

## Two New Members of the *Callerya* Group (Fabaceae) Based on Phylogenetic Analysis of *rbcL* Sequences: *Endosamara racemosa* (Roxb.) Geesink and *Callerya vasta* (Kosterm.) Schot

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(Manuscript received 2 May, 2003; accepted 29 May, 2003)

**ABSTRACT:** Two new members of *Callerya* group in Fabaceae, *Endosamara racemosa* (Roxb.) Geesink and *Callerya vasta* (Kosterm.) Schot, are identified based on phylogenetic analyses of chloroplast *rbcL* sequences. These taxa joined with other previously identified taxa in the *Callerya* group: *Afgekia*, *Callerya*, and *Wisteria*. These genera are resolved as a basal subclade in the Inverted Repeat Lacking Clade (IRLC), which is a large legume group that includes many temperate and herbaceous legumes in the subfamily Papilionoideae, such as *Astragalus*, *Medicago* and *Pisum*, and is not close to other Millettieae. *Endosamara* is sister to *Millettia japonica* (Siebold & Zucc.) A. Gray, but only weakly linked with *Wisteria* and *Afgekia*.

**KEY WORDS:** *Endosamara*, *Callerya*, Millettieae, *Millettia*, *rbcL*, Phylogenetic analysis.

### INTRODUCTION

Recent molecular phylogenetic studies of the tribe Millettieae have revealed that the tribe is polyphyletic and several taxa are needed to be segregated from the core Millettieae group. One of the major segregates from Millettieae is the *Callerya* group, comprising species from *Callerya*, *Wisteria*, *Afgekia*, and *Millettia japonica* (Siebold & Zucc.) A. Gray. The group is considered to be part of the Inverted-Repeat-Lacking Clade (IRLC; Wojciechowski *et al.*, 1999) including many temperate herbaceous legumes. Such result is consistent and supported by chloroplast inverted repeat surveys (Lavin *et al.*, 1990; Liston, 1995) and phylogenetic studies of the phytochrome gene family (Lavin *et al.*, 1998), chloroplast *rbcL* (Doyle *et al.*, 1997; Kajita *et al.*, 2001), *trnK/matK* (Hu *et al.*, 2000), and nuclear ribosomal ITS regions (Hu *et al.*, 2002). So far, 16 species of former Millettieae members have been identified in the *Callerya* group. Despite that none of the above studies included all of the 16 taxa in the analysis, the different data sets all have overlapped taxa and show the same basic topology that the *Callerya* group is likely the basal group in the IRLC.

The members of *Callerya* group have quite diverse morphology and show little autapomorphies, it is necessary to carry out a comprehensive survey among the Millettieae taxa, in order to identify other possible members in the *Callerya* group. One of the candidates is *Endosamara* from Millettieae. This genus was established by Robert Geesink, who elected the monotypic section *Bracteatae* of *Millettia* to genus level (Geesink, 1984). The type species, *Endosamara racemosa*, is very unique in having lomentation of the endocarp that separates from its exocarp, and forms a samaroid layer connecting the seeds. Comparisons of *Endosamara* and other *Millettia* species will be discussed below. The plant is a liana found in South India, Indo-China, to the Philippines. Geesink (1984) stated that this genus is related to *Callerya* and *Sarcodum*, but did not specify which characters linking these taxa together.

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Doyle *et al.* (1997) and Kajita *et al.* (2001) have constructed a large data set with 242 *rbcL* sequences representing 194 genera of legumes. Therefore it is useful to incorporate newly identified sequences into the *rbcL* data set to determine their putative phylogenetic position. In this study, we examined the chloroplast *rbcL* sequences of *Endosamara racemosa* (Roxb.) Geesink and a previously unsampled *Callerya* species, *Callerya vasta* (Kosterm.) Schot, to determine if they belong to the *Callerya* group.

## MATERIALS AND METHODS

### DNA extraction and amplification of the *rbcL* region

Total genomic DNA of *Endosamara racemosa* and *Callerya vasta* was isolated from dried materials using the standard CTAB extraction method (Doyle and Doyle, 1987). Voucher specimen information of the new sequences is listed in Table 1. Double stranded DNA copies of chloroplast *rbcL* sequence were amplified from genomic DNA using the polymerase chain reaction (PCR) as described in Hu *et al.* (2002).

Table 1. Voucher information of new samples used in this study. The deposition place of the specimen is indicated after collection numbers (L: Rijksherbarium, Leiden).

Taxa	Voucher specimen	GenBank Accession number
<i>Endosamara racemosa</i>	J. F. Maxwell 90-202 (Thailand: Chiang Mai) (L)	AY308805
<i>Callerya vasta</i>	W. Meijer 33899 (Indonesia: Borneo, Ranav) (L)	AY308806

Primers for PCR amplification and sequencing are listed in Table 2. They were designed according to Olmstead *et al.* (1992), but with minor modification compared to consensus sequences of published legume sequences. VioTaq DNA Polymerase (Viogene Biotek Corp., Taipei, Taiwan) was used in the PCR. Nucleotide sequences of PCR products were determined using automated cycle-sequencing and an ABI 377 DNA Sequencer (PE Applied Biosystems, Foster City, California, USA). The consensus sequences were assembled and edited using Sequencher™ 4.0 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Table 2. Primer design for amplifying *rbcL* sequences.

Primer	Sequences (from 5' to 3')	Direction to gene
<i>rbcL1</i>	ATG TCA CCA CAA ACA GAG ACT AAA GC	Forward
<i>rbcL334F</i>	TCT GTT ACW AAY ATG TTT ACT TC	Forward
<i>rbcL691F</i>	GAA ACA GGT GAA ATC AAA GGG CAT TA	Forward
<i>rbcL979R</i>	AAT ATG ATC TCC ACC AGA CAA ACG TAA	Reverse
<i>rbcL1303R</i>	TCC CTC ATT ACG AGC TTG TAC ACA	Reverse

### Sequence alignment and phylogenetic analyses

Sequences from total of 73 taxa in Fabaceae were used in the data set, including 34 taxa of Millettieae. Most of the *rbcL* sequences are directly obtained from the GenBank and the accession numbers are listed in Kajita *et al.* (2001). The data matrix includes all Millettieae taxa sampled by Kajita *et al.* (2001), but excludes some of basal Papilionoideae taxa from their complete data set. No more than four taxa from each tribe were used in the data matrix in order to save computation time. All sequences were aligned manually since the *rbcL* region contains very little indels. Neighbor-joining (NJ) and maximum parsimony (MP) analyses were performed with PAUP\* (Swofford, 2002). For all analyses, gaps were treated as missing data, and no sites containing insertion/deletions were excluded. Species from the tribes

Sophoreae (*Sophora jaubertii* Spach ex Jaubert & Spach, *Sophora davidii* Kom. ex Pavlov, and *Sophora flavescens* Aiton), were used as outgroups in the phylogenetic analysis according to the results of Doyle *et al.* (1997) and Kajita *et al.* (2001).

Two approaches of tree searching using parsimony criteria were employed, one is to set a smaller number of random addition starting point, the other is to perform a “two-step” tree searching method, which is used to explore more tree space (*sensu* Soltis & Soltis 1997). For the first approach, parsimony search options for 73-taxa data set invoked 10 random addition sequences, tree bisection-reconnection branch-swapping, and retention of multiple parsimonious trees. For the second approach, 50,000 random additions were used to start the tree searching, with NNI, but no Multree in effect. All of the 50,000 saved trees were input to the second round of tree searching by TBR, Multrees, and steepest decent. The internal support was evaluated by bootstrap analyses (Felsenstein, 1985) and decay indices (Bremer, 1988, 1994). In parsimony analysis, 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. Decay indices (Bremer support) were calculated by incorporating AutoDecay (Eriksson, 1998) and PAUP\* (Swofford, 2002), which quantify the extra length needed to collapse a branch in the consensus of near-most-parsimonious trees (Bremer, 1988, 1994).

A smaller data set was generated containing 17 selected taxa from *Callerya* group (including *Endosamara racemosa*) and other Hologalegina taxa (*sensu* Wojciechowski *et al.*, 1999). The purpose is to perform maximum likelihood (ML) and other more intense analysis that consumes considerable computation time, i.e. branch-and-bound search instead of heuristic search. *Robinia pseudoacacia* L. (Robinieae) was used as outgroup based on the results of previous molecular phylogenies (Doyle *et al.*, 1997; Kajita *et al.*, 2001; Hu *et al.* 2000, 2002). Branch-and-bound option was used in the smaller data set when performing parsimony search, and also used in the bootstrap and decay index analyses with 1000 replicates. An additional NJ search was also performed. For ML settings, HKY85 was chosen as nucleotide substitution model, Ti/Tv ratio and rate distribution is set to gamma, shape parameter is set to estimate, and molecular clock was not enforced. The sequence addition was set to as-is in the heuristic search under ML criteria.

A Kishino-Hasegawa test (Kishino & Hasegawa, 1989) was conducted to examine the differences among the trees obtained from different tree search methods. Tree lengths were used as scores to distinguish the optimal and suboptimal trees based on parsimony method, and  $2(-\ln L_1 + \ln L_2)$  values were used for ML method as for likelihood ratio test. The ML model used is HKY85 with rate shape parameter gamma estimated (HKY85+ $\Gamma$  model).

## RESULTS

The 73-taxa data matrix contains 1366 characters, of which 264 are parsimony informative. There are 5059 most parsimonious trees found in the heuristic search with tree length = 1331 from 10 replicates for sequence addition on tree searching. The two-step tree searching approach generated 1387 MP trees with only 43 trees that were not found in the first run of one-step tree searching. The consensus of all 5102 trees is shown in Figure 1, and is very similar to the NJ tree in general appearance, although the overall bootstrap values and decay indices only show moderate support. Nonetheless, it is clear that the newly sequenced *Endosamara racemosa* and *Callerya vasta* are both in the Hologalegina clade, and *Endosamara racemosa* is sister to *Millettia japonica*.

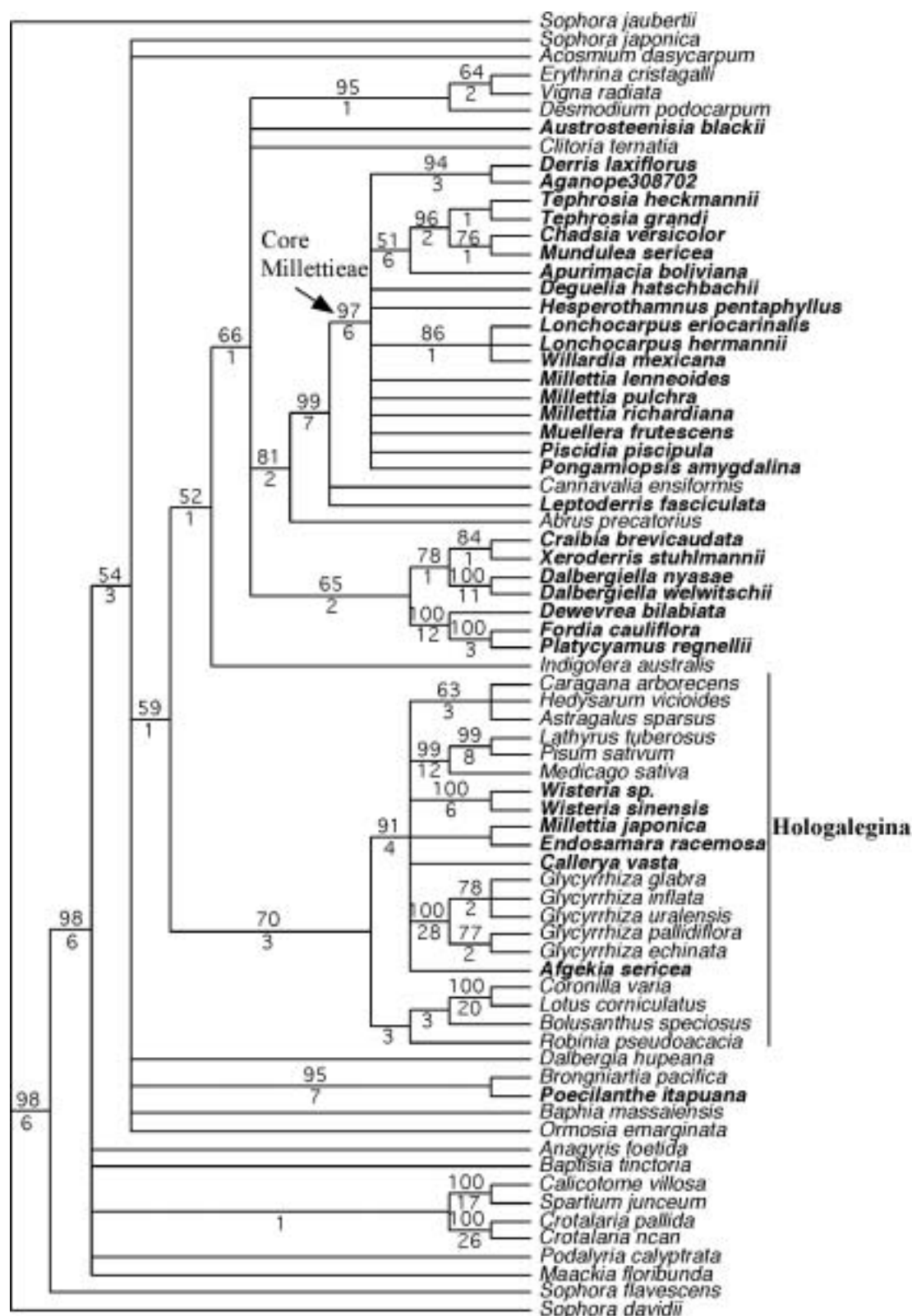


Fig. 1. Strict consensus of 5102 most parsimonious trees for 73-taxon data set. The numbers above the branches are bootstrap values from 1000 replicates using NJ criteria (only >50 are shown); the numbers below are decay indices. All Millettieae taxa are shown in bold face.

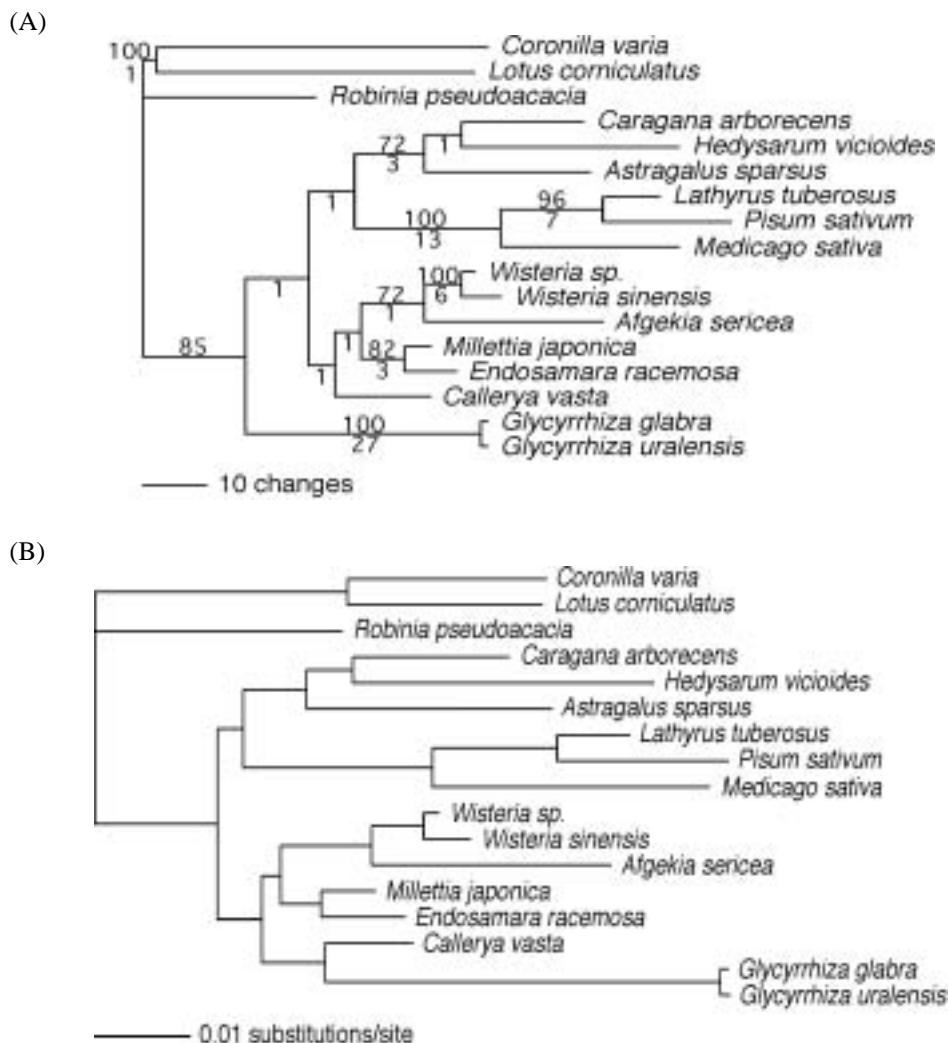


Fig. 2. Phylogenetic relationship among *Callerya* group by 17-taxa analysis. (A) The most parsimony tree obtained by branch-and-bound search. The numbers above the branches are bootstrap values from 1000 replicates using MP criteria (only >50 are shown); the numbers below are decay indices. (B) Maximum likelihood tree ( $-\ln L = 4438.63124$ ).

The 17-taxa data set contains 143 parsimony informative characters. Only one tree with tree length = 460 was obtained by branch-and-bound searching using parsimony criteria (MP tree), with tree CI = 0.64 and RI = 0.60. The obtained tree is identical to the NJ tree and is shown in Figure 2(A), with bootstrap and decay indices shown on the branches. The ML tree ( $-\ln L = 4438.63124$ ) shows similar relationships to the MP/NJ tree, but differs in position of the two *Glycyrrhiza* species (see Fig. 2). In MP/NJ tree, *Glycyrrhiza* is sister to the rest of Hologalegina, whereas in ML tree, *Glycyrrhiza* is sister to *Callerya vasta*. In either case, *Endosamara racemosa* and *Millettia japonica* are sister groups.

Table 3 shows the result of Kishino-Hasegawa test comparing MP tree and ML tree. Tree lengths using parsimony scores of MP and ML trees only differ in one step, and no significance was found. However, the  $-\ln L$  is significantly better for the ML tree than the MP tree. That is, under HKY85+ $\Gamma$  model of substitution, MP tree is rejected as alternative phylogeny.

Table 3. Result of Kishino-Hasegawa test comparing the MP and ML trees in Fig. 2 under the null hypothesis of no difference between the two trees. *L* is likelihood score. Asterisk denotes statistic significance of *P* value.

	MP		ML	
	Length	<i>P</i>	-ln <i>L</i>	<i>P</i>
MP tree	460	-	4443.65144	0.000*
ML tree	461	0.7631	4438.63124	-

## DISCUSSION

The relationships among all the *Millettieae* species are mostly congruent with the tree from the analysis by Kajita *et al.* (2001), with only a few differences, but are only in the nodes receiving low bootstrap support. It is apparent that the newly sampled *Endosamara racemosa* and *Callerya vasta* belong to the *Callerya* group, but it is uncertain for the relationships among the 18 currently known *Callerya* group members, since many taxa used in the nrITS and chloroplast *trnK/matK* data were not sampled in the *rbcL* data set. The result marks the fifth genera of *Millettieae* to be included in the IRLC based on molecular evidence. It would be interesting to examine the chloroplast inverted repeat condition and the chromosome number of *Endosamara racemosa*, as well as *Afgekia pilipes* and *Callerya vasta*.

The result of 17-taxa analysis shows a monophyletic *Callerya* clade, despite the somewhat peculiar position of *Glycyrrhiza*. The incongruence between MP and ML trees is probably due to the long branch leading to the two *Glycyrrhiza* sequences (long branch attraction, Felsenstein, 1978). Kishino-Hasegawa test indicated that the incongruence could be significant using maximum likelihood scores, but it is not significant if using parsimony as tree searching criteria. Although the support of monophyly of *Callerya* group is not high, all the former *Millettieae* members, *Callerya*, *Wisteria*, *Afgekia*, *Endosamara*, and *Millettia japonica*, are likely closely related. All of the above taxa are distributed in the Old World, range from East Asia to northeastern Australia, except for *Wisteria*, which is northeastern Asia and northeastern America distributed. It states again that the polymorphic genus *Millettia* is now urged for a revision.

It is indeed not very surprised that the two newly sampled taxa fell into *Callerya* group since they have both placed in the polymorphic *Millettia*, and show several morphological similarities with other *Callerya* species. *Callerya vasta* is no doubt part of *Callerya* group since it has all the diagnostic features that characterize *Callerya* (Schot, 1994), i.e. true panicle and diadelphous stamens. In comparison, *Endosamara* is quite distinct in having some unique characters that make it difficult to speculate its phylogenetic relationships with other *Millettieae* taxa. Below we briefly review some of the taxonomic history regarding to *Callerya* and *Endosamara*, with some notes on the current status of *Millettia*.

There are 16 sections of *Millettia* recognized in the first and only monograph by Dunn (1912). Three of the sections are now placed in the *Callerya* clade, and are distantly related with other *Millettia*. They are sections *Eurybotryae* (~12 species), *Austro-millettia* (three species) and *Bracteatae* (one species), the former two were treated under *Callerya* and the later under *Endosamara* by Geesink (1984). Section *Albiflorae* (four species) of *Millettia* was moved to *Imbralyx* (Geesink, 1984), which was later listed as a synonym of *Fordia* Hemsley by Buijsen (1988) and Schot (1991). Several other small taxa were moved in and out of *Millettia*, but most are within the "*Millettia* allies" group (Table 4).

Table 4. Current status of the *Milletia* sections. Section names are based on Dunn (1912) and the revision of Gillett (1961), who established Sect. *Berrebera*. Several key features delimit the sections are listed.

Section	Current status	Approximate spp. no.	Geographic distribution	Inflorescence	Upper filament free	Brachyblast
<i>Typicae</i>		8	India, Indo-China, and Taiwan	Pseudoraceme	No	Present
<i>Eurybotryae</i>	Moved under genus <i>Callerya</i> (Geesink 1984)	13	India, Indo-China, and Taiwan	Panicle	Yes	Absent
<i>Austromilletia</i>	Moved under genus <i>Callerya</i> (Geesink 1984)	3	N. E. Australia	Panicle	Yes	Absent
<i>Bracteatae</i>	Raised as genus <i>Endosamara</i> (Geesink 1984)	1-2	South India, Indo-China, to the Philippines	Panicle	Yes	Absent
<i>Podocarpae</i>		5	S. China to Indo-China	Pseudoraceme	Yes	Present
<i>Macraspermae</i>		4	S. China to S. E. Asia	Pseudopanicle/ Pseudoraceme	No	Present
<i>Fragiliflorae</i>		16	S. E. Asia	Pseudoraceme	No	Present
<i>Quosema</i>		11	S. E. Asia	Pseudopanicle/ Pseudoraceme	Yes/No	Present
<i>Albiflorae</i>	Raised as genus <i>Imbralyx</i> (Geesink 1984)	4	S. China to S. E. Asia	Panicle/ pseudopanicle	No	Present
<i>Efulgentes</i>		32	Tropical West Africa	Pseudoraceme	No	Present
<i>Compressogemmatae</i>		7	E. to S. Africa	Pseudoraceme	Yes/No	Present
<i>Afroscandentes</i>		9	West Africa	Pseudoraceme	No	Present
<i>Truncaticalyces</i>		6	Tropical Africa	Pseudopanicle/ Pseudoraceme	No	Present
<i>Sericanthae</i>		7	Tropical Africa	Pseudoraceme	No	Present
<i>Polyphyllae</i>		4	Madagascar & Congo	Pseudoraceme	Yes/No	Present
<i>Robustiflorae</i>		1	Congo & Angola	Pseudoraceme	Yes	Present
<i>Berrebera</i>		14	Africa	Pseudoraceme	No	Present
Unplaced		89	Asia (46), Africa (43)	Pseudoraceme	-	Present

Many new species of *Millettia* have been described in regional floras since Dunn's (1912) revision. In fact, there are 221 currently accepted names under *Millettia*, excluding the four sections that have been moved out of the genus (Hu, 2000). The newly updated species number is more than twice the usual estimates, e.g. 100 species by Geesink (1981) and 90 species by Geesink (1984). This simply reflects the fact that many of the published names are only familiar to local taxonomists, and there is no comprehensive work of this complex genus for the last century.

Among the 221 *Millettia* species, ~57% are found in Africa, ~43% are found in Asia (China, India, and Southeast Asia), and 5 species (under *Hesperothamnus*) are in Mexico. The seven *Millettia* sections distributed in Africa are: *Efulgentes*, *Compresso-gemmatae*, *Afroscandentes*, *Truncaticalyces*, *Sericanthae*, *Polyphyllae*, and *Robustiflorae*. The other five *Millettia* sections are distributed exclusively in Asia: *Typicae*, *Podocarpae*, *Macrospermae*, *Fragiliflorae*, and *Otosema* (Table 4).

Wei (1985a, 1985b) rearranged several sections in his revisions of Chinese *Millettia* and recognized the paniculate *Millettia* species (= *Callerya sensu* Schot, 1994) as a group, and separated this group into three sections: *Corynecarpae*, *Eurybotryae* (*sensu stricto*), and *Curvistylae*. He placed species of Dunn's (1912) section *Eurybotryae*, which having a deflexed stigma, to a new section *Curvistylae*. The deflexed stigma, however, was not considered as a distinct feature in Schot's (1994) revision for *Callerya*. On the other hand, the deflexed stigma can also be found in some *Millettia* species. In studies of African *Millettia*, Gillett (1961) redefined *Millettia* section *Sericanthae* to include only species with glabrous petals and a style-tip sharply bent inwards, in which the stigma faces down. The rest of the species in section *Sericanthae* were then moved to a new section *Berrebera* (Gillett, 1961). In contrast, that the styles in the sect. *Eurybotryae* are not "abruptly bent" as in sect. *Berrebera*. Therefore, this feature might be due to convergent evolution.

The basic difference to distinguish *Callerya* from other *Millettia* species is that the *Callerya* inflorescence does not form a brachyblast, a shortened branch with flowers in the axil of a bract. All other members of *Millettia* have this structure, sometimes as a short peduncle with 2-5 flowers, or reduced to a wart with a few flowers, in which case it is called a pseudoraceme or pseudopanicule. Table 4 shows several morphological features delimiting the sections of *Millettia*. Currently all the taxa with paniculate inflorescence have been moved out of the genus *Millettia*, and show their affinity to IRLC legumes. It should be noted that *Endosamara racemosa*, like several other *Callerya* species, has "leafy" panicle that the terminal inflorescence sometimes bears leaves at basal position. This is probably the reason why some authors described it as having racemous inflorescence (e.g. Panigrahi & Mishra, 1985).

The free upper filament in *Callerya* is one of the major features to separate them from *Millettia* since most of the typical *Millettia* species show adnated upper filament to other stamens. Interestingly, *Endosamara racemosa* also has diadelphous stamens as described by Dunn (1912) and Geesink (1984). However, two species closely related to *E. racemosa* show monadelphous stamens: *Millettia orissae* Panigr. et S. C. Mishra (Panigrahi & Mishra, 1985) and *Millettia pseudo-racemosa* Thoth. et Ravi. (Thothathri & Ravikumar, 1997). They may represent the polymorphic situation in the filament fusion of this group if all three taxa indeed belongs to *Endosamara*. However, careful examination of filament fusion is needed for future study as some *Millettia* species show different degree of the filament cohesion during the whole maturation process (Dunn, 1912), and at times the upper filament could be pushed apart by ovary/style after pollinator stepped onto the wing/keel petals.



There are several other Millettieae taxa showing panicle inflorescence and diadelphous stamens, such as *Craibia*, *Dewevrea*, *Kunstleria*, *Ostryocarpus*, *Platycyamus*, *Behaimia*, *Sarcodum*, and *Endosamara*. The first five taxa are included in either the core-Millettieae clade or the “satellite” group at the base of Millettieae-Phaseoleae clade based on the *rbcL* data (Kajita *et al.* 2001 and this study, Fig. 1) and other analyses (see Hu *et al.*, 2002). For the rest of them, they are all potential groups linked to the Hologalegina clade. The most likely candidates are *Sarcodum* and *Antheroporum*, based on overall morphological similarity and the geographic distribution, although the later does not show free upper stamen. Another phylogenetic study using nuclear ribosomal ITS sequences is being carried out to further examine the relationships among *Callerya* group and allies (Hu, unpublished data).

### ACKNOWLEDGEMENTS

The authors thank Frits Adema at National Herbarium Nederland, University of Leiden, to help obtaining the plant materials.

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利用葉綠體 *rbcL* 的譜系分析鑑定豆科雞血藤群的兩個新成員：  
*Endosamara racemosa* (Roxb.) Geesink 和 *Callerya vasta* (Kosterm.) Schot

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(收稿日期：2003 年 5 月 2 日；接受日期：2003 年 5 月 29 日)

摘 要

本研究利用葉綠體 *rbcL* 的 DNA 序列進行譜系分析，鑑定出兩個豆科雞血藤群 (*Callerya* group) 的新成員：*Endosamara racemosa* (Roxb.) Geesink 和 *Callerya vasta* (Kosterm.) Schot。先前對於雞血藤群的研究顯示這個類群還包含了另外三個從前也在崖豆族 (*Millettieae*) 的屬：*Afgekia*，*Callerya* (雞血藤屬)，和 *Wisteria* (紫藤屬)。由目前所得的資料來看，這些屬的親源關係都和大部份的溫帶草本豆科植物較近，而同屬於一個 IRLC 群 (Inverted Repeat Lacking Clade，因其葉綠體基因體中少了一份反向重覆序列，故名之)。而雞血藤群是位在 IRLC 群的基群位置。結果並顯示 *Endosamara* 是 *Millettia japonica* 的姐妹群，而與紫藤屬和 *Afgekia* 相近。

關鍵詞：*Endosamara*、雞血藤屬、崖豆族、崖豆屬、*rbcL*、譜系分析。

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