoideae.

Members of tribe Acacieae are generally trees or shrubs, they are distinguished by an indefinite number of stamens (more than 10) that are usually free or united only at the base and valvate calyx (Elias, 1981; Vassal, 1981). Individual flowers are arranged in inflorescences that may be either globular heads or cylindrical spikes. However, these features are not only unique to the tribe. In

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fact. no single morphological character distinguishes Acacieae from other tribes in subfamily Mimosoideae, and this has called into question the monophyly of Acacieae and the other tribes in the Mimosoideae (Chappill and Maslin, 1995). The main character that distinguishes the Acacieae from the Ingeae, are free filaments of the stamens while the Ingeae have united filaments. However, this is not entirely maintained in all taxa, some Acacia species have filaments shortly united at the base (Vassal, 1981; Miller et al., 2003; Murphy et al., 2010). Additionally, numerous stamens and eight polyads per anther are other characters also shared between the two tribes (Chappill and Maslin, 1995). The close relationship of the Ingeae and Acacieae has already been observed (Guinet, 1981; Vassal, 1981) and the conflicting character states have made classification that is solely based on morphological characters difficult.

Furthermore, the relationship of the Faidherbia genus with Acacia is controversial. The genus has shortly united stamens and pollens similar to some taxa of the Ingeae but was placed in the Acacieae (Guinet, 1981). Hence, ensuing some ongoing debate as to whether Faidherbia is better placed in the tribe Ingeae or Acacieae (Elias, 1981; Guinet, 1990; Lewis and Rico-Arce, 2005). The non-monophyly of Acacieae and Ingeae are particularly problematic, with the recognition of monophyletic taxa requiring the generic revision of Acacia s.l. (Miller et al., 2003b). This has contributed to a highly controversial debate about the application of the name Acacia (Pedley, 1986; Maslin et al., 2003a; b; Luckow et al., 2005b).

Albeit, because the tribe Acacieae constitute a large and morphologically heterogenous group, an infrageneric classification based on phylogenetic relationships has become imperative and cardinal for other studies such as: host-parasite coevolution (Crespi *et al.*, 2004; McLeish *et al.*, 2007); plant physiology (Pohlman *et al.*, 2005; Warwick and Thukten, 2006); atmospheric nitrogen fixation in relation to rhizobial interactions (Brockwell *et al.*, 2005); and insects' radiations associated with *Acacia* (Austin *et al.*, 2004) amongst others.

Therefore, the objective of this study is to elucidate on the phylogenetic relationship of the tribe Acacieae in order to test the monophyly of the tribe and establish relationships between its

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two genera in relation to tribe Ingeae. Similarly, the former, broadly circumscribed genus Acacia Mill. is polyphyletic and has been segregated into five genera, Acacia s.s., Acaciella Britton & Rose, Mariosousa Seigler & Ebinger, Senegalia Raf. and Vachellia Wight & Arn (Maslin, 2015). This study also tests the monophyly of the two groups of Acacia s.l. (Senegalia and Vachiellia) that abound in Africa employing molecular systematic techniques and data from three chloroplast regions (rbcL, matK, trnL-F) and the nuclear ITS region.

MATERIALS AND METHODS

Plant material. Thirty-two samples of Acacia species representing six species were randomly collected from the arid and semi-arid region of Nigeria while sequences of two other species (A. karroo & A. tortilis) were downloaded from GenBank. Two outgroup species Albizzia lebbeck and Albizzia zygia were included. These outgroup taxa were selected based on results of previous studies, which indicate members of the Ingeae as sister to Acacia (Brown et al., 2008). Collected samples were identified and authenticated at the University of Lagos Herbarium (LUH). The number, GenBank voucher and other information about samples are given in Appendix 1.

DNA extraction and amplification. Total genomic DNA was isolated from approximately 0.0300 g of silica-gel dried and 0.0180 g of herbarium plant material following a modified 2X CTAB protocol of Doyle and Doyle (1987). Herbarium samples were precipitated for one week while silica dried for 1hr. Extracted DNA was stored at -20°C prior subsequent use. Polymerase chain reaction (PCR) was performed in 50 µl reaction mixtures containing 25 µl biomix, 1 μl BSA, 2 μl DMSO, 1.75 μl of 10 μM of each primer, 17.5 µl of Millipore H2O and 1 µl of 30-50 ng template DNA. The Kim matK primers were used to amplify *matK* region while primers according to Sun et al. (1994), Olmstead et al. (1992) and Taberlet et al. (1991) were used for ITS, rbcL and trnL-F regions respectively. PCR profiles run for each region are given on Table 1. Amplifications were run on a Veriti® 96 well

thermal cycler. Each PCR product was run on 1% agarose gel stained in ethidium bromide and successful amplified products were sent to Source Bioscience (UK) for bidirectional sequencing using the same primer used in PCR.

Phylogenetic analysis. Chromatographic traces and contiguous alignments were edited using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, Michigan). Any uncertain base positions, generally located close to the priming sites, and regions of uncertain alignment were excluded from the phylogenetic analysis. Sequences were aligned and edited in Bioedit (Hall, 1999). Informative insertion/deletion events (indels) were identified and coded as binary characters, and gaps were treated as missing data. All four regions were analyzed separately, a combined data matrix of three chloroplast regions and the combined data matrix of the four regions were also analysed. Less than 1% of the data were scored as missing.

Parsimony analyses were performed on the aligned sequences using heuristic search in PAUP 4b10 (Swofford 2002) with nucleotide substitutions equally weighted and unordered. Heuristic search was used with tree-bisection-and-reconnection and random sequence addition. Bootstrap analysis was also performed to test the robustness of each clade with random addition of sequences 1000 replicates. A Bayesian analysis (Ronquist *et al.*, 2011) was carried out by first

determining the optimal substitution model using MrModeltest v2.3 (Nylander, 2004) and the Akaike information criterion. The general reversible model with a gamma shape (GTR + G)was selected for the trnL-F region; Hasegawa-Kishono-Yano with a proportion of invariable sites (HKY+I) for matK; Hasegawa-Kishono-Yano with a gamma shape and a proportion of invariable sites (HKY+I+G) for rbcL region Hasegawa-Kishono-Yano with a whereas gamma shape (HKY+G) was specified for the nuclear ITS region. Four discrete states were used for the gamma substitution. The data were therefore partitioned into two for the Bayesian analysis and the correct substitution model as specified by MrModeltest was specified for each partition. The partitions were unlinked so that each parameter could be specified separately. Analysis was run for 30,000,000 generations with sampling every 30,000 generations. Metropolis coupling with four chains, one cold and three heated were used with the two independent runs running simultaneously. The runs, however stopped (split standard deviation set at 0.01) after 7705000 generations and 7705 sampled trees in each run. Approximately 24.7% (7, 410, 000) trees were used as burnin in summarizing the parameters and tree. The robustness of each clade was ascertained based on the posterior probability as an inference on the validity of the tree.

Table 1. Amplification promes.								
Region	Initial denaturing	Denaturation	Annealing	Extension	Final extension	No. of		
	Temp./time	Temp./time	Temp./time	Temp./time	Temp./time	cycles		
ITS	97°C/2:00	97°C/1:00	55°C/0:45	72°C/0:45	72°C/7:00	30		
matK	94°C/5:00	94°C/0:40	48°C/0:40	72°C/0:40	72°C/7:00	30		
rbcL	96°C/0:50	96°C/0:50	53°C/0:50	72°C/2:00	72°C/7:00	30		
trnL-F	94°C/2:00	94°C/1:00	55°C/1:00	72°C/2:00	72°C/10:00	30		

Table 1. Amplification profiles

RESULTS

In this present study, eight *Acacia* s.l. and two outgroup (*Albizia*) species were examined for phylogenetic patterns employing 3 chloroplast regions and the nuclear ITS region. A total of 29 taxa were successfully sequenced for *matK* and *trnL-F* regions, 26 taxa for *rbcL* region while 23 taxa for the nuclear ITS region. Results of parsimony analysis and tree description for each examined gene region is summarized on Table 2. The strict consensus tree of all combined chloroplast region (Figure 1) revealed an unresolved basal clade of *A. auriculiformis* with outgroup taxa but at high support value of 99%. It also depicted a closer relationship of *Faidherbia albida* with the outgroup taxa, the monophyly of *Senegalia* and *Vachellia* was also observed. The topology of all the combined data (Figure 2) is congruent with the combined chloroplast regions. An unresolved basal entity and close relationship of A. auriculiformis and F. albida with the outgroup species is revealed. The strict consensus tree of ITS region Figure 3 revealed the base of the tree is well resolved with a paraphyletic F. albida and distinct clades of Senegalia and Vachellia species. There are good bootstrap support values of >70%for each monophyletic group. The strict consensus *matK* gene tree (Appendix 2a) revealed an unresolved paraphyletic clade of outgroup taxa and A. auriculiformis at a low support value of Vachellia, Senegalia and Faidherbia taxa 51.7%. exhibited a monophyletic relationship at high bootstrap value. However, some low support values were recorded. rbcL strict consensus gene tree (Appendix 2b, Supplementary Data) revealed an unresolved paraphyletic base at low support value of 35.11%, A. auriculiformis was clustered with the outgroup taxa whereas Vachellia, Faidherbia and Senegalia taxa were monophyletic. Relationships were mostly depicted at low support values. The last chloroplast *trnL*-F strict consensus gene tree depicted an unresolved basal clade (Appendix 2c,). Other Acacieae species, Senegalia, Vachellia and Faidherbia are monophyletic whereas A. auriculiformis was clustered with the outgroup taxa at low support value of 30.13%.

Table 2. Parsimony profiles a	and tree	description.
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Robustness of most clades were at high bootstrap support values of >70%.

Bayesian analysis showed a higher resolution of species cluster at distinct node with a quite high posterior probability. A similar topology was observed with the parsimony results. Although the trees from the Bayesian analysis are somewhat similar in topology from the parsimony analysis. Bayesian phylogram of the combined chloroplast and all combined dataset (Figures 4 and 5) established a closer association of A. auriculiformis with the outgroup taxa at 0.6 BI and likewise a close relationship of F. albida. ITS gene tree Bayesian analysis (Figure 6) revealed a well resolved basal taxa and a close relationship of F. albida with the outgroup taxa. matK gene tree (Appendix 2d) revealed a monophyletic Senegalia and Vachellia at high bayesian inference of 1.00. However, an unresolved basal entity was observed. Bayesian phylogram of rbcL region (Appendix 2e) showed an unresolved basal relationship of A. auriculiformis and the outgroup taxa at 0.92 BI. However, members of the Vachellia taxa were closer to the outgroup taxa than F. albida. trnL-F bayesian phylogram (Appendix 2f) also revealed an unresolved basal entity at 0.50 BI, and a close relationship of F. albida with the outgroup clade.

Gene region	Total number of characters	Constant characters	Variable characters	Parsimony informative characters	Tree length	Restriction index (RI)	Homoplasy index (HI)	Consistency index (CI)	Rescaled consistency index (RC)
ITS	783	486	59	238	531	0.9301	0.2147	0.7304	0.7304
matK	935	877	20	38	62	0.9895	0.0323	0.9677	0.9576
rbcL	1331	1249	33	49	107	0.9298	0.1963	0.8037	0.7473
trnL-F	1168	1049	41	81	147	0.9571	0.1429	0.8571	0.8204
matK+rbcL+ trnL-F	3434	3172	94	168	340	0.9306	0.2776	0.7224	0.7445
ITS+ <i>matK</i> + rbcL+trnL-F	1427	3658	153	406	871	0.9303	0.2090	0.7910	0.7359



Figure 1. Maximum parsimony phylogram of the original tree (*matK+rbcL+trnL-F*); numbers at node indicates bootstrap support values.



Figure 2. Maximum parsimony phylogram of the original tree (ITS+*matK*+*rbcL*+*trnL*-*F*); numbers at node indicates bootstrap support values.



Figure 3. Maximum parsimony phylogram of the original tree (ITS); numbers at node indicates bootstrap support values.



Figure 4. Phylogram inferred by Bayesian analysis (*matK+rbcL+trnL-F*); numbers at node indicates posterior probability values.



Figure 5. Phylogram inferred by Bayesian analysis (ITS+*matK*+*rbcL*+*trnL*-*F*); numbers at node indicates posterior probability values.



Figure 6. Phylogram inferred by Bayesian analysis (ITS); numbers at node indicates posterior probability values.

DISCUSSION

The phylogenetic pattern of the tribe Acacieae based on three chloroplasts (*matK*, *rbcL*, *trnL-F*) region and the nuclear ITS region was elucidated in order to test the monophyly of the tribe and establish relationships between its two genera in

relation to tribe Ingeae. Combined data matrix of the three chloroplast regions and a combined data matrix of the four regions were also analyzed. Based on both parsimony and bayesian methods, results revealed the polyphyly of tribe Acacieae. An unresolved basal paraphyletic clade of *A*. *auriculiformis* with the outgroup taxa was shown in all the datasets, at mostly low support values. Results support the monophyly of *Vachellia*, *Senegalia* and *Faidherbia* taxa.

Our results are in support to some previous studies on the phylogeny of the Acacieae. The polyphyly of the tribe Acacieae were equally found in some previous studies: Luckow et al. (2000, 2003) based on plastid trnL, trnK intron and matK gene sequences; Miller et al. (2003) based on trnK, matK, psbA-trnH, and trnL-F sequence data. Considerable attention has also been devoted in recent phylogenetic studies to unravel the nonmonophyly of Acacia s.l. Numerous analyses have established clear support for at least five independent lineages scattered widely across the mimosoid clade: Vachellia, Acacia s.str., Acaciella, Senegalia and Mariosousa (Miller and Bayer, 2001, 2003; Luckow et al., 2003; Maslin et al., 2003; Seigler et al., 2006b; Bouchenak-Khelladi et al., 2010; Miller and Seigler, 2012). These studies suggest a non-monophyletic Senegalia. However, based on our dataset, Senegalia is monophyletic. It exhibited a close association with Faidherbia albida.

The affinities of the monotypic genus *Faidherbia* has been controversial. Grimes (1999) found *Faidherbia* as sister to the *Acacia* subg. *Acacia* whereas, Maslin (1995), presented two conflicting relationships of *Faidherbia* nested within either the Ingeae or *Acacia* subg *Aculeiferum* grade. Polhill (1994), Maslin and Stirton (1997), Robinson and Harris (2000), and Maslin *et al.* (2003) classified *F. albida* to be closer to the Ingeae. However, in our analysis *F. albida* is nested within the *Senegalia* grade while *A. auriculiformis* (*Acacia* s.s) was the closest taxon to the outgroup taxa. Results from these analyses support the current classification of *F. albida* within the tribe Acacieae.

In our study, the polyphyly of *Albizia* was observed. This support some previous studies of Grimes (1999), Polhill (1994), Luckow *et al.* (2000, 2003), Sulaiman *et al.* (2003), Lewis *et al.* (2005), and Shinwari *et al.* (2014), which found a lack of resolution and support in the *Acacia* sstr and *Albizia*. Although the genus *Albizia* was selected as the outgroup taxa, the unresolved cluster of the basal outgroup taxa with *Acacia* sstr (*A. auriculiformis*) could likely be attributed to the polyphyly of the *Albizia* (Luckow, 2003). This could probably be as a result of our limited taxon sampling of *Acacia* s.s. A powerful solution will likely be found in a denser species sampling and highly variable character selection (Doyle *et al.,* 1997; Wojciechowski *et al.,* 2004; Lavin *et al.,* 2005). A key finding of this study is the polyphyly of *Albizia* and its close association with *Acacia* s.s. *Acacia.*

In summary, the phylogeny based on both chloroplast and nuclear DNA confirms the polyphyly of Acacieae and paraphyly of tribe results show that Ingeae. Our Ingeae and Acacia s.s. together form an unresolved basal entity. Furthermore, the monophyly of Vachellia and Senegalia taxa at high support values were also indicated. However, the relationship between the Ingeae and Acacia s.s still remain unresolved. This study is a maiden attempt to resolve the intergeneric relationships of tribe Ingeae and Acacia s.s. however, based on traditional generic concepts and recent molecular phylogenetic findings, some large genera e.g. Albizia are potentially polyphyletic. Phylogenetic patterns of additional gene regions should be explored as well more detailed taxon sampling as а of morphologically variable genera.

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