Phylogenetic Relationships of Tribe Crotalarieae (Fabaceae) Inferred from DNA Sequences and Morphology

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Abstract—Tribe Crotalarieae is a large and diverse group of papilionoid legumes that largely occur in Africa. A systematic study of generic relationships within the tribe was undertaken using nucleotide sequences from the internal transcribed spacer (ITS) of nuclear ribosomal DNA, the plastid gene rbcL, and morphological data. The Crotalarieae are supported strongly as monophyletic and sister to the tribe Genistaeae. Lebeckia, Lotononis, and Wiborgia are all paraphyletic in the molecular analyses and morphological data support the division of Lebeckia into three more natural genera (one of which includes the monotypic North African Spartidium). Four major lineages were identified within the tribe based on sequence data: the “Cape” group, comprising Aspalathus, Lebeckia, Rafnia, Spartanium, and Wiborgia; the Lotononis group, comprising Lotononis pro parte, Pearsonia, Robynsiophyton, and Rothia; a group comprising Lotononis section Lephis, L. section Listia, and allies; and the Crotalaria genus, comprising Bolusia, Crotalaria, and Lotononis hirsuta (Lotononis section Eucrotonis). Morphological analysis yields a similar topology, except that Lotononis is monophyletic if L. hirsuta were excluded. When the molecular and morphological data sets are combined, the same major clades are retrieved as in the molecular analysis, with the notable exception that Lotononis and Lebeckia senso stricto are supported as monophyletic. The results from this study have important implications for the classification of the tribe Crotalarieae and present an important step towards a natural and phylogenetic generic classification for the tribe.

Keywords—Crotalarieae, Fabaceae, genistoid legumes, ITS, morphology, phylogeny, rbcL.

Crotalarieae (Benth.) Hutch. is a tribe of legumes that currently comprises 11 genera and ca. 1204 species (van Wyk 2005). It represents the largest tribe of papilionoid legumes in Africa and also within the genistoid alliance, comprising about 51% of genistoid legumes (species totals in Lewis et al. 2005 used for calculation), largely due to the fact that the genus Crotalaria contains ca. 600 species (Polhill 1982). Some of the species in the tribe are important commercially such as Aspalathus linearis, which is used for the production of Rooibos tea (van Wyk et al. 1997), and Lotononis bainesii Bak., an important fodder plant (Bryan 1961). Some Crotalaria and Lotononis species have been reported to have medicinal properties (van Wyk 2005) and a few are used as what is called ’Musa-pelo’ in Lesotho traditional medicine to cure or ease a broken heart (Moteetee and van Wyk 2007), while others from the same genera are poisonous (van Wyk et al. 2002).

The Crotalarieae are subendemic to Africa, with only a few species of Crotalaria, Lotononis, and Rothia occurring on other continents. Aspalathus, Rafnia, and Wiborgia are endemic to the Cape Floristic Region, while Lebeckia (as currently circumscribed) is distributed throughout the Cape and extends into the south-western and central parts of Namibia. Generic circumscriptions and relationships within the tribes are known to be complicated, with evidence of reticulation and convergence (Dahlgren 1970a; Polhill 1976, 1981; van Wyk 1991a). The most recent evaluations of generic relationships in the tribe are those of Polhill (1976, 1981), van Wyk (1991a), and van Wyk and Schutte (1989, 1995). According to van Wyk (1991a), two main groups can be identified within the tribe, namely the “Cape” group comprising Aspalathus, Lebeckia, Rafnia, and Wiborgia, and the Lotononis group comprising Lotononis, Pearsonia, Robynsiophyton, and Rothia. The placement of Bolusia, Crotalaria, and Spartidium within these two groups is not clear, but a close relationship between the former two genera has been suggested. Recent revisionary studies of Lebeckia (the first since Harvey’s treatment of 1862) have shown that the genus is unlikely to be monophyletic (Le Roux 2006; Le Roux and van Wyk 2007; Boatwright, in prep.).

Several molecular systematic studies of Fabaceae and specifically the genistoid legumes (sensu Polhill 1976, 1981) have been conducted in recent years (Käss and Wink 1995, 1996, 1997; Crisp et al. 2000; Doyle et al. 2000; Kajita et al. 2001; Wink and Mohamed 2003; Wojciechowski et al. 2004; Boatwright et al. 2008). These studies place Crotalarieae within the “core” genistoid clade together with, among others, the South African tribes Podalyrieae Benteth. and Genistaeae (Bonn) Dumort., and confirm the exclusion of the Argyrolobium group (Argyrolobium, Dichilus, Melobolobium, Polhilia) and the rest of the Genistea from Crotalarieae (van Wyk and Schutte 1995). A sister relationship between Crotalarieae and Genistaeae, with Podalyrieae successively sister to these, is shown by the molecular studies cited above, but sampling limitations did not allow detailed evaluations at the generic level.

The present study is aimed at exploring generic circumscriptions and relationships within Crotalarieae, using nrDNA ITS sequences, plastid rbcL sequences, and morphological data, based on a sample of 135 species representing all of the 11 genera currently recognized, as well as major infrageneric groups within some of the genera. The aim was to assess the monophyly and relationships of the individual genera.

Materials and Methods

Plant Accessions and Choice of Outgroups—A total of 175 sequences of ITS (88% of the total ITS matrix) and 207 sequences for rbcL (91% of the total rbcL matrix) were produced from taxa of the Crotalarieae and combined with previously published sequences from the tribes Genistaeae and Podalyrieae as outgroups (van Wyk and Schutte 1995; Crisp et al. 2000; Boatwright et al. 2008). Sequences of both the ITS and rbcL regions were available for 161 taxa of the Crotalarieae and these were used for the combined molecular analysis. A morphological matrix was compiled for those species included in the combined molecular analysis, based on 31 characters (including a few chemical and cytological characters) that were
scored from examining specimens from BM, BOL, GRA, JRAU, K, NBG (including SAM and STE), P, PRE, S, UPS, and WIND for *Lebeckia*, *Robynsiophyton*, *Rothia*, and *Spartidium* and from literature for the other genera (Polhill 1974, 1976, 1982; Dahlgren 1975, 1988; Schutte and van Wyk 1988; van Wyk 1991b; van Wyk and Schutte 1995; Campbell and van Wyk 2001; van Wyk 2003). These data, along with sequences of the same species, were used to perform a combined molecular/morphological analysis.

DNA Extraction, Amplification, and Sequencing—Sequencing of the gene regions was carried out in two independent laboratories, therefore the strategies differed slightly. The gene sequencing methods of Kass and Wink (1997) were followed at University of Heidelberg, whereas sequences generated at the University of Johannesburg were gathered according to the methods described above. DNA was extracted from silica-dried or herbarium leaf material using the CTAB (cationic tetrahexylammonium chloride) method of Doyle and Doyle (1987) and purified through QIAquick silica columns (Qiagen Inc., Hilden, Germany). Sources of material used in the study are listed in Appendix 1. The internal transcribed spacers (ITS) of nuclear rDNA were amplified using the primers of White et al. (1990) and Sun et al. (1994), while for *rbcL* those of Fay et al. (1997) were used. Amplification reactions were carried out using polymerase chain reactions (PCR), in 25 μl reactions containing; 22.5 μl Abgene 1.1x PCR Mastermix (Abgene House, Billingham Road, Epsom, Surrey, KT19 9AP, U.K.), consisting of 1.25 units Thermoprobe Plus DNA Polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% (v/v) Tween® 20, 0.2 mM each of deoxyribonucleotide triphosphates (dNTPs); 0.5 μl of both forward and reverse primers (0.1 ng/μl); 0.8 μl 0.004% bovine serum albumin (BSA); 1.2% dimethyl sulfoxide (DMSO; for ITS only); 20-50 ng DNA template; and sterile distilled water to make up a final volume of 25 μl. The DMSO was added to the amplification reactions as this may improve sequencing of ITS due to relaxation of the template secondary structure during amplification (Álvarez and Wendel 2003). The PCR cycles and cycle sequencing reactions followed Boatwright et al. (2008).

**Sequence Alignment and Phylogenetic Analyses**—Complementary strands of the sequenced genes were edited in Sequencher v. 3.1.2 (Gene Codes Corporation), and aligned manually in PAUP* version 4.0b10 (Swofford 2002) and using the programs listed in the materials and methods section. Insertions and deletions (indels) of nucleotides were scored as missing data and thus did not contribute to the combined analysis, but an additional search with gaps from the ITS data set coded as binary characters was performed (no gaps were present in the *rbcL* dataset). Gaps were coded in SeqState version 1.32 (Müller 2005) using simple indel coding as described by Simmons and Ochoterena (2000). Maximum parsimony analyses (MP; Fitch 1971) and Bayesian MCMC analysis (BI; Yang and Rannala 1997; Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; MRBAYES version 3.1.2) were performed as described in Boatwright et al. (2008). Internal support for MP was estimated with 1,000 bootstrap replicates (Felsenstein 1985) using tree-bisection-reconnection (TBR) and holding 10 trees per replicate. The following scale for bootstrap support percentages (BP) was used: 50–74%, low; 75–84%, moderate; 85–100%, strong. Congruence of the separate datasets was assessed by examining the individual bootstrap consensus trees in order to compile a combined ITS and *rbcL* matrix for the 161 taxa where both sequenced regions were available. The bootstrap trees were considered incongruent only if they displayed ‘hard’ (i.e., strong bootstrap support) rather than ‘soft’ (i.e., low bootstrap support) incongruence (Seelan et al. 1997; Wiens 1998). In addition, incongruence length difference tests (ILD, Farris et al. 1995) were performed using the partition-homogeneity test of PAUP*. The test was implemented with 1,000 replicate analyses, using the heuristic search option with simple addition of taxa, and the MULTREES option on in effect.

The GTR + I + G model [selected by MODELTEST v. 3.06 (Posada and Crandall 1998) using the corrected AKAIE information criterion (AICc)] was implemented for the BI analysis and a total of three million generations were performed with a sampling frequency of 10. A majority rule consensus tree was produced to determine the posterior probabilities (PP) of all observed bipartitions (only PPs above 0.5 are reported on the tree). The following scale was used to evaluate the PPs: 0.50–0.84, moderate; 0.85–1.0, strong.

Despite repeated attempts, we were only able to amplify ITS1 for some samples of *Lotononis marlothii*, *L. pentaphylla*, *L. platycarpa*, and *L. rostrata*. We could also amplify only the first half of *rbcL* for some samples of *Aspalathus lacticifolia* subsp. *lacticifolia*, *A. shawii* subsp. *shawii*, *Lotononis fulgens*, and *Spartidium saharae*. Rhamnosiphonium shotgun, *Rothia hisruta*, *Spartidium saharae*, and *Wiborgia humilis*. Missing data represented 4.3% of the entire combined molecular matrix due to the fact that both ITS and *rbcL* sequences were not available for some of the Genistaeae included as outgroups.

**Morphological Analysis**—Much debate currently surrounds the inclusion of morphological characters in phylogenetic analyses (e.g., Scotland et al. 2003). However, Wiens (2004) discusses the importance of morphological characters in phylogenetics, suggesting that their implementation in separate and combined analyses may provide more rigorous phylogenies, while improved resolution has been shown by Wortley and Scotland (2006) when morphology is combined with molecular data. Characters and character states used for the cladistic analysis are given in Appendix 2. The character states were polarised using the method of outgroup comparison. Phylogenetic analyses were performed in PAUP* (Swofford 2002) with the characters treated as unordered and equally weighted (Fitch 1971). A heuristic search with 1,000 random addition sequences, TBR, and the MULTREES option off was performed with a limit of 10 trees held per replicate. Internal support was assessed using 1,000 bootstrap replicates (Felsenstein 1985) as described above. *Dichlas gracilis*, a suffrutex, was selected as the outgroup for the analysis. A second analysis was performed where *Genista tinctoria* L., a shrub, was used as outgroup to test whether polarising the shrubby habit as ancestral had an effect on the topology, but no difference was found in this analysis (tree not shown).

**RESULTS**

**rbcL Data Set**—The *rbcL* matrix consisted of 1,296 aligned positions with 222 variable and 167 parsimony informative characters. Tree searches produced 2,650 equally parsimonious trees of 527 steps, a consistency index (CI) of 0.50, and a retention index (RI) of 0.92. Overall the strict consensus tree (not shown) is poorly resolved with a few supported clades. A clade consisting of *Lebeckia section Calobota* (Eckl. & Zeyh.) Benth., *Lotonia* *Stiza* (E.Mey.) Benth., and *Spartidium saharae* is weakly supported (60 BP). *Lotononis s.s.* (*L. section Lotoninon* and allies) is moderately supported (74 BP), while the rest of the genus is unresolved except for *L. section Listia* (E.Mey.) B.-E.van Wyk, which has high support (89 BP). The *Crotalaria* clade is strongly supported (88 BP) and the sister relationship between *Bolusia* and *Crotalaria* is also strongly supported with a BP of 90. Both these genera are well-supported to be monophyletic (*Bolusia* 98 BP; *Crotalaria* 90 BP) and they are successively sister to *Lotononis hisruta* [*L. section Euchlora* (Eckl. & Zeyh.) B.-E.van Wyk]. Tribe Crotalarieae as a whole is strongly supported as monophyletic (94 BP) and sister to the Genistaeae (100 BP). Genistaeae and Podalyrieae are both supported as monophyletic (89 BP and 78 BP, respectively).

**ITS Data Set**—The analysis of the ITS data set included 560 characters, of which 353 were variable and 256 parsimony informative. Analysis resulted in 5,970 equally parsimonious trees with a tree length (TL) of 1,031 steps, a CI of 0.51, and a RI of 0.85. The analysis in which gaps were coded resulted in identical clad resolution as the analysis without coded gaps, although some clades had stronger or weaker support (not shown). The analysis with coded gaps resulted in 2,540 trees (TL = 1,340; CI = 0.51; RI = 0.84). The strict consensus tree based on ITS data (not shown) is better resolved than that of *rbcL*, with several well-supported clades. *Aspalathus* was weakly supported as monophyletic (64 BP), although this support percentage is higher when the gap characters are included (78 BP). *Lebeckia* and *Wiborgia* are both paraphyletic. *Lebeckia section Viborgioides* Benth. including *Wiborgia humilis* is moderately supported (80 BP), while the positions of *L. inflata* and *L. mucronata* are unresolved. The remaining species of *Wiborgia* form a weakly supported clade (61 BP; 79 BP with gap coding). *Rafinia* is weakly supported as monophyletic (75 BP) and *Lebeckia section Calobota* along with *L. section Stiza* and *Spartidium saharae* also has weak support (59 BP).
Lebeckia section Lebeckia is unresolved. Lotononis is left unresolved and L. hirsuta is well separated from the rest of the genus. Three main clades can be distinguished. The first consists of Lotononis s.s. (also supported in the rbcL tree) with moderate support (84 BP). The second clade, comprising taxa from Lotononis section Leptis (E.Mey. ex Eckl. & Zeyh.) Benth., L. section Listia, and their allies also has low support (64 BP). The third clade of Lotononis groups with Bolusia and Crotalaria with strong support (87 BP) and comprises one anomalous species, L. hirsuta, which makes up the monotypic section Euchlora. Crotalaria and Bolusia are weakly supported as sister taxa (64 BP) and Crotalaria is strongly supported to be monophyletic (99 BP). Pearsonia, Robynsiophyton, and Rothia form a strongly supported clade (98 BP) and the latter two are strongly supported to be sister genera (97 BP). The taxa of this clade all possess a 17 base-pair deletion at positions 179–196 in the aligned ITS matrix. The Crotalariaeae are strongly supported to be monophyletic (81 BP) and sister to Genisteae (100 BP).

Combined ITS/rbcL Data Set—Visual inspection of the bootstrap consensus trees resulting from separate analyses of ITS and rbcL sequences presented no strongly supported incongruent patterns. The ILD test indicated significant difference between the two datasets (p=0.001). Following the suggestions of Seelanan et al. (1997) and Wiens (1998), together with suggestions that the ILD test may be unreliable (Reeves et al. 2001; Yoder et al. 2001) these datasets were combined directly. The combined ITS and rbcL matrix consisted of 1,854 included positions, of which 540 were variable and 404 parsimony informative. The MP analysis produced 560 equally parsimonious trees (Fig. 1; TL = 1,473; CI = 0.50; RI = 0.86). 

In both the MP and BI analyses, the same major clades...
could be observed: the “Cape” group consisting of Aspalathus, Lebeckia, Rafnia, Spartidium, and Wiborgia (53 BP; PP 1.0); the Lotononis group consisting of Lotononis s.s., Pearsonia, Robynsiophyton, and Rothia (PP 0.94); a second Lotononis clade consisting of Lotononis section Leptis, L. section Listia, and allies (77 BP; PP 1.0); and the Crotalaria group consisting of Bolusia, Crotalaria, and Lotononis hirsuta/L. section Euclora (99 BP; PP 1.0). Lebeckia, Lotononis, and Wiborgia are all paraplyletic and Lebeckia section Lebeckia is unresolved in both the analyses.

Generic relationships are unresolved within the “Cape” group and represented as a polynomy at the base of this clade in the bootstrap consensus tree of the parsimony analysis (not shown). In the BI tree, Aspalathus (88 BP; PP 1.0) and Wiborgia (70 BP; PP 1.0) are sister to each other, with L. mucronata (PP 0.96) and Lebeckia inflata included in this clade. These positions are unresolved in the MP analysis. Lebeckia section Calobota (including L. section Stiza and Spartidium saharae; 93 BP; PP 1.0) is sister to Aspalathus and Wiborgia, followed by L. section Viborgioides (including Wiborgia humilis; 64 BP; PP 1.0) and Rafnia (94 BP; PP 1.0). Lebeckia section Lebeckia is unresolved in both the MP and BI trees.

Lotononis is paraphyletic in both the MP and BI trees. The Lotononis group is moderately supported in the BI tree (PP 0.94) and consists of those species of Lotononis from the sections Aulacinthus (E.Mey.) Benth., Buchenroderia (Eckl. & Zeyh.) B.-E. van Wyk, Cleistogamia B.-E. van Wyk, Kresbia (Eckl. & Zeyh.) Benth., Lotononis, Monocarpa B.-E. van Wyk, Oxydium Benth., and Polylobium (Eckl. & Zeyh.) Benth. (100 BP; PP 1.0). These are sister to a strongly supported clade (BP 100; PP 1.0) consisting of Pearsonia (98 BP; PP 1.0), Robynsiophyton, and Rothia. The sister relationship of the latter two genera has high bootstrap support (97%) and PP (1.0).

The next clade consists of species of Lotononis section Listia (100 BP; PP 1.0) and sections Digitata B.-E. van Wyk, Leboroea (Del.) Benth., Leptis, Lipozygis (E.Mey.) Benth., and Synclistis B.-E. van Wyk (99 BP; PP 1.0), and these are moderately to strongly supported as sister (77 BP; 1.0 PP).

The Crotalaria clade consists of Bolusia and Crotalaria (100 BP; 1.0), strongly supported as sister groups (92 BP; PP 1.0), and Lotononis hirsuta (corresponding to Lotononis section Euclora). This clade receives high support in both the MP and BI analyses (99 BP; PP 1.0).

The Crotalarieae are strongly supported as being monophyletic (97 BP; PP 1.0) and sister to Genisteae (100 BP; PP 1.0). The monophyly of the latter tribe receives low to strong support (65 BP; PP 1.0). The Podalyrieae s.s. are strongly supported as monophyletic (93 BP; PP 1.0) and the relationships within the tribe conform to those of previous studies (Boatwright et al. 2008).

Morphological Analysis—The morphological matrix included 31 polarised characters, 29 of which were parsimony informative. Parsimony analysis yielded 261 trees (not shown) of 101 steps (CI = 0.36, RI = 0.93). In the strict consensus tree (not shown), Aspalathus and Rafnia group together, albeit without support. Aspalathus (excluding A. crenata and A. perfoliata) and Rafnia are weakly supported as monophyletic (55 BP and 71 BP, respectively). Lebeckia section Viborgioides, L. mucronata, and Wiborgia s.s. group together, while L. inflata groups with Lebeckia section Lebeckia, but without support. Only Wiborgia is weakly supported as monophyletic (78 BP). Lebeckia section Lebeckia is moderately supported as monophyletic (84 BP), but there is no resolution within this group. Lotononis hirsuta is not included in Lotononis, but groups with Crotalaria (63 BP) and Bolusia as in the molecular analysis. The rest of Lotononis is monophyletic (56 BP) in the morphological analysis as opposed to paraphyletic in the combined molecular analysis, but relationships within the genus are largely unresolved. Pearsonia, Rothia, and Robynsiophyton form a strongly supported clade (87 BP) and a sister relationship between Robynsiophyton and Rothia is strongly supported (88 BP).

Combined ITS/rbcL/Morphological Data Set—The bootstrap consensus trees from the combined analysis of ITS and rbcL and the morphological analysis showed no strongly supported incongruent patterns, although an ILD test indicated significant difference between the data sets (\(p=0.002\)). The suggestions of Seelanan et al. (1997) and Wiens (1998) were followed and the data sets combined directly. The combined matrix consisted of 1,885 characters, 1,405 of which were constant, 480 variable, and 303 parsimony informative. The MP analysis resulted in 370 trees (TL = 1,166, CI = 0.53, RI = 0.84; Fig. 2).

The trees resulting from this analysis are similar to those from the combined molecular analysis, except that Lotononis and Lebeckia section Lebeckia are monophyletic. The “Cape” group (73 BP), Lotononis group (including Pearsonia, Robynsiophyton, and Rothia), and Crotalaria group (100 BP) found in the molecular analysis were also retrieved in the combined data set of molecular plus morphological characters. Within the “Cape” group, Aspalathus, Lebeckia section Calobota (including L. section Stiza and Spartidium saharae), L. section Lebeckia, L. section Viborgioides (including Wiborgia humilis), Rafnia, and Wiborgia (excluding Wiborgia humilis) received moderate to strong support as monophyletic (99 BP; 95 BP; 77 BP; 87 BP; 98 BP; 94 BP, respectively). Lotononis is monophyletic (75 BP; excluding L. hirsuta) and the groups retrieved within the genus are identical to the separate clades found in the molecular analysis, namely Lotononis s.s. (100 BP). Lotononis section Listia and allies (100 BP), and Lotononis section Listia (100 BP). The sister relationship between the latter two is moderately supported with a BP of 88. Pearsonia is strongly supported as monophyletic (99 BP) and sister to Robynsiophyton and Rothia (100 BP). The sister relationship between Robynsiophyton and Rothia is strongly supported (99 BP). The Crotalaria group is strongly supported (100 BP) and consists of Bolusia, Crotalaria and Lotononis hirsuta. Crotalaria is strongly supported to be monophyletic (100 BP) and is sister to Bolusia (94 BP).

Discussion—The phylogenetic hypotheses presented in this study are based on a complete sample of Crotalarieae at the generic level and a representative sample of most of the taxonomic and morphological variation within these genera. The data were based not only on DNA sequences but also on salient morphological characters that were carefully polarised and proved to be informative at the generic level. The low resolution within the Crotalarieae based on morphology and some chemical and cytological characters reflects the somewhat reticulate relationships within the tribe (also mentioned by van Wyk 1991a; van Wyk 2005) with remarkable examples of parallelism, convergence, and analogy with regard to vegetative and reproductive morphology as outlined by Dahlgren (1970a). The presence of
seemingly identical apomorphic states in unrelated genera (e.g., hairy petals in *Lebeckia* section *Calobota* and *Lotononis* section *Leptis*) complicates the cladistic analysis. In the absence of single apomorphies with diagnostic value, natural groups can only be delimited using combinations of characters. Our results (both molecular and morphological) support some of the current generic concepts within the tribe, but provide novel insights into as yet phylogenetically unstudied groups such as *Lebeckia* and the smaller genera of the Crotalarieae, namely *Robynsiophyton*, *Rothia*, and *Spartidium*.

Previous studies on relationships within the Crotalarieae have all suggested a close relationship between the “Cape” genera (viz., *Aspalathus*, *Lebeckia*, *Rafnia*, and *Wiborgia*), as well as the genera that have a zygomorphic calyx (viz., *Lotononis*, *Pearsonia*, *Robynsiophyton*, and *Rothia*; Dahlgren 1963, 1967, 1970a; Polhill 1981; van Wyk 1991a). The placement of *Bolusia*, *Crotalaria*, and *Spartidium* has been uncertain. A sister relationship between the former two genera was suggested by Polhill (1976) and van Wyk (1991a), while *Spartidium* was thought to either be closely allied to *Genisteae* or to *Lebeckia*, but the affinities remained unclear (Polhill 1976). The results of the current study indicate four major lineages within the Crotalarieae comprising the “Cape” group (*Aspalathus*, *Lebeckia*, *Rafnia*, *Wiborgia*, including *Spartidium saharae*), two lineages comprising genera with zygomorphic calyces (*Lotononis*, *Pearsonia*, *Robynsiophyton* and *Rothia*), plus *Bolusia*, *Crotalaria*, and *Lotononis* section *Euchlora*.

The “Cape” Group—The lack of resolution between genera in this group is caused by the low sequence divergence among these taxa. *Aspalathus* and *Rafnia* are both strongly supported as monophyletic and indicated as sister taxa by the morphological data because they share sessile leaves and an asymmetrical upper suture of the pod (Campbell and van Wyk 2001). *Aspalathus* is placed closer to *Wiborgia* by both the MP and BI analyses. Although the sample of *Aspalathus* is not at all comprehensive, some clades of interest can be noted.
Aspalathus linearis and A. pendula both have simple, terete leaves and this clade corresponds to the Lebeckia clade (Dahlgren 1988), named for its similarity to Lebeckia section Lebeckia. Aspalathus loricifolia, A. hirta, A. hystrich, and A. shauni all have spine-tipped leaves (corresponding to the Laterales group of Dahlgren 1988) and form a clade, which is well-supported in the BI tree. The Borboniae group (Dahlgren 1988) represented by A. perfoliata and A. crenata form a weak to strongly supported clade. Within Rafnia, two clades can be identified that correspond to the two sections of the genus described by Campbell and van Wyk (2001): the R. acuminata clade representing R. section Rafnia, and the R. spicata clade representing R. section Colobotropsis E.Mey. These two sections differ mainly in the rostrate keel petals and unequal calyx lobes of R. section Colobotropsis.

It is clear from both the MP and BI analyses that Lebeckia and Wiborgia are not monophyletic. Lebeckia is represented by several smaller clades within the “Cape” group, while Wiborgia humilis is nested within the Lebeckia leipoldtiana clade and well-separated from the main Wiborgia clade.

The species of Lebeckia section Calobota form a well-supported clade with those of L. section Stiza and the monotypic genus Spartidium. These species are all shrubs with trifoliolate or unifoliolate leaves and green mature stems. The petals are usually pubescent and they possess a 5 + 4 + 1 anther configuration (five short dorsifixed anthers, four long basifixed anthers, and an intermediate carinal anther). Some of these character states also occur in Lotononis species and are thus not informative in the morphological analysis. The species of L. section Stiza and L. section Calobota occur in the Cape and those of the latter also extend northwards into Namibia. Spartidium sahareae is a North African plant that has had uncertain affinities within the tribe. The close relationship of this species to L. section Calobota has also been demonstrated independently by Edwards and Hawkins (2007) based on ITS data.

Wiborgia and species of Lebeckia section Viborgioides are superficially very similar in their rigid, woody habit, glabrous petals and highly reflexed standard petals in most species. However, they differ markedly in the winged, few-seeded samaras that are typical of Wiborgia species (Dahlgren 1975). However, Wiborgia humilis has thin-walled, inflated pods that lack the dorsal wing characteristic of Wiborgia (Dahlgren 1970b). The fruits of Lebeckia section Viborgioides are typically turgid, thin-walled, many-seeded, and wingless. Wiborgia humilis is the only species of Wiborgia with a 6 + 4 anther arrangement (6 short dorsifixed and 4 long basifixed anthers) instead of the 5 + 4 + 1 arrangement found in all other species. These characters therefore support the placement of W. humilis in the L. section Viborgioides clade.

Lebeckia section Lebeckia is unresolved in the BI and MP analyses. This section is morphologically very distinct from the other sections in the genus and its monophyly is supported by four apomorphies, namely the suffrutescent habit, acicular leaves, a 5 + 5 anther arrangement, and rugose seeds (Le Roux 2006; Le Roux and van Wyk 2007). This is clear from the moderate support this group received in the morphological analysis as well as the combined morphological and molecular analysis. Lebeckia lotonoides and L. inflata are the only other species of Lebeckia with rugose seeds, but these species share characters with L. section Calobota and L. section Viborgioides, respectively (Boatwright and van Wyk 2007; Boatwright, pers. obs.).

The positions of both L. inflata and L. mucronata are unresolved in the combined ITS and rbcl analysis. Lebeckia mucronata is currently placed within L. section Calobota. Lebeckia inflata was described by Bolus (1887) without any reference to its position in the infrageneric systems of Bentham (1844) and Harvey (1862). Both of these species, however, share several anatomical and morphological characters with species of L. section Viborgioides, such as a 6 + 4 anther arrangement, glabrous petals, and the dorsiventral leaves with mucilage cells. When morphological characters were combined with the molecular ITS and rbcl data, L. inflata was included in a clade along with L. section Viborgioides and Wiborgia humilis, while the position of L. mucronata remained unresolved.

The Lotononis Clades—Based on the molecular data, Lotononis is paraphyletic and consists of three clades. Lotononis s.s. groups with the Pearsonia clade (albeit without support in the MP analysis) and is strongly supported as monophyletic. Two groups were noted within this clade. The first consists of representatives of L. section Oxydium and the second of representatives of L. sections Aulacanthus, Buchenroedera, Cleistogama, Kresbia, Lotononis, Monocarpa, and Polylobium. Lotononis section Oxydium is strongly supported to be monophyletic in the molecular analyses and currently consists of 35 species (accommodated in 14 subsections as described by van Wyk 1991b) distinguished from other sections by several characters, including single stipules at each leaf base and glabrous wing and keel petals. In the second group, L. section Cleistogama is sister to L. section Monocarpa, while L. sections Aulacanthus, Lotononis, and Polylobium form a clade without support. The resolution in the rest of the group is low and relationships not clear. In the molecular analysis, Lotononis s.s. and the Pearsonia clade clearly share similarities in their sequences but morphologically Lotononis s.s. allies with the rest of the genus to form a monophyletic assemblage. From these results it is clear that some generic apomorphies support the current concept of the genus. When the molecular data were combined with the morphology, Lotononis remained monophyletic, although with moderate support. It is interesting to note the close agreement between the sectional classification proposed by van Wyk (1991b) and the results of this study. Pearsonia, Robynsiophyton, and Rothia share several morphological characters that are unique among the genera of Crotalarieae, such as the uniform anthers, straight styles, and presence of angulate esters of hydroxylpanine. The generic concepts of Robynsiophyton and Rothia, based mainly on anther characters, have been doubtful and Robynsiophyton was thought to be a local derivative of Pearsonia (van Wyk 1991a). Taxonomic studies of the former two genera and a thorough examination of their morphology and anatomy revealed that these generic concepts are indeed sound and emphasizes the value of androecial characters in the Crotalarieae (Boatwright and van Wyk, unpubl.; Boatwright et al., unpubl.).

The second clade comprises those species from Lotononis section Leptis, Lotononis section Listia, and allies. This clade is moderately to strongly supported in the molecular analysis but lacks resolution in the morphological analysis. The concept of the hitherto monotypic Listia was broadened by van Wyk (1991b) and treated as a section of Lotononis with several distinct characters. This broadened concept is supported in the analyses presented in this study. The absence of suitable material of the rare Lotononis macrocarpa Eckl. & Zeyh. (an anomalous species within this group) prevented its inclusion.
in this study. Special efforts should be made to obtain DNA from this species in order to evaluate its placement within L. section Listia. van Wyk (1991b) mentions that no apomorphies exist for L. section Leptis, which is clear from the morphological analysis where this section is left unresolved. The molecular results, however, indicate a close relationship between this section and L. sections Digitata, Leboreda, Lipozygis, and Synclistis, all of which are successively sister to L. section Listia. The third clade comprises L. hirsuta, which groups with the Crotalaria clade.

The Crotalaria Clade—The close relationship between Crotalaria and Bolusia has been mentioned by both Polhill (1982) and van Wyk (1991a) and these authors suggested that Bolusia could merely be a local derivative of Crotalaria. Data from the present study indicates that Bolusia is sister to and not embedded within Crotalaria (rbcL analysis with multiple representatives), so that its generic status seems justified. Bolusia differs from Crotalaria mainly in its glabrous style and strongly coiled keel petals. Also included in this clade is Lotononis hirsuta, representing the monotypic section Euclora. This species differs from all other Lotononis species in its peculiar tuberous habit and from most others in the very large, inflated pods, equally lobed calyx, and very large seeds with smooth surfaces. It shares with Crotalaria and Bolusia the strongly inflated pods.

Implications for Generic Classification—The results generated in this study have important implications for the generic classification system of the Crotalarieae. Detailed studies of character variation in the tribe over a period of more than 23 yr are now nearing completion, allowing us to make an informed evaluation of the congruence between morphological and molecular patterns. The results clearly show that the genera Calobota Eckl. & Zeyh. and Euclora Eckl. & Zeyh. should be reinstated. A new genus should be described that will include Lebeckia sect. Viborgioides, as well as L. inflata and L. mucronata. Spartodium will be transferred to Calobota and Viborgia humilis to the new genus (Boatwright et al., unpubl.). These changes, about to be formalized, will clearly result in a more practical and predictable generic classification system for the tribe Crotalarieae.

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LITERATURE CITED


**APPENDIX 1.** Voucher information and GenBank accession numbers of the taxa sampled in this study. Voucher specimens are deposited in the following herbaria: Australian National Herbarium (CANB), University of Johannesburg Herbarium (JRAU), Kew Herbarium (K), National Herbarium, Pretoria (PRE), Southern Cape Herbarium (SCHG). The information listed follows: accessions: rbcL ITS Voucher specimen.

<table>
<thead>
<tr>
<th>Character State</th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Present</td>
<td>1</td>
</tr>
</tbody>
</table>

APPENDIX 2. List of morphological characters and character states used in the morphological analysis.

1. Habit— herbs or suffrutices = 0; shrubs = 1. 2. Persistence— perennial = 0; annual = 1. 3. Young twigs— without bark formation (bark formation late or absent) = 0; with bark formation (bark formation early) = 1. 4. Leaves— dicate = 0; unifoliolate = 1; simple or phyllocladous = 2. 5. Lamina— flat = 0; terete (acicular) = 1. 6. Petiole presence— present = 0; absent (leaves sessile) = 1. 7. Petiole base— normal or leaves sessile = 0; tuberculate or with persistent spur = 1. 8. Stipules presence— present = 0; absent = 1. 9. Stipules symmetry— symmetrical or absent = 0; asymmetrical (dissimilar in size or shape) = 1. 10. Bracteole presence— present = 0; vestigial or absent = 1. 11. Standard petal vestiture— hairy at least along the dorsal midrib = 0; totally glabrous = 1. 12. Keel shape— not rostrate = 0; markedly rostrate or helically coiled = 1. 13. Calyx lower lobes— with trifid lower lip = 0; without trifid lower lip = 1. 14. Calyx lateral lobes— not fused higher up = 0; fused higher up = 1. 15. Anthers— dimorphic = 0; uniform = 1. 16. Carpinal anther— resembling basified anthers = 0; intermediate = 1; resembling dorsiﬂux anthers = 2. 17. Gynoecium base— sessile or subsessile = 0; stipitate = 1. 18. Style— upcurved = 0; straight = 1; helically coiled = 2. 19. Style vestiture— glabrous = 0; hairy = 1. 20. Fruit type— not a samara = 0; fruit an ovoid, winged samara = 1. 21. Fruit upper suture— upper suture straight or symmetrically convex = 0; upper suture asymmetrically convex = 1. 22. Fruit— many-seeded = 0; few-seeded (1–2) = 1. 23. Funicle length— normal length = 0; exceptionally long = 1. 24. Seed shape— transversely oblong = 0; oblong = 1. 25. Seed surface— smooth = 0; rugose = 1. 26. Chromosome base number—7 = 0; 8 = 1; 9 = 2. 27. Cyanogenesis— absent = 0; present = 1. 28. Quinolizidine and piperidyl alkaloids (Lysine pathway)— present = 0; absent = 1. 29. Sarpeine— present = 0; absent = 1. 30. Lupanine type esters— absent = 0; present = 1.