Phylogenetic Analysis of Nuclear Ribosomal ITS/5.8S Sequences in the Tribe Millettieae (Fabaceae): *Poecilanthe-Cyclolobium*, the core Millettieae, and the *Callerya* Group

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Communicating Editor: Jerrold I. Davis

Abstract. The taxonomic composition of three principal and distantly related groups of the former tribe Millettieae, which were first identified from nuclear phytochrome and chloroplast *trnK/matK* sequences, was more extensively investigated with a phylogenetic analysis of nuclear ribosomal DNA ITS/5.8S sequences. The first of these groups includes the neotropical genera *Poecilanthe* and *Cyclolobium*, which are resolved as basal lineages in a clade that otherwise includes the neotropical genera *Brongniartia* and *Harpalyce* and the Australian *Templetonia* and *Hovea*. The second group includes the large millettioid genera, *Millettia*, *Lonchocarpus*, *Derris*, and *Tephrosia*, which are referred to as the "core Millettieae" group. Phylogenetic analysis of nuclear ribosomal DNA ITS/5.8S sequences reveals that *Millettia* is polyphyletic, and that subclades of the core Millettieae group, such as the New World *Lonchocarpus* or the pantropical *Tephrosia* and segregate genera (e.g., *Chadsia* and *Mundulea*), each form well supported monophyletic subgroups. The third lineage includes the genera *Afgekia*, *Callerya*, and *Wisteria*. These genera are resolved as a basal subclade in the inverted-repeat-lacking clade, which is a large legume group that includes the many well known temperate and herbaceous legumes, such as *Astragalus*, *Medicago* and *Pisum*, but not any other Millettieae.

Phylogenetic analyses of *trnK/matK* DNA sequences have revealed new and well supported higher level relationships within Papilionoideae subfamily of Fabaceae (e.g., Hu et al. 2000; Wojciechowski et al. 2000; Lavin et al. 2001a). In particular, the large tribe Millettieae has been shown to comprise at least three distantly related lineages: a *Poecilanthe-Cyclolobium* group, a "core Millettieae" group, and a *Callerya-Wisteria* group (Hu et al. 2000). The genera *Callerya* and *Wisteria*, for example, actually belong to the inverted-repeat-lacking clade (IRLC; Wojciechowski et al. 1999), a clade comprising legumes lacking one copy of the 25-kb inverted repeat in the chloroplast genome. Notably, this relationship also has been suggested by studies of the chloroplast inverted repeat (Lavin et al. 1990; Liston 1995), the nuclear phytochrome gene family, and the chloroplast *rbcL* analysis (Doyle et al. 1997). Even chromosome numbers of Millettieae species (Hu 2000a) suggest that *Callerya* and *Wisteria* are indeed distinct from other Millettieae. Whereas the *Callerya* and *Wisteria* taxa examined all have $x = 8$ or 16, these numbers are uncommon in the other Millettieae genera such as *Millettia*, *Lonchocarpus*, and *Derris*.

Regardless of the above advances into the higher level relationships of Millettieae, it remains unclear how many other Millettieae taxa are part of the *Callerya-Wisteria* group. Furthermore, it is uncertain whether all species currently assigned to the genera *Callerya* and *Wisteria* group occur within this clade. In addition, many species of Millettieae have yet to be surveyed for chromosome numbers, especially in genera that are morphologically similar to *Callerya* and *Wisteria*, such as *Dewevrea* M. Micheli, *Endosamara* Geesink, *Ostryocarpus* Hooker, and *Sarcodum* Loureiro.

In order to obtain a more complete phylogeny for *Callerya* and other Millettieae, sequence variation from the nuclear ribosomal DNA internal transcribed spacer (ITS) region was sampled and analyzed for more of the relevant species than have been sampled in previous studies of this tribe that involved other loci (Lavin et al. 1998; Hu et al. 2000). This study was motivated by the high sequence variability in the ITS region, which has been shown to provide many informative sites for phylogenetic analysis and to be amenable to exhaustive taxon sampling at and below the genus level (Baldwin et al. 1995; Wojciechowski et al. 1999; Lavin et al. 2001a, 2001b).

Materials and Methods

DNA Extraction and Amplification of the ITS Region. Total genomic DNAs were isolated from fresh or dried materials using the standard CTAB extraction method or a protocol designed for rain forest species (Doyle and Doyle 1987; Scott and Playford 1996). Double stranded DNA copies of the ITS region were amplified from genomic DNA using the polymerase chain reaction (PCR) and used the same reagents and conditions described in Hu et al. (2000), but with shorter annealing (1 min) and extension (1 min 30 sec) time. Amplification and sequencing primers of the ITS region were the same as used by Wojciechowski et al. (1997, 1999). Vent$_R$ DNA Polymerase (New England Biolabs) was used in most of the reactions due to its higher fidelity, but in some recalcitrant samples, *Taq* polymerase (Promega Corp., Madison, Wisconsin) was used to increase the product yield. Nucleotide sequences of PCR products were determined using automated cycle-sequencing and an ABI 377 DNA Sequencer (PE Applied Biosystems, Foster

City, California) at the University of California, Davis. To minimize errors associated with PCR and sequencing, two or more independent PCR amplifications were employed for each taxon and sequenced separately for both strands. Ambiguous sites were confirmed by a third run of PCR and sequencing if there was any conflict in the consensus sequences from the first two runs of sequencing. The consensus sequences were assembled and edited using Sequencher® 3.0 (Gene Codes Corp., Ann Arbor, Michigan).

Sequence Alignment and Phylogenetic Analyses. All sequences were aligned manually with the aid of Se-Al (Rambaut 1996). The aligned data set has been deposited in TreeBase (Accession #S760). Missing data represented 6.7% of the data set involving *Cyclolobium* and *Poecilanthe* (largely because of the missing 5.8S sequence from several taxa and the ITS2 sequence of *Harpalyce brasiliana*), 0% of the core Millettieae data set, and 2.7% of the data set involving *Callerya* and *Wisteria*. Parsimony analyses of the individual data sets were performed with PAUP* (Swofford 2001). For all analyses, gaps were treated as missing data, no sites containing insertion/deletions were excluded, and search options invoked 100 random addition sequences, tree bisection-reconnection branch-swapping, and retention of multiple parsimonious trees. Bootstrap analyses (Felsenstein 1985) were used to assess clade stability. Each of 1,000 bootstrap replicates was analyzed with the heuristic search option invoking one random addition replicate each, and not invoking the retention of multiple parsimonious trees.

Taxon Sampling. Although this ITS analysis follows the basic sampling design implemented in the analysis of the *trnK/matK* sequences of Millettieae (Hu et al. 2000), it differs in having an increased representation of species from genera such as *Callerya*, *Wisteria*, *Lonchocarpus*, *Millettia*, and *Tephrosia*. This phylogenetic study is constrained by ITS sequence variation, which is limited to comparisons among fairly closely related legume genera. This is because ITS sequence comparisons across distantly related legume tribes render many regions of the alignments ambiguous. This is in contrast to the *trnK/matK* sequences of legumes that are easily aligned across all of the legume family (Wojciechowski et al., unpubl. data).

All major subgroups of the tribe Millettieae detected in the *trnK/matK* study of Hu et al. (2000) were sampled. Sampling of the large genera, such as *Callerya*, *Wisteria*, *Tephrosia*, *Millettia*, and *Lonchocarpus* was more comprehensive than in previous studies. Four sequences were sampled from *Poecilanthe* and *Cyclolobium*, 17 sequences of *Afgekia*, *Callerya*, and *Wisteria*, and 56 sequences representing the remaining genera traditionally placed in the tribe Millettieae. The source of specimens from which DNA was isolated is detailed in the Appendix. Also targeted were the closest relatives of each of the three main lineages of Millettieae. The molecular phylogenetic analyses of Crisp et al. (2000) and Hu et al. (2000) suggest that *Cyclolobium* and *Poecilanthe* are part of the genistoid legume radiation; therefore, outgroups and putatively close relatives of these two genera were selected based on these analyses. Similarly, selection of outgroups used in the analysis of the ITS sequence data from *Afgekia*, *Callerya*, *Wistera*, and other IRLC members was guided by the *trnK/matK* analyses of Wojciechowski et al. (2000). The phytochrome (Lavin et al. 1998) and *trnK/matK* (Hu et al. 2000) analyses of Millettieae samples guided the selection of outgroups for the ITS study of the core Millettieae clade. Sequence alignments are available at http://gemini.oscs.montana.edu/~mlavin/data/ITSmill.htm.

RESULTS

The first analysis, involving *Poecilanthe* and *Cyclolobium* and thirty-eight related taxa, included 330 parsimony-informative sites. A broad array of species from the genistoid legumes was used as outgroups in this phylogenetic analysis. The four equally most parsimonious trees each have a length of 1882, a CI of 0.42, and an RI of 0.58. The topology of these trees suggests that *Cyclolobium* and *Poecilanthe* together are the sister
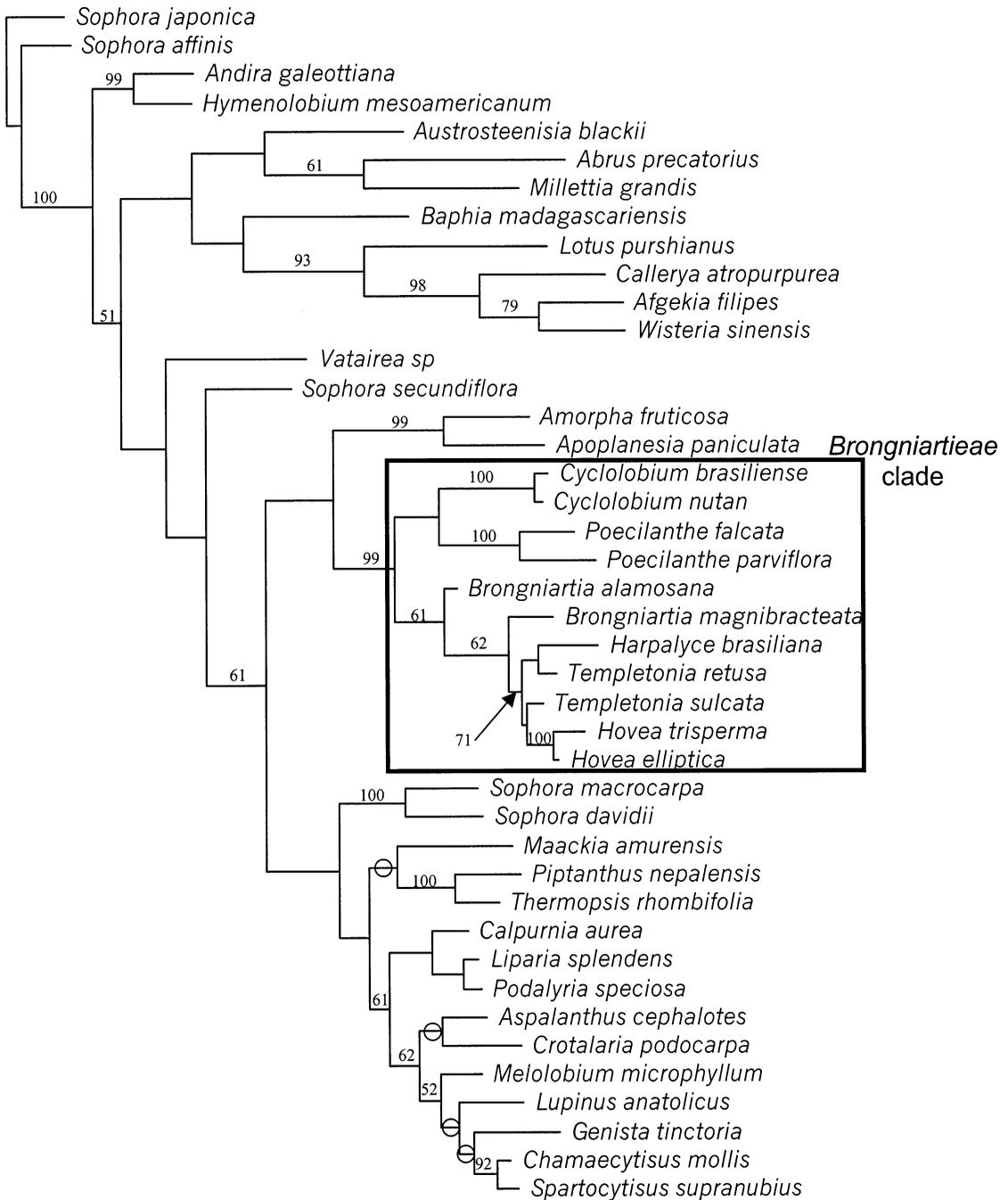
of a clade that otherwise includes *Brongniartia*, *Harpalyce*, *Templetonia*, and *Hovea* (Fig. 1). This ''Brongniartieae clade'' is very well supported as monophyletic, whereas the basal branching order of *Cyclolobium* and *Poecilanthe* within the Brongniartieae clade is moderately supported.

The data set involving the core Millettieae includes 72 taxa and 398 parsimony-informative sites. Species from the tribe Indigofereae and Phaseoleae were used as outgroups in this phylogenetic analysis. The ten equally most parsimonious trees each have a length of 2907, a CI of 0.34, and an RI of 0.65. The best supported of the larger clades involves one that stems from the most recent common ancestor of *Millettia ichthyochtona* and *Piscidia mollis* (Fig. 2). The core Millettieae clade, which is moderately supported by the ITS analysis, is actually slightly more inclusive than this best supported clade and stems from the most recent common ancestor of *Millettia lasianthus* and *Millettia leptobotrya*. Within the core Millettieae clade, two of the very well supported monophyletic subclades are: a clade containing New World *Lonchocarpus*, and another containing *Tephrosia* and the segregate genera *Chadsia* Bojer and *Mundulea* (DC.) Bentham. Two genera of the former tribe Millettieae, *Austrosteenisia* and *Dalbergiella*, are placed in a basal grade leading to the core Millettieae clade. Four other genera of the former Millettieae, *Aganope*, *Craibia*, *Ostryocarpus*, and *Platycyamus*, are resolved in basal branching order within the weakly supported clade containing the Phaseoleae outgroups (Fig. 2).

The data set containing samples of *Afgekia*, *Callerya*, and *Wisteria* includes 40 terminal taxa and 303 parsimony-informative sites. Species from the tribes Sophoreae, Millettieae, Robinieae, Loteae, and Coronilleae were used as outgroups in this phylogenetic analysis. The two equally most parsimonious trees each have a length of 1613, a CI of 0.44, and an RI of 0.61. The inverted-repeat-lacking clade (IRLC) is resolved as monophyletic in the strict consensus with moderate bootstrap support. The former genera of Millettieae, *Afgekia*, *Callerya*, and *Wisteria*, are resolved as a basal weakly supported clade within the IRLC (Fig. 3). The strict consensus also resolves the three *Glycyrrhiza* L. species as a well-supported monophyletic clade, which forms the weakly supported sister clade to the remaining IRLC (Fig. 3). The five *Wisteria* samples form a well-supported clade nested within *Callerya*, which is rendered paraphyletic. *Afgekia filipes* is sister to a clade comprising *Callerya megasperma* and *Callerya australis*. Together these three taxa form a well supported sister clade of *Wisteria*. *Callerya atropurpurea* is grouped with low support as sister to the rest of the *Callerya* clade.

DISCUSSION

The results of the ITS sequence analyses are in strong agreement with the higher level relationships
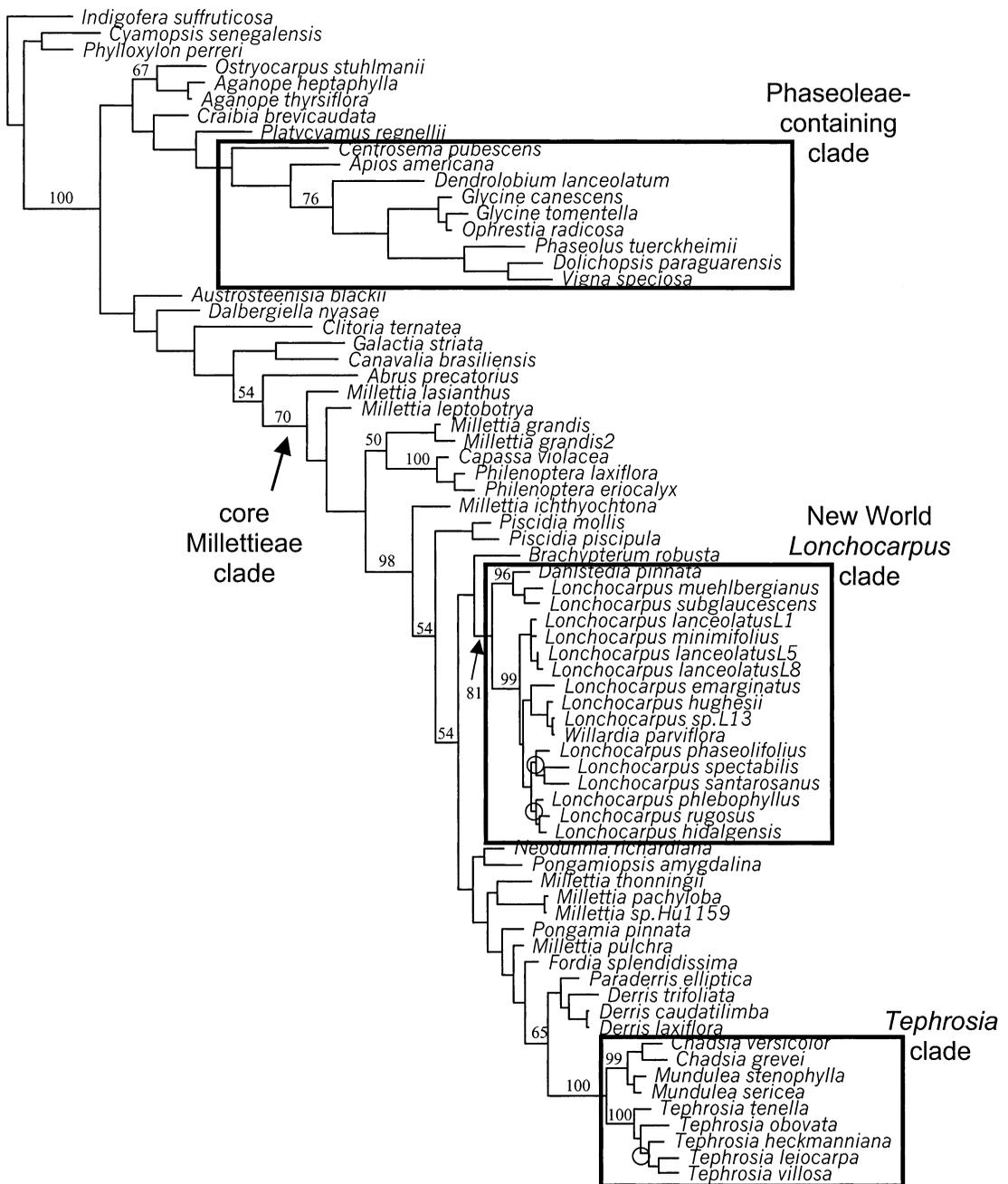
FIG. 1.   One of four equally most parsimonious trees derived from the Brongniartieae ITS data set that comprises 42 sequences and 633 aligned sites (6.7% missing data), 330 of which are parsimony informative. This tree has a length of 1882, a consistency index of 0.42, and a retention index of 0.58. Values above the nodes are bootstrap values for clades resolved in the strict consensus. Nodes with a superimposed open circle are collapsed in the strict consensus.

resolved with phytochrome (Lavin et al. 1998) and *trnK/matK* sequences (Hu et al. 2000). An extrapolation of our sampling from both the ITS and *trnK/matK* studies strongly suggest that the large majority of for-

mer Millettieae species now reside in the core Millettieae clade, which contains the largest genera, *Lonchocarpus* (ca. 150 spp.), *Derris* (ca. 40 spp.), *Millettia* (ca. 200 spp.), and *Tephrosia* (ca. 400 spp.; Geesink 1984; Hu

FIG. 2. One of ten equally most parsimonious trees derived from the core Millettieae ITS data set that comprises 72 sequences and 773 aligned sites (0% missing data), 398 of which are parsimony informative. This tree has a length of 2907, a consistency index of 0.34, and a retention index of 0.65. Values above the nodes are bootstrap values for clades resolved in the strict consensus. Nodes with a superimposed open circle are collapsed in the strict consensus.

2000a). In contrast to previous taxonomies, these large genera are now viewed as composing a very closely related group. Members of this clade are neither most closely related to Dalbergieae nor to Galegeae, as suggested by various authors (e.g., Bentham; Polhill 1981; Sousa and de Sousa; Geesink 1984). Also because of the more extensive sampling, this study more strongly suggests that all species currently assigned to the genera *Afgekia*, *Callerya*, and *Wisteria* are part of an early branching clade nested within the IRLC.

The increased taxon representation this study has provided for the core Millettieae and *Callerya-Wisteria*
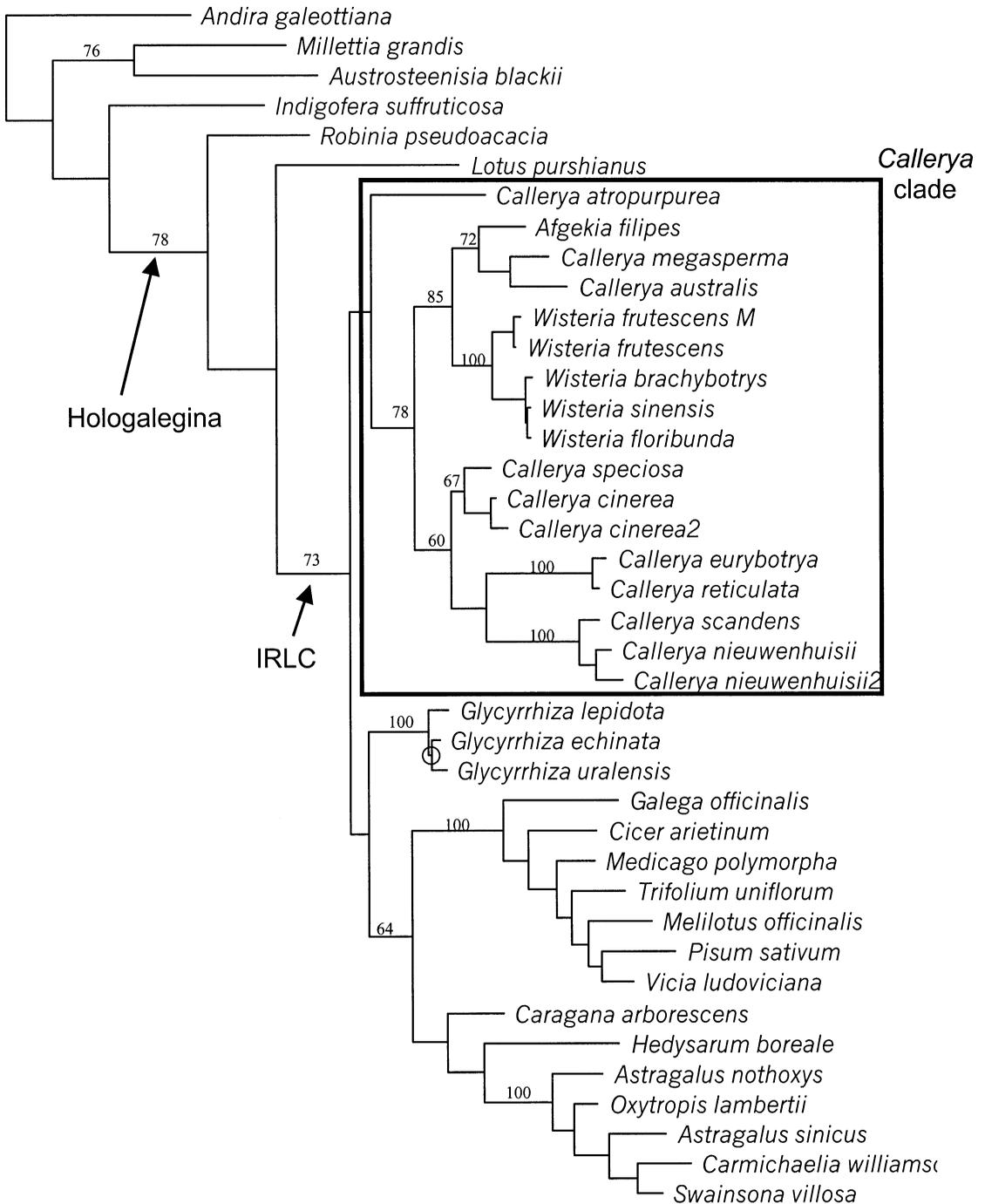
FIG. 3. One of two equally most parsimonious trees derived from the *Callerya* ITS data set that comprises 40 sequences and 624 aligned sites (2.7% missing data), 303 of which are parsimony informative. This tree has a length of 1613, a consistency index of 0.44, and a retention index of 0.61. Values above the nodes are bootstrap values for clades resolved in the strict consensus. The node with a superimposed open circle is collapsed in the strict consensus. IRLC designates the inverted-repeat-lacking clade. The "M" in *Wisteria frutescens* refers to variety *macrostachya*.

clades not only increases confidence with regard to the predicted membership of these clades, but also an increased confidence with respect to those non-molecular traits that the *trnK/matK* study suggested to be the most phylogenetically informative. The accumulation of non-protein amino acids other than canavanine in the core Millettieae clade contrasts with the accumulation of canavanine in seeds of all *Afgekia*, *Callerya*, and *Wisteria*, and most of the hologalegina (Wojciechowski et al. 2000), the papilionoid clade in which *Callerya* and relatives is nested (Bell et al. 1978; Evans et al. 1985; Hu et al. 2000). The inflorescences of all core Millettieae have nodes bearing fascicled flowers, which contrasts with the single flowers at inflorescence nodes in *Afgekia*, *Callerya*, and *Wisteria*, a similarity shared among these three genera and hologalegina (Hu 2000b; Hu et al. 2000). Pod morphology may be the least phylogenetically informative class of characters in this group of Papilionoideae, even though such traits have most greatly influenced previous taxonomies (e.g., Bentham 1860; Polhill 1981; Sousa and de Sousa 1981; Geesink 1984). For example, indehiscent pods no longer suggest a relationship among the genera *Lonchocarpus*, *Derris*, and the genera of Dalbergieae. Likewise, dehiscent pods are no longer viewed as indicative of a relationship of *Millettia* and *Tephrosia* with genera of the hologalegina, and winged pods no are longer viewed as homologous among *Derris* and the genera of Dalbergieae (Hu et al. 2000).

The Brongniartieae Clade. Both ITS and *trnK/matK* suggest that *Poecilanthe* and *Cyclolobium* should be excluded from the tribe Millettieae. The history of *Poecilanthe* and its various placements within the tribes Dalbergieae, Millettieae, and Robinieae has been reviewed by Lavin et al. (1998) and Hu et al. (2000). The genus *Cyclolobium* has been placed in Dalbergieae (Allen and Allen 1981; Bentham 1860; Burkart 1952; Hoehne 1940) and Millettieae (Geesink 1984; Lewis 1987). The ITS phylogeny confirms the chloroplast *trnK/matK* result (Hu et al. 2000) in showing that *Cyclolobium* and *Poecilanthe* are closely related within a Brongniartieae clade and distant from the clades containing Dalbergieae, Robinieae, or other Millettieae (Fig. 1). This same finding was made by Crisp et al. (2000) in an analysis of another ITS data set that contained fewer representatives of this Brongniartieae clade. Placement of *Poecilanthe* in the Brongniartieae clade has also been suggested by pod morphology and alkaloid chemistry, as reviewed by Lavin (1987), Greinwald et al. (1995), Hu (2000b), and Hu et al. (2000).

The **Tephrosia** *Clade.* The ITS phylogeny suggests that *Tephrosia* is a monophyletic group. Although only five out of about 400 species were sampled from this genus, the result is significant because of the very high support for this clade that comprises species from the Americas, Africa, and Asia (Fig. 2). The samples of

Chadsia and *Mundulea* form the well supported sister clade to *Tephrosia*. The genera *Requienia* DC., *Ptycholobium* Harms, *Caulocarpus* E. G. Baker, and *Lupinophyllum* Hutch. are considered to be very closely related to *Tephrosia*, but were not sampled in this study. The *Tephrosia* clade very likely includes these genera given the high degree of morphological similarity shared among all of them. For example, all of the genera have leaflets with penniparallel secondary venation, which is unique. Geesink (1984) points out that *Chadsia* is marked by digitately and pinnately arranged leaflets, which are very similar to the digitately compound leaves of *Ptycholobium*, *Caulocarpus*, and *Lupinophyllum*.

The *Tephrosia* clade is resolved with weak support as sister to a subclade that includes *Derris* and *Paraderris* species (Fig. 2), which contrasts with its well supported placement as an early branching clade within the core Millettieae, according to the phytochrome (Lavin et al. 1998) and *trnK/matK* analyses (Hu et al. 2000). The exact relationship of the *Tephrosia* clade within the core Millettieae clade thus remains unresolved. This issue can only be addressed by a future study that implements taxon sampling in a more thorough and strategic manner.

The American **Lonchocarpus** *Clade.* Genera of Millettieae with indehiscent pods (e.g., *Derris*) or with biflorous pseudoracemose inflorescences (e.g., *Lonchocarpus*) have been referred to subtribe Lonchocarpinae and considered closely related to the genera of Dalbergieae (Bentham 1860; Polhill 1981; Sousa and de Sousa 1981). No molecular data support this and the ITS phylogeny groups *Lonchocarpus* and *Derris* species well within the core Millettieae clade (Fig. 2). In particular, the genus *Lonchocarpus* sensu stricto is resolved as monophyletic. This genus is confined to the New World tropics, except for the amphi-Atlantic *Lonchocarpus sericeus* (Sousa and Delgado 1993), and is characterized by pseudoracemes with nodes bearing usually two flowers on top of a single pedicel (''biflorous'' inflorescences), which is otherwise found among Millettieae only in the Asiatic genus *Paraderris* (Geesink 1984). The ITS phylogeny suggests that *Lonchocarpus* species form a well supported clade along with the samples of other New World species, such as *Dahlstedtia* and *Willardia* (Fig. 2).

Within the New World *Lonchocarpus* clade, there are two well supported subclades. One includes *Dahlstedtia pinnata* and two *Lonchocarpus* species, *L. muehlbergianus* and *L. subglaucescens*. *Dahlstedtia pinnata* has red petaled flowers and pseudopaniculate inflorescences similar to those of the African *Philenoptera* (Geesink 1984). Similarly, *Lonchocarpus muehlbergianus* and *L. subglaucescens* also possess pseudopaniculate inflorescences. Two other yet unsampled South American species of *Lonchocarpus*, *Lonchocarpus praecox* Martius ex Bentham and *L. araripensis* Bentham, also have pseu-

dopaniculate inflorescences, and thus differ from the rest of New World *Lonchocarpus*, which have pseudoracemes. These four pseudopaniculate *Lonchocarpus* species are referred to as ''American *Philenoptera*'' (Geesink 1984). Definitely, the American *Philenoptera* are part of the American *Lonchocarpus* clade and not most closely related to the African *Philenoptera* (e.g., *Philenoptera laxiflora* and *Philenoptera eriocalyx* in Fig. 2).

The second sub-clade in the New World *Lonchocarpus* clades comprises species with pseudoracemose inflorescences, contrasting with the pseudopaniculate sister clade. Using the subgeneric classification of *Lonchocarpus* by Sousa and Delgado (1993) and Palomino and Sousa (2000), the only infrageneric taxon found to be monophyletic is section *Willardia*, with *Willardia parviflora*, *Lonchocarpus hughesii*, and *Lonchocarpus* sp.L13. The ITS phylogeny thus strongly supports the inclusion of *Willardia* within *Lonchocarpus* (Fig. 2).

The **Philenoptera** *Clade.* An increase in taxon sampling among the core Millettieae continues to resolve a *Philenoptera* clade (represented by *Millettia grandis*, *Capassa violacea*, *Philenoptera eriocalyx*, and *Philenoptera laxiflora*). *Philenoptera* has been referred to as the ''African *Lonchocarpus*'' because of its overall similarity to the American *Lonchocarpus* except for the pseudopaniculate inflorescence of this genus (Hepper 1958; Gillett et al. 1971; Lock 1989). The identity and phylogenetic position of the *Philenoptera* clade was first revealed by the *trnK/matK* sequence analysis as a well supported basal-most subclade in the core Millettieae clade (Hu et al. 2000). *Philenoptera* and *Capassa* form a very well supported group in the ITS phylogeny, and *Millettia grandis* is resolved as the weakly supported sister group (Fig. 2). Whereas the *trnK/matK* phylogeny suggests that *Millettia leptobotrya* is part of the *Philenoptera* clade (Hu et al. 2000), the ITS phylogeny suggests that *Millettia leptobotrya* and *Millettia lasianthus* are the earliest branching lineages in the core Millettieae clade. This conflict is likely an artifact of insufficient sequence variation in ITS, as inferred from the very low bootstrap values in this part of the ITS phylogeny (Fig. 2).

The **Callerya** *Clade.* The relationship of *Callerya* and *Wisteria* to other legumes that lack one copy of the chloroplast DNA inverted repeat is consistent with chromosome number counts (Goldblatt 1981; Hu 2000a), chloroplast inverted repeat surveys (Lavin et al. 1990; Liston 1995), and studies of the phytochrome gene family (Lavin et al. 1998), *rbcL* (Doyle et al. 1997), and chloroplast *trnK/matK* (Hu et al. 2000). The genus *Callerya* comprises about 22 species previously classified in the genera *Padbruggea* Miq., *Whitfordiodendron*, and *Millettia* sections *Eurybotryae* and *Austromillettia*. The genus is distinguished from *Millettia* by a combination of characters, such as highly branched panicles with nodes bearing one flower each, diadelphous stamens, and conspicuous bracts and bracteoles (Schot

1994). Schot (1994) originally recognized 19 *Callerya* species, 16 of them distributed in southeastern Asia (seven in South China), and the other three in northeastern Australia and Papua New Guinea (*Callerya australis*, *Callerya megasperma*, and *Callerya pilipes* (F. M. Bailey) Schot). Loc (1996) added *Callerya bonatiana* (Pampanini) P. K. Loc, and resurrected two species, *Callerya championii* (Bentham) P. K. Loc and *Callerya dielsiana* (Harms) P. K. Loc, that had been included within *Callerya cinerea* (Schot 1994). When Geesink (1984) first recognized this genus, he did not emphasize the relationships between *Callerya* and other Millettieae, but rather noted only that *Philenoptera* and *Ostryocarpus* are closer to *Callerya* than to *Millettia* sensu stricto. All molecular evidence (Lavin et al. 1990, 1998; Liston 1995; Doyle et al. 1997; Hu et al. 2000) places *Callerya* firmly in the IRLC rather than among genera such as *Millettia* or *Philenoptera*.

Loc (1986) transferred *Millettia dielsiana* Harms from synonymy under *Callerya cinerea* (Schot 1994) to a distinct species as *Callerya dielsiana* on account of its flat pods. *Callerya cinerea* comprises morphologically distinct ecotypes (Wei 1985a, 1985b), but many specimens with intermediate morphologies between *Callerya cinerea* and *Callerya dielsiana* can be found (Schot 1994). *Callerya cinerea* and *Callerya dielsiana* are at least sister species, as suggested not only by this nomenclatural history but also by the ITS phylogeny (Fig. 3), where *Callerya dielsiana* is represented by ''*Callerya cinerea*2.''

Callerya atropurpurea occupies the basal-most branching position within the *Callerya* clade (Fig. 3), but this position is very weakly supported. Ferguson and Skvarla (1981) show that the pollen of *Callerya atropurpurea*, then reported as *Whitfordiodendron atropurpureum* (Wall.) Merrill, is similar to that of *Afgekia* in having a well-developed endexine and very thick tectum. They suggested that no other Millettieae taxa share this similarity. This observation is consistent with the phytochrome DNA evidence (Lavin et al. 1998) that links *Callerya atropurpurea* as part of the *Callerya* clade, in spite of the ITS (Fig. 3) and *trnK/matK* evidence (Hu et al. 2000) that suggests *Callerya atropurpurea* is a genetic outlier with respect to the main *Callerya* clade.

The ITS phylogeny indicates that *Wisteria* is nested within a paraphyletic *Callerya* (Fig. 3). *Wisteria*, one of the few temperate representatives of traditional Millettieae, comprises six species in China and Japan, and one or two in North America (Gillis 1980; Valder 1995). The three samples of eastern Asian *Wisteria* (*W. brachybotrys*, *W. sinensis*, *W. floribunda*) form a well-supported clade that is sister to the two samples of the eastern North American *W. frutescens*. Stritch (1984) transferred all Asian *Wisteria* to the genus *Rehsonia* Stritch mostly because of the geographical distinction. No morphological difference follows this geographical split, and all *Wisteria* species are very similar to each

other, as well as to species of *Millettia*. Indeed, *Wisteria* species differ from those of *Millettia* mainly by deciduous leaves and by the production of racemes instead of pseudoracemes. One species not sampled here, *Millettia japonica* (Siebold & Zucc.) A. Gray, is sometimes placed in *Wisteria*, but is undoubtedly part of this IRLC clade as suggested by other molecular data (see Kajita et al. 2001). This is not unanticipated given the temperate habitat of this species and its similarity to at least the North American *Wisteria* (see discussion of *Wisteria* in Geesink 1984).

A sample of *Afgekia filipes* also groups within the *Callerya* clade. This is not a novel association because all *Callerya* and *Afgekia* species were at one time placed in the genus *Padbruggea* (Geesink 1984). *Afgekia filipes* was thought to be an oddity in this genus because of its elongated funicle and hilum, putative lack of bracteoles, and putative pseudopaniculate inflorescence. Wei (1994) notes that this species (then treated as *Whitfordiodendron filipes* Dunn) has reduced, not absent, bracteoles. The ITS phylogeny strongly suggests that *Afgekia filipes* is closely related to the Australian *Callerya megasperma* and *Callerya australis* (Fig. 3). This tropical Austral-Asian clade forms a notable association with *Wisteria*, which has a temperate distribution in the northern hemisphere.

The two other species of *Afgekia* are very likely closely related to *Callerya* as well. A recent survey of chromosome numbers of *Afgekia sericea* Craib and *Afgekia mahidolae* Burtt & Chermsirivathana reveal that both species have $2n = 16$ (Prathepha 1994), the same number as most of *Callerya* and *Wisteria*. In addition, *Afgekia sericea* accumulates canavanine, a non-protein amino acid, as do *Callerya*, *Wisteria*, and most other members of the IRLC.

Zhu (1994) established the monotypic tribe Wisterieae by arguing that *Wisteria* is unique in basic chromosome number, phytochemistry, embryology, morphology, geographic distribution, and in the lack of one copy of the chloroplast DNA inverted repeat. Zhu further suggested the isolated position of *Wisteria* because of its velvet pods and pollen with a broad apocolpia that were different from nine other samples of Papilionoideae that she included in her analysis involving scanning electron microscopy. Zhu included *Callerya reticulata* (as *Millettia reticulata*) in her study. She suggested that this species is similar to *Derris*, *Pongamia*, and *Craspedolobium* Harms because they all share a fine striae on the surface of the muri of the pollen grains, a feature that is indeed not distinct in her published photos. Most importantly, Zhu's arguments are rendered indefensible because she was unaware of the similarity of base chromosome numbers and chloroplast genome structure in *Wisteria* and *Callerya reticulata* (and indeed other *Callerya* species).

Taking the phytochrome, *trnK/matK,* and ITS sequence data together, in combination with non-molecular data mentioned above, the genera *Afgekia*, *Callerya*, and *Wisteria* are likely most closely related to each other within the IRLC. Because none of these three genera can be classified conveniently into any of the existing tribes in the IRLC, a new tribe for this group may be eventually justified. Until a comprehensive review of the tribes of Papilionoideae is undertaken, in addition to a thorough molecular and non-molecular sampling of the species of *Afgekia*, *Callerya*, and *Wisteria*, the informal ''*Callerya* clade'' is retained here.

Millettia *and Allies.* The ITS phylogeny suggests that *Millettia* is polyphyletic, which agrees with the results of the phytochrome (Lavin et al. 1998) and *trnK/matK* studies (Hu et al. 2000). The *Millettia* species sampled in this study, including the segregate genera *Pongamia* and *Neodunnia*, are scattered throughout the core Millettieae clade (Fig. 2). The additional sampling presented in this ITS analysis reinforces the conclusions of Lavin et al. (1998) that species assigned to *Millettia* are genetically and phenotypically the most diverse compared to any other traditionally recognized genus of Millettieae, as originally suggested in the pioneer monograph of *Millettia* by Dunn (1912). Because of this, species of *Millettia* are most in need of study if relationships within the core Millettieae clade are to be further resolved and understood.

This study of ITS sequence variation, combined with the chloroplast *trnK/matK* study (Hu et al. 2000), resolves some important issues in the legume group formerly referred to as tribe Millettieae. Whereas *trnK/matK* resolved relationships at the higher levels, ITS sequence variation is more amenable to sampling and making comparisons within and among species of closely related genera. The data presented here suggest that exhaustive taxon sampling within Brongniartieae (including the neotropical *Cyclolobium*, *Poecilanthe*, *Harpalyce*, and *Brongniartia*, and the Australian *Templetonia* group; Crisp et al. 2000), among *Tephrosia* and segregate genera, among the American *Lonchocarpus* species, and among the species of the *Callerya* clade potentially will provide the phylogenetic resolution needed for a revised taxonomic classification, as well as for a biogeographic analysis of these clades. ITS sequence variation also promises to be very informative for comparisons among the approximately 200 species of *Millettia* (Hu 2000a), the most problematic of all the Millettieae genera, if only because most of these species should very likely belong to the clade containing the Asiatic genus *Derris* and the American genus *Lonchocarpus*.

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APPENDIX

Voucher specimen information of the taxa analyzed for ITS sequence variation. [a]Abbreviations used for accession identification: DLEG, Desert Legume Program (Boyce Thompson Southwestern Arboretum and The University of Arizona), Tucson, Arizona USA.; USDA, U.S. Department of Agriculture Plant Introduction accession numbers. [b]Based on van Wyk and Schutte. Data are provided in the following sequence: taxon; voucher; source[a] and geographic region; GenBank accession number.

PAPILIONOIDEAE

Tribe Abreae

Abrus precatorius L.; Hu 1136; Taiwan; AF467015

Tribe Amorpheae

Amorpha fruticosa L.; Lavin 6221 (BH); Wyoming, USA; U59890. *Apoplanesia paniculata* C. Presl; Hughes 254 (FHO); Guatemala; AF187093

Tribe Brongniartieae

Brongniartia alamosana Rydb.; Hu 1120; DLEG 89–0398, Sonora, Mexico; AF467022. *Brongniartia magnibracteata* Schltdl.; Lavin 5795 (CANB); AF287638. *Harpalyce brasiliana* Bentham; Plowman et al. 8132; Brazil; AF204711, AF204733. *Hovea elliptica* (Sm.) DC.; Crisp 8924 (CANB); AF287640. *Hovea trisperma* Bentham; Crisp 8925 (CANB); AF287639. *Templetonia retusa* R. Br.; Crisp 8996 (CANB); AF287636. *Templetonia sulcata* Bentham; Crisp 9057b (CANB); AF287635

Tribe Carmichaelieae

Carmichaelia williamsonii Kirk; Sanderson 1550; New Zealand; U50520, U50521

Tribe Cicereae

Cicer arietinum L.; AJ237698

Tribe Crotalarieae

Crotalaria podocarpa DC.; AF007469. *Melolobium microphyllum* (L.f.) Ecklon & Zeyher; T. Edwards 470; Lesotho, South Africa; Z95576, Z95560

Tribe Dalbergieae

Andira galeottiana Standl.; Lavin & Delgado 8214 (MEXU); Veracruz, Mexico; U59889. *Hymenolobium mesoamericanum* H.C. Lima; Pennington 614 (E); Costa Rica; AF187087. *Vatairea* sp.; Pennington 587 (E); Costa Rica; AF187088

Tribe Desmodieae

Dendrolobium lanceolatum (Dunn) Schindl.; Hu 1092; USDA PI 316214, Australia; AF467044

Tribe Galegeae

Astragalus nothoxys Gray; Wojciechowski & Sanderson 177 (ARIZ); Arizona, USA; AF121688, AF126979. *Astragalus sinicus* L.; Wojciechowski & Sanderson 408 (ARIZ); USDA PI 150557, China; U50502, U50503. *Caragana arborescens* Lam.; Wojciechowski & Sanderson 413 (ARIZ); USDA PI 310390, former USSR; L10798, L10799. *Galega officinalis* L.; M. Smejkal (A); Moravia, Czechoslovakia; U50760, U50761. *Glycyrrhiza echinata* L.; Liston 258 (OSC); U55999, U56000. *Glycyrrhiza lepidota* (Nutt.) Pursh; Toolin 1572 (ARIZ); U50758, U50759. *Glycyrrhiza uralensis* Fisch.; Hu 1142; Nursery specimen; AF467050. *Oxytropis lambertii* Pursh; Wojciechowski 155 (ARIZ); Arizona, USA; AF121753. *Swainsona villosa* J. Black; NSW 233473; Australia; AF113867

Tribe Genisteae

Aspalanthus cephalotes Thunb.; Heidrich 373; Cape Prov., South Africa; Z72308, Z72309. *Chamaecytisus mollis* (Cav.) Greuter & Burdet; RBG, Kew serial # 84327; AF007472. *Genista tinctoria* L.; RBG-Kew 51334; AF007471. *Lupinus anatolicus* W. Swiecicki & W.K. Swiecicki; AF108085. *Spartocytisus supranubius* (L.) Webb & Berth.; Käss 347; Pico del Teide, Tenerife; Z72250, Z72251

Tribe Hedysareae

Hedysarum boreale Nutt.; Wojciechowski & Sanderson 131 (ARIZ); Colorado, USA; U50482, U50483

Tribe Indigofereae

Cyamopsis senegalensis Guill. & Perr.; Hu 1099; USDA PI 263525, Senegal; AF467040. *Indigofera suffruticosa* Mill.; Hu 1102; USDA PI 404341, Brazil; AF467051. *Phylloxyon perreri* Drake; Labat et al. 2663 (K); Madagascar; AF274685

Tribe Liparieae

Liparia splendens (Burm.f.) Bos & De Wit; Heidrich 384; Cape Prov., South Africa; Z72324, Z72325

Tribe Loteae

Lotus purshianus (Bentham) Clements; Wojciechowski 707 (DAV); California, USA; AF467067

Tribe Millettieae

Afgekia filipes (Dunn.) R. Geesink; Maxwell 90–246 (L); Thailand; AF467016. *Aganope heptaphylla* (L.) Polhill

= *Ostryocarpus*; Forman & Blewett 1127 (L); Borneo; AF467017. *Aganope thyrsiflora* (Bentham) Polhill = *Ostryocarpus*; Sidiyasa 640 (L); Indonesia; AF467018. *Austrosteenisia blackii* (F.Muell.) Geesink; Pedley 5005 (K); Australia; AF467020

Brachypterum robusta (Roxb.) Geesink; Hu 1182; Davis (cultivated); AF467021

Callerya atropurpurea (Wall.) Schot; Liston 876 (OSC); Singapore; AF467023. *Callerya australis* (Endl.) Schot; Beesely 1053; AF467024. *Callerya cinerea* (Bentham) Schot; Pi-Chiang Team 257 (KUN); Yunnan, China; AF467025. *Callerya cinerea* (Bentham) Schot, #2; Hu 1196; Yunnan, China; AF467026. *Callerya eurybotrya* (Drake) Schot; Tao 578 (KUN); Yunnan, China; AF467027. *Callerya megasperma* (F. Muell.) Schot; Liston *s. n.* (OSC); AF467028. *Callerya nieuwenhuisii* (J.J. Sm.) Schot; Ambriansya & Arifiu 293 (L); Borneo; AF467029. *Callerya nieuwenhuisii* (J.J. Sm.) Schot, #2; Chew1013 (UC); Sarawak, Malaysia; AF467030. *Callerya reticulata* (Bentham) Schot; Liston 877 (OSC); Nursery specimen; AF467031. *Callerya scandens* (Elmer ex Dunn) Schot; Ridsdale 416 (UC); Palawan, Philippines; AF467032. *Callerya speciosa* (Champ.) Schot; Yu-Xi Team 1029 (KUN); Yunnan, China; AF467033. *Capassa violacea* Klotzsch = *Philenoptera*; Hu 1087; DLEG 91–0069, Transvaal, South Africa; AF467035. *Chadsia grevei* Drake subsp. *grevei*; Chase 6684 (K); Madagascar; AY009141. *Chadsia versicolor* Bojer; Schrire 2530 (K); Madagascar; AF467037. *Craibia brevicaudata* (Vatke) Dunn; Polhill & Robertson 5296 (K); AF467039. *Cyclolobium brasiliense* Bentham; Ratter et al. 7431 (E); Brazil; AF287637. *Cyclolobium nutans* Rizz. & Heringer; Lima *s. n.* (RB); Brazil; AF467041

Dahlstedtia pinnata (Bentham) Malme; Lima 4–1 (RB); Brazil; AF467042. *Dalbergiella nyasae* Baker f.; Muller 2686 (K); Africa; AF467043. *Derris caudatilimba* F.C. How; Hu 1156 (DAV); Yunnan, China; AF467045. *Derris laxifora* Bentham; Hu 1081; Taiwan; AF467046. *Derris trifoliata* Lour.; Hu 1082; Taiwan; AF467047

Fordia splendidissima (Blume ex Miq.) Buijsen; Tangah *s. n.*; Sabah, Malaysia; AF467048

Lonchocarpus emarginatus Pittier; Hughes 1344 (FHO); Oaxaca, Mexico; AF467052. *Lonchocarpus hidalgensis* Lundell; Hughes 1598 (FHO); Queretaro, Mexico; AF467053. *Lonchocarpus hughesii* M. Sousa; Hughes 1718 (FHO); Comayagua, Honduras; AF467054. *Lonchocarpus lanceolatus* Bentham L1; Hughes 1723 (FHO); Oaxaca, Mexico; AF467055. *Lonchocarpus lanceolatus* Bentham L5; Hughes 160/92 (FHO); Oaxaca, Mexico; AF467056. *Lonchocarpus lanceolatus* Bentham L8; Hughes 144/92–2 (FHO); Oaxaca, Mexico; AF467057. *Lonchocarpus minimiflorus* Donn. Smith; Hughes 1223 (FHO); Zacapa, Guatemala; AF467058. *Lonchocarpus muehlbergianus* Hassl.; Tressens et al. 1992 (UC); Corrientes, Argentina; AF467059. *Lonchocarpus phaseolifolius* Bentham; Hughes 5 (FHO); Yoro, Honduras;

AF467060. *Lonchocarpus phlebophyllus* Standl. & Steyerm.; Hughes 1101 (FHO); Chiquimula, Guatemala; AF467061. *Lonchocarpus rugosus* Bentham; Lavin 8204 (BH); AF467062. *Lonchocarpus santarosanus* J. D. Smith; Hughes 1229 (FHO); Sonsonate, El Salvador; AF467063. *Lonchocarpus* sp.L13; Sonora, Mexico; AF467064. *Lonchocarpus spectabilis* F. J. Herm.; Hughes 899 (FHO); Guerrero, Mexico; AF467065. *Lonchocarpus subglaucescens* Mart. ex Bentham; Hatschbach 41090 (UC); Brazil; AF467066

Millettia dielsiana Harms ex Diels, see *Callerya cinerea* (Bentham) Schot #2. *Millettia grandis* (E. Mey.) Skeels; Lavin & Lavin *s. n.* (MONT); Sydney, Australia; AF467474. *Millettia grandis* (E. Mey.) Skeels, no. 2; Schrire 2597 (K); South Africa; AY009139. *Millettia ichthyochtona* Drake; Lu-Shi Team 1368 (KUN); China; AF467475. *Millettia lasiantha* Dunn; Pawek 5926 (UC); Nkhata Bay, Malawi; AF467476. *Millettia leptobotrya* Dunn; Hu 1164 (DAV); Yunnan, China; AF467477. *Millettia oosperma* Dunn, see *Callerya cinerea* (Bentham) Schot. *Millettia pachyloba* Drake; Hu 1163 (DAV); Yunnan, China; AF467478. *Millettia pulchra* var. *microphylla* Dunn; Huang 15567 (TAI); Taiwan; AF467479. *Millettia* sp.; Hu 1159; Yunnan, China; AF467480. *Millettia thonningii* Baker; Faden 74/81 (K); Ghana; AF467481. *Mundulea sericea* (Willd.) A. Chev.; Schrire 2529 (K); Madagascar; AF467482. *Mundulea stenophylla* R. Vig; Chase 6687 (K); Madagascar; AY009140

Neodunnia richardiana (Baillon) Geesink; Schrire 2555 (K); Madagascar; AF467483

Ostryocarpus stuhlmannii (Taub.) Geesink; Corby 2162 (K); Africa; AF467485

Paraderris elliptica (Roxb.) Bentham; Michigan State University (no voucher);? AF467486. *Philenoptera eriocalyx* subsp. *wankiensis* (Mend. & Sousa) Geesink; Hu 1090; DLEG 91–0067, Zimbabwe; AF467487. *Philenoptera laxiflora* (Guill. & Perr.) Rob.; Hu 1126; DLEG 91–0456, Senegal; AF467488. *Piscidia mollis* Rose; Hu 1117; DLEG 89–0063, Sonora, Mexico; AF467489. *Piscidia piscipula* (L.) Sarg.; Lavin & Luckow 5793a (TEX); Veracruz, Mexico; AF467490. *Platycyamus regnellii* Bentham; Lima *s. n.* (RB); Minas Gerais, Brazil; AF467491. *Poecilanthe falcata* (Vell.) Heringer; H. C. Lima 2 (RB); Brazil; AF467492. *Poecilanthe parviflora* Bentham; H. C. Lima *s. n.* (RB); Santa Catarina, Brazil; AF187089. *Pongamia pinnata* (L.) Pierre (=*Millettia*); Hu 1094; Taiwan; AF467493. *Pongamiopsis amygdalina* (Baill.) R. Vig.; Du Puy M575 (K); Madagascar; AF467494

Tephrosia heckmanniana Harms; Hu 1127; USDA 304576; AF467497. *Tephrosia leiocarpa* A. Gray; DLEG 880028, Arizona, USA; U50752, U50753. *Tephrosia obovata* Merr.; Huang 15568 (TAI); Taiwan; AF467498. *Tephrosia tenella* A. Gray; Jenkins 88–1 (ARIZ); Arizona, USA; U50754, U50755. *Tephrosia villosa* Pers.; Lavin 6219 (BH); Africa; AF467499

Whitfordiodendron myrianthus Dunn, see *Callerya nieu-*

wenhuisii (J.J. Sm.) Schot #2. *Willardia parviflora* Rose (annotated as *Lonchocarpus andrieuxii* M. Sousa); Sousa 9380 (UC); Mexico; AF467500. *Wisteria brachybotrys* Siebold & Zucc.; Muth 82125 (DAV); Kyushu, Japan; AF467501. *Wisteria floribunda* (Willd.) DC.; Muth 8433 (DAV); Honshu, Japan; AF467502. *Wisteria frutescens* (L.) Poiret; Moldenke & Moldenke 29243 (ARIZ); North Carolina, USA; U50750, U50751. *Wisteria frutescens var. macrostachya* Torr. & A. Gray; USDA 2774; U55997, U55998. *Wisteria sinensis* (Sims) Sweet; Hu 1125; (cultivated) California, USA; AF467503

Tribe Phaseoleae

Apios americana Medik.; Hu 1223; Maryland, USA; AF467019. *Canavalia brasiliensis* Mart. ex Bentham; Hu 1069; USDA PI 319336, Mexico; AF467034. *Centrosema pubescens* Bentham; Hu 1093; USDA PI 415699, Taiwan; AF467036. *Clitoria ternatea* L.; Hu 1068; USDA PI 322364, Sao Paulo, Brazil; AF467038. *Dolichopsis paraguariensis* Hassl.; Krapovickas 46512 (MEXU); Argentina; AF069116. *Galactia striata* (Jacq.) Urb.; Hu 1116 (DAV); USDA PI 538312, Dominican Republic; AF467049. *Glycine canescens* F.J. Herm.; IL434 ; USDA PI 440932, South Australia; AF023444. *Glycine tomentella* Hayata; isolated 046; AJ011338. *Ophrestia radicosa* var. *schliebenii* (Harms) Verdc.; Hu 1104; USDA PI 255748, Zambia; AF467484. *Phaseolus tuerckheimii* Donn. Sm.; INIFAP-URG-11633; Chiapas, Mexico; AF115248. *Vigna speciosa* (Kunth) Verdc.; CIAT 4070 (MONT); Quintana Roo, Mexico; AF069121

Tribe Podalyrieae

Calpurnia aurea (Ait.) Bentham[b]; Heidelberg Botanical Garden, Germany; Z95568, Z95569. *Podalyria speciosa* Eckl. & Zeyh.; Crisp 9044 (CANB) ; AF287671

Tribe Robinieae

Robinia pseudoacacia L.; Hu 1067; (cultivated) California, USA; AF467495

Tribe Sophoreae

Baphia madagascariensis C. H. Stirt. & Du Puy; DuPuy M554 (K); Madagascar; U59888. *Maackia amurensis* Ruprecht & Maxim.; Göttingen Botanical Garden, Germany; Z72336, Z72352. *Sophora affinis* Torr. & A. Gray; L. Escobar *s. n.* (MONT); Texas, USA; U59886. *Sophora davidii* Kom. ex Pavlov; Hu 1064; AF467496. *Sophora japonica* L. =*Styphnolobium japonicum* (L.) Schott.; Heidelberg Botanical Garden, Germany (cult.); Z72340, Z72341. *Sophora macrocarpa* Sm.; Wink 433; Concepcion, Chile; Z95563, Z95577. *Sophora secundiflora* (Gomez-Ortega) Lagerh. ex DC.; L. Escobar *s. n.* (MONT); Texas, USA; U59885

Tribe Thermopsideae

Piptanthus nepalensis D. Don; Heidelberg Botanical Garden, Germany; Z72320, Z72321. *Thermopsis rhombifolia* (Nutt.) Richards var. *ovata*; USDA PI 10173, Idaho, USA; AF007468

Tribe Trifolieae

Medicago polymorpha L.; Jenkins 91–8 (ARIZ); Arizona, USA; U50863, U50864. *Melilotus officinalis* (L.) Pallas; Wojciechowski 309 (ARIZ); Arizona, USA; U50764, U50765. *Trifolium uniflorum* L.; AZ4194, Margot Forde Forage Germplasm Centre; AF053179

Tribe Vicieae

Pisum sativum L.; Wojciechowski 398 (ARIZ); (cultivated) Arizona, USA; U50861, U50862. *Vicia ludoviciana* Nutt.; McLaughlin & Bowers 3185 (ARIZ); Arizona, USA; U51216, U51217