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PHYLOGENETIC ANALYSES OF TRIBES TRIFOLIEAE AND VICIEAE, BASED ON SEQUENCES OF THE PLASTID GENE *mat*K (PAPILIONOIDEAE: LEGUMINOSAE)

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Abstract

Tribes Trifolieae and Vicieae along with Cicereae and *Galega* (Galegeae) form a monophyletic group that has been designated informally as the "vicioid clade". There is good support from analyses of various molecular data for the clade itself, but relationships of genera within the clade are not fully understood nor has monophyly of the tribes and genera been fully tested. Sequences of the plastid gene *mat*K from 84 members of the vicioid clade were analysed using maximum parsimony. Results presented here provide strong support for a monophyletic Vicieae that includes *Vicia*, *Lathyrus*, *Pisum* and *Lens*. *Vicia* is paraphyletic with regard to other genera of Vicieae, but there is support for monophyletic groups of species of *Vicia*. *Pisum* is sister to a monophyletic *Lathyrus,* and *Lens* is sister to a small group of species of *Vicia*. A monophyletic *Trifolium* is sister to the Vicieae, and together they form a moderately supported monophyletic group. Similarly, a monophyletic *Ononis* is sister to genera of tribe Trifolieae including *Medicago*, *Trigonella* and *Melilotus*. *Medicago* is monophyletic and includes previously transferred species from *Trigonella*. *Medicago* and *Trigonella* are sister taxa, but *Melilotus* is nested within *Trigonella*. Morphological and biochemical features are considered as they support particular groups within the vicioid clade.

Introduction

The tribes of temperate herbaceous papilionoid legumes long considered related to Galegeae (Carmichaelieae, Cicereae, Hedysareae, Trifolieae, Vicieae; Polhill, 1994) comprise the species-rich clade marked by the loss of one copy of the inverted repeat in plastid DNA (the IR-lacking clade or "IRLC"). Although preliminary analyses that utilised plastid *rbc*L sequence data (Doyle *et al.*, 1997) did not resolve relationships within this clade, mainly due to the limited number of taxa that were sampled, subsequent and more extensive sampling from these tribes based on nrDNA internal transcribed spacer region (ITS) sequences (Sanderson and Wojciechowski, 1996) and plastid *mat*K sequences (Wojciechowski *et al.*, 2000) have contributed both support for the monophyly of this group of taxa and explicit hypotheses for generic and tribal relationships within this clade. Within the IRLC, three large clades,

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informally termed the "astragalean", "hedysaroid" and "vicioid", are also strongly supported although relationships among them, and several genera in Galegeae, are only partly resolved (Sanderson and Wojciechowski, 1996; Wojciechowski *et al.*, 2000). In these analyses, *Glycyrrhiza* (Galegeae) along with *Callerya* and *Wisteria* of tribe Millettieae Hutch. are strongly supported as sister to the rest of the IRLC, results now substantiated by further sampling and analyses of several molecular markers (Lavin *et al.*, 1998; Hu *et al.*, 2000; Kajita *et al.*, 2001; Hu *et al.*, 2002).

One of the strongly supported clades within the IRLC, the vicioid clade, consisting of the tribes Cicereae, Trifolieae, Vicieae and the genus *Galega* (Galegeae), was first suggested by analyses of the *rpo*C genes (Liston and Wheeler, 1994). Analyses of the vicioid clade based on nrDNA ITS (Sanderson and Wojciechowski, 1996) and *matK* (Wojciechowski *et al.*, 2000) data indicate that *Cicer* and *Galega* are sister to the Vicieae and Trifolieae, although support for the relative positions of *Galega* and *Cicer* is not strong, while *Parochetus*, sometimes placed in the Trifolieae, is sister to the vicioid clade. Although the latter analyses added to our understanding of relationships within Trifolieae and Vicieae, they included a relatively small number of taxa from these two tribes, and a few genera (*Lens* and *Vavilovia*) were not sampled.

The vicioid clade is well supported in molecular analyses (e.g., Sanderson and Wojciechowski, 1996; Wojciechowski *et al.*, 2000), but had not previously been recognised (on morphological grounds) as a group of related taxa. As pointed out by Kupicha (1977) the leaflets of *Cicer* and Trifolieae (including *Ononis*) have craspedodromous venation where the veins extend to the margin into teeth, if present, while members of the Vicieae have leaflets with brochidodromous venation where the veins do not extend to the margin. Craspedodromous venation is very uncommon within Papilionoideae, but is also found in *Galega* (Polhill, 1981). Thus, craspedodromous venation appears to be a morphological synapomorphy for the vicioid clade, but has been modified in Vicieae itself.

Most recent treatments of the Vicieae recognise the following genera, *Lathyrus*, *Lens*, *Pisum*, *Vavilovia* and *Vicia* (Kupicha, 1981a; Isely, 1998). Members of this tribe are widely distributed throughout northern temperate regions, primarily Eurasia, extending into Africa, but only *Vicia* and *Lathyrus* are found in North and South America (Kupicha, 1981a; Mabberley, 1997; Isely, 1998). Kupicha (1977, 1981a, b) who considered delimitation of the tribe as a whole, excluded *Cicer*, which had often been included within Vicieae (e.g. Gunn and Kluve, 1976), as a monotypic tribe, Cicereae. While each of the genera in the Vicieae has been considered to be a natural, monophyletic group, this has not explicitly been stated or tested by phylogenetic analyses of species representing all genera of Vicieae. Instead most phylogenetic analyses to date have concentrated on a single genus (e.g. Kupicha, 1976, Asmussen and Liston, 1998) or sections within a genus as in several studies within *Vicia* (Ladizinsky, 1975; Birch *et al.*, 1985; Maxted, 1993; van de Ven *et al.*, 1996). When intergeneric phylogenetic analyses of Vicieae have been conducted, they indicate that *Lathyrus*, *Vicia* and *Pisum* (the only genera sampled) form a monophyletic group, thus supporting the current circumscription of the tribe (Wojciechowski *et al.*, 2000). However, too few species were sampled to provide reliable information on relationships of genera within the tribes.

Similarly to the Vicieae, most genera of the Trifolieae are confined to temperate regions of Europe, Asia and Africa, and only *Trifolium* is found in North and South America (Heyn, 1981). Most recent treatments of the Trifolieae (Heyn, 1981; Lock and Simpson, 1991; Mabberley, 1997; Isely, 1998) include the following genera *Medicago*, *Melilotus*, *Trigonella*, *Trifolium*, *Parochetus* and *Ononis*, although *Ononis* has sometimes been placed in its own tribe (e.g. Hutchinson, 1964). Analyses of *mat*K sequence data support the inclusion of *Ononis* in the Trifolieae (Wojciechowski *et al.*, 2000). The placement of *Parochetus* in Trifolieae has been considered doubtful (e.g. Small, 1987b), and analyses of *mat*K sequence data (Wojciechowski *et al.*, 2000) do not support inclusion of the genus in Trifolieae as currently circumscribed (rather,

Parochetus is sister to the vicioid clade). The placement of *Factorovskya* in *Medicago* by Small and Brooks (1984) is recognised by most recent authors (e.g. Lock and Simpson, 1991; Polhill, 1994) and is supported by analyses of nrDNA ITS sequence data (Downie *et al.*, 1998) and *mat*K sequence data (Steele *et al.*, 1999). As with the Vicieae, most genera in the Trifolieae are thought to be monophyletic, although this has not been rigorously tested for most genera. Generic delimitation of *Trigonella* and *Medicago* has been especially problematic (e.g. Small, 1987a) and Small (1987b, 1989) moved approximately 23 "medicagoid" species from *Trigonella* to *Medicago* (sections *Buceras* and *Lunatae*) on the basis of floral characters related to the explosive pollination syndrome. Analysis of *mat*K sequence data (Wojciechowski *et al.*, 2000), although limited in sampling, indicated that *Medicago* (including "medicagoid" species) and *Trigonella* are monophyletic sister groups while *Melilotus* is either nested within *Trigonella* or sister to it. Analyses of the nrDNA ITS and external transcribed spacer region (ETS) sequence data (Bena, 2001) with a larger sample of species confirmed the relationship of the "medicagoid" *Trigonella* to *Medicago* and indicated that *Melilotus* is nested within *Trigonella*. *Medicago radiata* L. recognised as a monotypic genus, *Radiata*, by Yakovlev and colleagues (1996) is also included within *Medicago* (Bena, 2001). *Trifolium* has always been included within Trifolieae, but analyses of *mat*K sequence data suggested that it is sister to all genera within Vicieae rather than to other genera of Trifolieae, although this relationship is not strongly supported (Wojciechowski *et al.*, 2000). The relationships of segregates from *Medicago* (*Melilotoides*) and *Trifolium* (*Armoria*, *Chrysaspis* and *Lupinaster*), which have been recognised by Yakovlev *et al.* (1996), are unclear and have not been sufficiently sampled in previous analyses of molecular data.

Thus, preliminary hypotheses of relationships within the vicioid clade based on molecular data (summarised in Wojciechowski *et al.*, 2000) provide a basis for a more extensive phylogenetic analysis of Trifolieae and Vicieae that samples more species from the larger genera and samples each genus broadly both geographically and taxonomically. Our goal in this paper is to test hypotheses of tribal and generic monophyly and to provide an estimate of relationships among the genera in Trifolieae and Vicieae that would serve as a framework for further investigations.

In addition, a number of morphological and biochemical characteristics whose evolution within Trifolieae and Vicieae have long been considered to have taxonomic significance will be discussed in the context of the phylogeny of these tribes derived from our analyses of *mat*K sequences. Two interesting characters are the presence or absence of the non-protein amino acid canavanine and trifoliate leaves (Kupicha, 1976, 1981b; Bell *et al.*, 1978; Heyn, 1981). The distribution of the non-protein amino acids like canavanine has been thought to have some systematic significance in papilionoids (Birdsong *et al.*, 1960; Bell, 1971; Bell *et al.*, 1978) and their presence now appears to be a synapomorphy for a monophyletic group composed of two of four major clades within Papilionoideae (Wojciechowski, 2003). Canavanine is present in nearly all members of the IRLC; within the vicioid clade it is found in *Galega orientalis* and all tested species of *Medicago*, *Ononis*, *Trigonella* and *Melilotus* and *Trifolium*, but is absent in some species of *Vicia* and from all species of *Lathyrus*, *Pisum* and *Lens* (summarized in Bell, 1971). The presence of canavanine has not been tested in *Parochetus* nor in non-cultivated species of *Cicer* (it is absent from *Cicer arietinum* L., the cultivated chick pea). Trifoliate leaves are found in a number of lineages within Papilionoideae such as *Psoralea*, *Rupertia* and *Phaseolus* (Grimes, 1990) and in some members of the vicioid clade (*Parochetus* and genera in Trifolieae have primarily trifoliate leaves), although most genera within Papilionoideae and the IRLC, including *Cicer* and *Galega* have pinnately compound leaves (Heyn, 1981; Kupicha, 1976, 1981a, b; Polhill, 1981). Phylogenetic reconstruction of the vicioid clade will allow us to consider hypotheses about the presence or absence of canavanine and the origin of trifoliate leaves.

Materials and methods

Taxonomic Sampling

Total genomic DNA from species of Trifolieae and Vicieae was isolated from fresh material (as in Steele and Vilgalys, 1994) derived from plants grown from seeds (obtained from E. Small or the United States Department of Agriculture Plant Introduction Program) or from plants collected in the field. The sources of plant material for DNA isolation and GenBank accession numbers for *mat*K sequences from all taxa included in analyses in this paper are provided in Table 1. Seven sequences from a previous study (Wojciechowski *et al.*, 2000) were also included. Voucher specimens for all new taxa sampled are deposited at ASU.

Our sampling strategy for *mat*K sequence data was designed to represent all genera in the Trifolieae and Vicieae and includes the following number of representatives from each genus (# sampled/total number of species in genus; see reference): *Medicago* (19/86 species; Downie *et al.*, 1998); *Trigonella* (13/50 species; Small, 1987b), *Melilotus* (2/20 species; Isely, 1998); *Ononis* (3/70 species; Lock and Simpson, 1991); *Vicia* (10/140), *Lens* (2/5), *Pisum* (1/2) and *Lathyrus* (5/150) (Kupicha, 1986); and *Trifolium* (23/250 species; Zohary and Heller, 1984). A total of 78 ingroup taxa were sampled. Difficulties in amplifying complete *mat*K sequences from *Ononis* contributed to the low number of sequences from that genus included in our analyses. In addition, the genus *Vavilovia*, a monotypic segregate from *Pisum*, was not sampled for the present study.

Sampling for the phylogenetic analyses reported here had as its basis a more extensive study of relationships of species within the genus *Medicago* (Steele and Wojciechowski, unpublished data), thus we had more samples of *Medicago* than of any other genus. We chose to limit the number of sequences of *Medicago* in the analyses presented here and included species based on our results of analyses of a larger number of *mat*K sequences and studies of other workers (e.g. Small, 1987a; Small, 1989; Small and Jomphe, 1989; Downie *et al.*, 1998; Cook, 1999). Eight of the 19 *Medicago* species included have been placed in the genus *Trigonella* (Small, 1987a, b; Small, 1989; Small and Jomphe, 1989).

Molecular methods

Protocols for PCR amplification of the *mat*K coding region and the flanking 5' *trn*K intron were similar to those described previously (Steele and Vilgalys, 1994; Hu *et al.*, 2000). Primers designed for these two studies as well as additional primers are given in Table 2. Double stranded PCR products were purified using Millipore centrifugal filters (Ultrafree) and sequenced using cycle-sequencing protocols and fluorescently labelled dye terminator chemistries on a Perkin Elmer ABI 377 DNA Sequencer at California State University, Hayward, or by Davis Sequencing, Davis, CA (http://www.davissequencing.com/). Sequence output files were edited and assembled into 'contigs' using the program Sequencher 3.1and 4.1 (GeneCodes); sequences were aligned using that same program and adjusted manually, if necessary. The data matrix has been deposited in TreeBASE (http://www.treebase.org/treebase/index.html) and is available from the second author (http://ls.la.asu.edu/plantbiology/faculty/wojciechowski.html).

Phylogenetic analyses

All positions in the dataset having insertion and deletions ('indels') were excluded from analyses. As there were only a very small number of variable positions in the insertion sequences, little phylogenetic information was lost by this method of character coding. Most indels were autapomorphies, but a very few are shared among two or more species and may constitute synapomorphies for those taxa. The sequences used were complete with the exception of the sequence for *Trifolium dubium*, our only representative of section *Chronosemium*.

TABLE 1. Accession information for all taxa included in *mat*K gene analyses. Source column indicates the source of seeds or field collected material. Abbreviations used: USDA, seeds accessions obtained from US Department of Agriculture Plant Introduction program; Small, seed accessions obtained from E. Small (Ottawa, Canada). Voucher specimens for all new accessions obtained as part of this study deposited at ASU. GenBank accession numbers for *mat*K sequences are indicated.

Species	Source/voucher specimen	GenBank accession number
Cicer canariense A. Santos & G.P. Lewis	USDA 557453	AF522079
Cicer macracanthum Popov	USDA 599080	AF522080
Cicer pinnatifidum Jaub. & Spach	USDA 458555; *Wojciechowski & Sanderson* 409 (ARIZ)	AF522081
Cicer yamashitae Kitam.	USDA 504550	AF522082
Galega orientalis Lam.	USDA 325337; *Wojciechowski & Sanderson* 399 (ASU)	AF522083
Lathyrus aphaca L.	USDA 286527	AF522084
Lathyrus latifolius L.	*Wojciechowski* 543 (DAV)	AF522085
Lathyrus sativus L.	USDA 283562	AF522086
Lathyrus tingitanus L.	USDA 451858	AF522087
Lathyrus vestitus Nutt.	KPS, s.n.	AF522088
Lens culinaris Medik.	USDA 172938	AF522089
Lens ervoides (Brign.) Grande	USDA 572330	AF522090
Medicago biflora (Griseb.) E. Small	USDA 464827	AF522091
Medicago brachycarpa M. Bieb.	USDA 244326	AF522092
Medicago heyniana Greuter	*Small* M-839	AF522093
Medicago hypogaea E. Small	*Small* F-177	AF522094
Medicago italica (Miller) Fiori	USDA PI 385014	AF522095
Medicago lanigera C. Winkl. & B. Fedtsch.	*Small* M-1569	AF522096
Medicago medicaginoides (Retz.) E. Small	*Small* T-264	AF522097
Medicago monantha (C.A. Mey.) Trautv.	*Small* T-251	AF522098
Medicago monspeliaca (L.) Trautv.	*Small* T-252	AF522099
Medicago noena Boiss.	USDA 495404	AF5220100
Medicago orbicularis (L.) Bartal	*Small* M-316	AF5220101
Medicago platycarpa (L.) Trautv.	USDA 257499	AF5220102
Medicago polyceratia (L.) Trautv.	*Small* T-254	AF5220103
Medicago polymorpha L.	KPS , s.n.	AF5220104
Medicago prostrata Jacq.	USDA 577453	AF5220105
Medicago radiata L.	USDA 459140	AF5220106
Medicago ruthenica (L.) Ledeb.	*Small* T-263	AF5220107
Medicago sativa L.	KPS , s.n.	AF5220108
Medicago truncatula Gaertn.	*Small* T-264	AF5220109
Melilotus alba Medik.	KPS, s.n.	AF5220110
Melilotus indica (L.) All.	KPS, s.n.	AF5220111
Ononis arvensis L.	USDA 440578	AF5220112
Ononis biflora Desf.	USDA 244319	AF5220113
Ononis natrix L.	USDA 246743	AF5220114
Parochetus communis Buch.-Ham. ex D. Don	*A. Liston* (cultivated)	AF5220115
Pisum sativum L.	Boyer and Mullet (1988)	

TABLE 1 continued

Trifolium albopurpureum Torr. & A. Gray	KPS, s.n.	AF5220116
Trifolium burchellianum Ser.	USDA 369915	AF5220118
Trifolium caucasicum Tausch	USDA 597496	AF5220119
Trifolium cheranangiense J.B. Gillett	USDA 234413	AF5220120
Trifolium dubium Sibth.	KPS, s.n.	AF5220121
Trifolium fragiferum L.	KPS, s.n.	AF5220122
Trifolium gracilentum Torr. & A. Gray	KPS, s.n.	AF5220123
Trifolium hirtum All.	KPS, s.n.	AF5220124
Trifolium hybridum L.	USDA 184554	AF5220125
Trifolium incarnatum L.	KPS, s.n.	AF5220126
Trifolium lupinaster L.	USDA 604717	AF5220127
Trifolium microcephalum Pursh	KPS, s.n.	AF5220128
Trifolium nanum Torr.	University of Colorado EPOB collection 4520 (1992) (ARIZ)	AF5220129
Trifolium patulum Tausch	USDA 31403	AF5220130
Trifolium repens L.	KPS, s.n.	AF5220131
Trifolium resupinatum L.	KPS, s.n.	AF5220117
Trifolium semipilosum Fresen.	USDA PI 208956	AF5220132
Trifolium spumosum L.	USDA PI 141510	AF5220133
Trifolium striatum L.	KPS, s.n.	AF5220134
Trifolium subterraneum L.	KPS, s.n. q	AF5220135
Trifolium thallii Vill.	USDA PI 371858	AF5220136
Trifolium willdenovii Spreng.	KPS, s.n.	AF5220137
Trifolium wormskoldii Lehm.	KPS, s.n.	AF5220138
Trigonella arabica Delile	USDA PI 194476	AF5220139
Trigonella balsanae Boiss. & Reut.	USDA PI 222221	AF5220140
Trigonella bicolor (Boiss. & Balansa) Lassen	*Small* L-309	AF5220141
Trigonella calliceras Fisch. ex Bieb.	*Small* T-239	AF5220142
Trigonella caerula (L.) Ser.	*Small* T270	AF5220143
Trigonella caerulescens (Bieb.) Halácsy	USDA 314398	AF5220144
Trigonella corniculata (L.) L.	USDA 220123	AF5220145
Trigonella cretica (L.) Boiss.	USDA 415833	AF5220146
Trigonella foenum-graecum L.	USDA 567879	AF5220147
Trigonella gladiata Steven ex Bieb.	USDA 203474	AF5220148
Trigonella kotschyi Benth.	USDA 206775	AF5220149
Trigonella macrorrhyncha Boiss.	USDA 222232	AF5220150
Trigonella spruneriana Boiss.	USDA 352710	AF5220151
Vicia americana Willd.	KPS, s.n.	AF5220152
Vicia articulata Hornem.	USDA 206390	AF5220153
Vicia bengalensis L.	USDA 393833	AF5220154
Vicia gigantea Hook.	KPS, s.n.	AF5220155
Vicia grandiflora Scop.	USDA 602377	AF5220156
Vicia hirsuta (L.) Gray	KPS, s.n.	AF5220157
Vicia ludoviciana Nutt.	*McLaughlin & Bower* 3185 (ARIZ)	AF5220158
Vicia lutea L.	USDA 199265	AF5220159
Vicia sativa L.	KPS, s.n.	AF5220160
Vicia villosa Roth	KPS, s.n.	AF5220161

TABLE 2. Primers used for both polymerase chain reaction amplification and direct sequencing of the chloroplast *mat*K gene and flanking *trn*K intron regions. All primer sequences are shown 5' to 3'; unless specified (as in "R", for reverse) all are forward primers with respect to direction of transcription of the *mat*K gene, i.e., from 5' *trn*K exon toward 3' *trn*K exon.

trnK1L	CTCAATGGTAGAGTACTCG
trnK2R*	CCCGGAACTAGTCGGATGG
matK4L	CTTCGCTACTGGGTGAAAGATG
matK4La	CCTTCGATACTGGGTGAAAGAT
trnK685F	GTATCGCACTATGTATCATTTGA
trnK708R	TCAAATGATACATAGTGCGATAC
matK1100L	TTCAGTGGTACGGAGTCAAATG
matK1932Ra	CCAGACCGGCTTACTAATGGG

Results of previous analyses of subfamily Papilionoideae and of the temperate herbaceous tribes (Lavin *et al.*, 1998; Hu *et al.*, 2000; Wojciechowski *et al.*, 2000; Kajita *et al.*, 2001; Hu *et al.*, 2002) were used as a basis for determining outgroups for analyses of Trifolieae and Vicieae. *Parochetus communis*, four species of *Cicer* (Cicereae; representing three of the four sections within the genus (van der Maesen, 1987)) and *Galega orientalis* (Galegeae) were used as outgroups.

Maximum parsimony analyses were performed using heuristic search strategies, as implemented in PAUP* 4.0b8 (Swofford, 2001), and the following options: SIMPLE sequence addition, TBR branch swapping, and an automatic increase in the maximum number of trees to be retained while branch swapping. All characters were unweighted and unordered. Relative level of support for individual clades was estimated by bootstrap analysis (Felsenstein, 1985) as implemented in PAUP* 4.0b8 (100 replicate samples) using identical heuristic searches (Swofford, 2001). The maximum number of trees to be retained per replicate was limited to 360 for bootstrap analyses (the number of most parsimonious trees produced).

Results

The aligned *mat*K data set consists of 84 *mat*K sequences with a total length of 1614 bp (78 ingroup taxa and six outgroups), of which 171 were excluded as indels. A total of 335 parsimony informative characters (23%) were present. Analyses using maximum parsimony resulted in 360 most parsimonious trees with a length of 910 steps. Figure 1 is a majority-rule bootstrap consensus tree resulting from parsimony analysis with values above 50% indicated on branches. Selected subgeneric taxa for the genera *Medicago* (Small, 1987b), *Trifolium* (Zohary and Heller, 1984), and *Vicia* (Kupicha, 1977) are also indicated. The ingroup consists of two groups corresponding to the Trifolieae and Vicieae with one important exception. One group (supported at the 71% level) includes *Trifolium* as sister to all sampled genera of Vicieae, *Vicia*, *Lens*, *Pisum* and *Lathyrus*. Another group (supported at the 77% level) has *Ononis* as sister to a well-supported group formed by *Medicago*, *Melilotus* and *Trigonella*. These latter three genera have been recognised as tribe Trigonelleae by Schultz (1901) and subtribe Trigonellinae by Small (1987b).

Trigonella and *Medicago* each form well-supported clades that are sister to one another. Two species of *Melilotus* form a group that is nested within *Trigonella*. Thus *Trigonella* is paraphyletic with regard to *Melilotus*. Species of *Medicago* in the sections *Lunatae*, *Buceras* and *Platycarpae* that were formerly in *Trigonella* (Small 1987a, 1989) are included with all other species of *Medicago* (95% bootstrap support). Species in section *Buceras* and *Medicago radiata* (66% bootstrap support) are sister to all other species of *Medicago*, a group with 93% bootstrap support. Within that latter group sections *Platycarpae* and *Lunatae*, *M. hypogaea* and *M. lanigera* form a group with 65% bootstrap support that is sister to a well-supported group of the remaining species of *Medicago* (91% bootstrap support).

All genera traditionally classified in Vicieae form a monophyletic group, supported at the 100% bootstrap level in all analyses. Three groups of species and a single species (*Vicia gigantea*) form a polytomy within a group with weak bootstrap support (52%) that is sister to two species *V. articulata* and *V. hirsuta*. One group (clade 1) is that formed by species of *Lathryus* and *Pisum* (100% bootstrap support). Species of *Lathyrus* form a monophyletic group (92% bootstrap support) which is sister to *Pisum sativum*. Another group (clade 2) is formed by two species of *Lens* which are basal to four species of *Vicia*, three from subgenus *Vicia* section *Vicia* (*V. grandiflora*, *V. lutea* and *V. sativa*), and *V. americana*, subgenus *Vicilla*, section *Americanae*. This modestly supported group of six species (72% bootstrap support) is also supported by the presence of a unique nine base pair deletion in the *mat*K coding region. A third group consists of *Vicia benghalensis*, *V. ludoviciana* and *V. villosa*, all of which are in subgenus *Vicilla*, section *Cracca* (Kupicha, 1977) (100% bootstrap support). Note that species in the genus *Vicia* do not form a monophyletic group in any of our analyses, the genus is paraphyletic with *Lathyrus*, *Pisum* and *Lens* nested within it.

Species of *Trifolium* form a well supported monophyletic group (96% bootstrap support). *Trifolium dubium* is sister to all other species of *Trifolium* with *T. lupinaster* being sister to the remainder of species of *Trifolium* (99% bootstrap support) that form two well-supported groups of species (each with 100% bootstrap support). Members of one of the groups consists of 5 species in section *Trifolium* plus *T. subterraneum* (section *Trichocephalum*). The other group consists of species in sections *Lotoidea*, *Vesicaria*, *Mistyllus* and *Involucarium*.

Discussion

Vicieae, supported by 100% bootstrap values in all analyses, have many morphological synapomorphies including a base chromosome number of $n = 7$, a pubescent style (with a pollen brush), hypogeal germination and cotyledons that are fleshy storage organs, seed coat pattern, and an unusual vascular anatomy (Kupicha, 1975, 1977, 1981a, b; Lavin and Delgado, 1990; Lersten, 1979; Lersten and Gunn, 1982). Thus morphological and molecular data strongly support the Vicieae as currently delimited consisting of *Vicia*, *Lathyrus*, *Pisum* and *Lens*, with the exclusion of *Cicer* (Kupicha, 1977, 1981a, b). Within Vicieae there is no support for the monophyly of the genus *Vicia*, a result anticipated by Lavin and Delgado (1990) who stated "*Vicia*, in addition to showing the most variation in pollen brush morphology, lacks any diagnostic generic characters and may be paraphyletic with respect to the other genera." But there is support for groups of species of *Vicia* (clade 2 and section *Cracca*). Clade 2 consists of three species in subgenus *Vicia* with *Vicia americana* being sister to that group and with two species of *Lens* sister to the above four species; this clade has modest bootstrap support, but is also supported by a unique nine bp deletion in the coding region of *mat*K. Five of the six species in clade 2 (*V. americana* has not been tested) lack canavanine, a non-protein amino acid that is present in nearly all outgroup taxa, all members of Trifolieae, and in some species of *Vicia* (summarised in Bell *et al.*, 1978). Loss of

canavanine may be a synapomorphy for clade 2. Species in subgenus *Vicia* (within clade 2) are united by several morphological characters including presence of stipular nectaries and an inflorescence much shorter than the subtending leaf (Kupicha, 1976). Species sampled from subgenus *Vicilla* section *Cracca* form a well-supported group (Fig. 1). Nearly all species in section *Cracca* are united by the possession of a laterally compressed style, a trait found outside the section only in *Vicia dennesiana* (not sampled and possibly extinct) (Kupicha, 1976). But, *Vicia hirsuta*, also placed in section *Cracca* by Kupicha (1977), forms a strongly supported group with *V. articulata* (section *Ervoides*). Note that *V. hirsuta* has a terete style with evenly distributed pubescence unlike that of other species in section *Cracca*, thus morphological characteristics and results shown in Fig. 1 do not support its placement in that section. The similarity among the small-flowered ("ervoid") species of *Vicia* (approximately 14 species) may be due to convergence and thus they were placed in different sections by Kupicha (1976); however, the two species that we sampled (*Vicia articulata* and *V. hirsuta*) do form a monophyletic group. Species of *Lens* and a few of the small-flowered species of *Vicia* (including *Vicia hirsuta*) have been segregated as the genus *Ervum* (reviewed by Gunn, 1969), but *Lens* and *V. hirsuta* do not form a monophyletic group in our analyses. While the groups discussed above are of interest, more species of *Vicia* need to be sampled to determine the monophyly of groups of species or sections within the genus.

Within Vicieae, the group formed by *Lathyrus* and *Pisum* (clade 1) is supported by the presence of pubescence only on the adaxial side of the style (Kupicha, 1977, 1981b); this is the introse pollen brush of Lavin and Delgado (1990). However, *Vicia ervilia* and *V. koeieana* (not sampled) and species in the genus *Lens* also have this type of style pubescence (Kupicha, 1981b). *Lathyrus* and *Pisum* accumulate pisatin, a phytoalexin, that is not found in other members of Vicieae (Ingham, 1981; Bisby *et al.*, 1994). In addition, both genera lack canavanine; its loss in this clade may have occurred independently from that in clade 2, but the lack of resolution among groups within Vicieae makes it unclear if *Lathyrus*, *Pisum* and *Lens* and the species of *Vicia* that lack canavanine all form a monophyletic group. Within Vicieae ptyxis (leaf posture in the bud) varies among genera; it is conduplicate or folded in *Vicia*, *Lens* and *Pisum*, but is supervolute or rolled in *Lathyrus* and *Vavilovia* (Kupicha, 1981b). The supervolute condition would be a synapomorphy for the latter two genera and we would predict that if *Vavilovia* were to be sampled and included in our *mat*K analyses it would be sister to *Lathyrus* and not to *Pisum*. The species of *Lathyrus* sampled so far always form a strongly supported monophyletic group. These results support the monophyletic nature of the genus, although increased sampling of species of both *Lathyrus* and species of *Vicia* considered to be similar would be useful to confirm the monophyly of *Lathyrus*.

The monophyly of genus *Trifolium* is strongly supported by analyses of *mat*K data (Fig. 1) and presence of a 5 bp insertion in the 5' *trn*K intron as well as by analyses of sequences of the nrDNA ITS region (unpublished data; Liston *et al.*, 2001; A. Liston, Oregon State University, pers. comm.). The monophyly of the genus is also supported by a number of morphological features such as the generally palmately trifoliate leaves, stipules adnate to the petiole and sheathing the stem, wings and keel with claws partially adnate to the base of the staminal column (Heyn, 1981; Isely, 1998; Small and Jomphe, 1989; Zohary and Heller, 1984). One of the main features that has linked *Trifolium* with *Medicago* and *Trigonella* is trifoliate leaves, but based on the molecular data, this leaf morphology has more likely evolved independently in *Trifolium*. In our analyses, *Trifolium* is never placed in a monophyletic group with other genera of Trifolieae, but instead is sister to all genera in Vicieae, a tribe that has only pinnately compound leaves (or leaves thought to be derived from pinnate leaves). This suggests that trifoliate leaves may have arisen independently in the lineage leading to *Trifolium*.

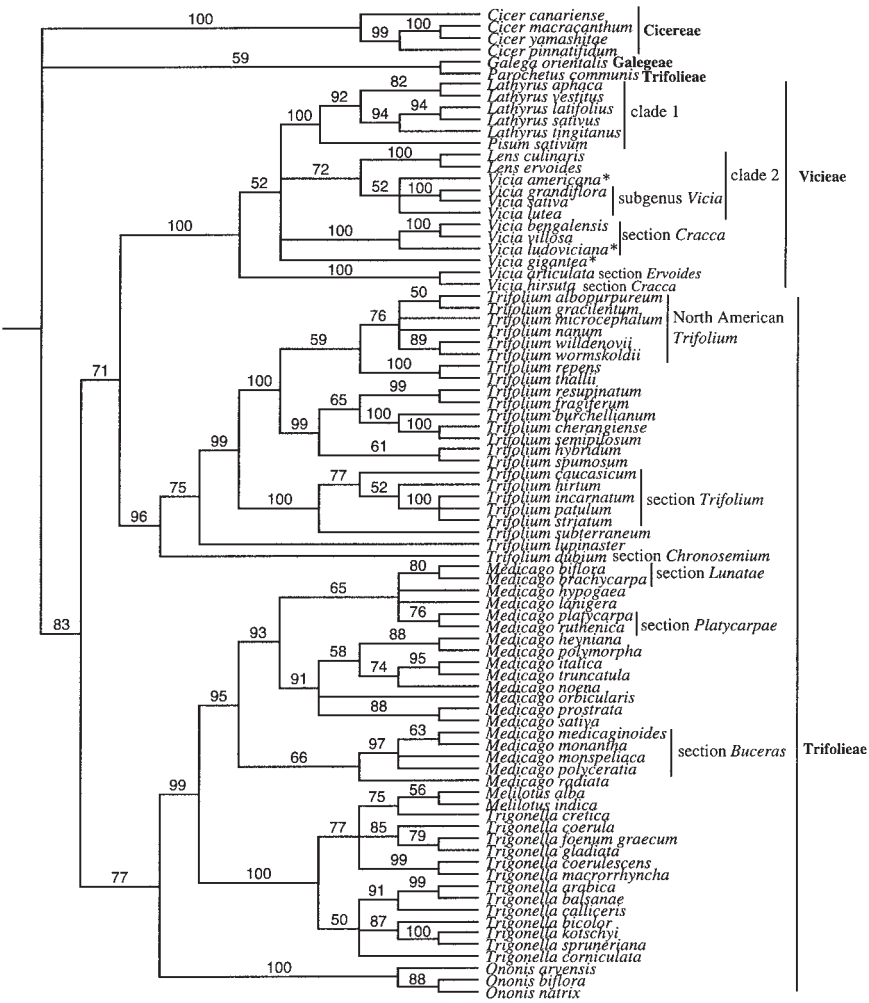

FIG. 1. Bootstrap majority rule tree from maximum parsimony analyses of *mat*K sequence data. Numbers above branches are bootstrap support values (100 replicates). Selected infrageneric taxa discussed in the text are indicated for *Vicia* (Kupicha, 1976), *Trifolium* (Zohary and Heller, 1984) and *Medicago* (Small and Jomphe, 1989). Two monophyletic groups of species and/or genera within Viceae are indicated as clades 1 and 2. North American species of *Vicia* and *Lathyrus* are indicated with an asterisk; a clade of North American *Trifolium* is directly indicated.

Reflecting a different taxonomic philosophy and perhaps a particular interest in certain genera some authors (e.g., Yakovlev *et al.*, 1996) have recognised a number of segregate genera usually included within *Trifolium*, including *Armoria*, *Chrysaspis* and *Lupinaster* with the remainder of species in *Trifolium*. The tree shown in Fig. 1 does include a strongly supported group (sects. *Trifolium* and *Trichocephalum*) corresponding to a limited *Trifolium* as recognised by Yakovlev and colleagues, but sampled species in *Armoria* (*T. fragiferum*, *T. hybrium*, *T. spumosum* and *T. repens*) do not form a monophyletic group. We only sampled one species of *Chrysaspis* (equivalent to section *Chronosemium*), but Watson and colleagues (2000) found species in section *Chronosemium* as part of a monophyletic group that is sister to the remainder of *Trifolium* when plastid restriction site data were analysed (although not when nrDNA ITS sequences were analysed). Liston and colleagues (2001; pers. comm.) analysed a large number of nrDNA ITS sequences: they found a monophyletic group of species in section *Chronosemium* to be sister to the remainder of *Trifolium*. We sampled only one species of *Lupinaster* (equivalent to section *Lotoideae* subsection *Lupinaster*) and so cannot comment on the monophyly of the segregate genus *Lupinaster*. In summary, since *Trifolium s.l.* is strongly supported to be monophyletic based on both morphological and molecular data but relationships within the genus are not completely understood, it is perhaps best to recognise *Trifolium s.l.* and to consider recognition of monophyletic groups within it at the infrageneric level.

Ononis is sister to a clade comprised of *Medicago*, *Trigonella* and *Melilotus* (Fig. 1). The latter three genera are united in having stipules that are adnate to the petiole, but that do not sheath the stem as in *Trifolium*. All other taxa in the vicioid clade and the trifoliate *Parochetus* have free stipules (Heyn, 1981; Small, 1989). The relationship of the genus *Ononis* to other genera in Trifolieae has been controversial, primarily because *Ononis* has a number of autapomorphies, including stamens that are monadelphous with dimorphic anthers, viscid leaves, and are species generally polyploid (but do appear to have a base chromosome number of $n = 8$) (Heyn, 1981; Kupicha, 1977). Results of analyses of *mat*K data support the placement of *Ononis* within a Trifolieae lacking *Trifolium*. *Ononis* is undoubtedly monophyletic; our data and all morphological data support that hypothesis. It is likely that trifoliate leaves arose independently in *Ononis*; *Ononis* includes species characterised by pinnate leaves as well as species with trifoliate leaves and some species with unifoliate leaves. In addition, some species have the lowermost leaves pinnately compound, with the upper leaves being trifoliate (Ivimey-Cook, 1968). Trifoliate leaves have most likely evolved within the genus *Ononis* and independently in the common ancestor of *Medicago*, *Trigonella* and *Melilotus* (the latter three genera have exclusively pinnately trifoliate leaves), although a larger number of species of *Ononis* need to be included in a phylogenetic analysis to determine the pattern of distribution of leaf type within the genus. Overall, *Ononis* is similar to *Trifolium* in that both are distinctive lineages with a number of autapomorphies that are sister to very strongly supported groups.

A group formed by *Medicago*, *Trigonella* and *Melilotus* has been recognised as subtribe Trigonellinae by Small (1989) and is united by the consistent presence of pinnately trifoliate leaves. This group is always present in analyses of *mat*K data and has high bootstrap support (99%). Likewise, *Medicago* and *Trigonella* are strongly supported as sister taxa in all analyses. Species of *Medicago* are united by a number of morphological features including a number of floral features associated with the explosive pollination syndrome (Small and Jomphe, 1989) and the presence of hemolytic saponins (Jurzysta *et al.*, 1988). The high level of bootstrap support for *Medicago* provides strong support for an expanded *Medicago* that includes 23 species formerly included in *Trigonella* (the "medicagoid" *Trigonella)*, and the inclusion of *M. hypogaea* formerly recognised as *Factorovskya aschersoniana* (Small, 1987b; Small and Brooks, 1984; Small and Jomphe, 1989). Analyses of nrDNA ITS and ETS data also support the inclusion of the "medicagoid" *Trigonella* in the genus *Medicago* (Bena,

2001). Two segregate genera *Radiata* and *Melilotoides* are recognised by Yakovlev and colleagues (1996), the former includes two species (both segregates of *Medicago radiata* L.), while the latter includes species placed by Small into sections *Platycarpae* and *Lunatae*. Yakovlev and colleagues (1996) retain species of section *Buceras* in *Trigonella*. Species of *Melilotoides* (*M. biflora*, *M. brachycarpa*, *M. platycarpa* and *M. ruthenica*) form a weakly supported monophyletic group, but one that includes a number of other species of *Medicago* (Fig. 1). Although *M. radiata* is part of a weakly supported group basal to all other species of *Medicago*, it is included in a monophyletic group of all species of *Medicago* supported at the 95% bootstrap level. Overall, the delimitation of the genus *Medicago* by Small and colleagues (e.g. Small and Jomphe, 1989) is the one best supported by the analyses of *mat*K data presented here and by ITS and ETS data (Bena, 2001).

Species of *Trigonella* (and *Melilotus*) form a monophyletic group in our analyses (100% bootstrap support), although there are no known synapomorphies for *Trigonella* and it has been regarded as an "umbrella" genus that included any species which could not be conventionally placed into either *Medicago* or *Melilotus* (Small, 1987b). Unfortunately, we were unable to sample species of *Trigonella* section *Ellipticae*. These perennial species are found in the mountains of Afghanistan and nearby areas; even herbarium specimens of these species are rare. Thus section *Ellipticae* should be sampled before ruling out the possibility that *Trigonella* is paraphyletic with regard to *Medicago*. The two species of *Melilotus* sampled form a monophyletic group that is nested within *Trigonella*; this is consistent with movement of species between the two genera (Small, 1987b), earlier analyses of *mat*K data (Wojciechowski *et al.*, 2000) and analyses of subtribe Trigonellinae using nrDNA ITS and ETS data (Bena, 2001). A close relationship between the two genera is supported by a number of characters, for example, some species of *Trigonella* and nearly all species of *Melilotus* release coumarin upon maceration of leaf tissue, while species of *Medicago* and *Trifolium* are coumarin negative (Ingham, 1981). *Melilotus* is defined on the basis of few or single-seeded, indehiscent fruits and flowers in slender racemes of small flowers (Isely, 1998). A reduction in number of seeds per fruit has occurred independently within a number of lineages in the vicioid clade, most notably in *Medicago* and *Trifolium*. In those two genera, some groups of species with one or two seeds per fruit are recognised at the sectional level, sections *Lupularia* and *Trifolium* respectively.

Of the 12 genera in the vicioid clade only three of them (*Trifolium*, *Vicia* and *Lathyrus*) have representatives that are endemic to North and South America as well as in Eurasia and Africa (Heyn, 1981; Kupicha, 1981a, b; Polhill, 1981). Interestingly, these three genera are placed in one clade, although the presence of each in the Americas must be independent of the other. Within *Trifolium*, the North American species comprise a monophyletic group (Watson *et al.*, 2000; Liston *et al.*, 2001, pers. comm.; Fig. 1). Unfortunately, only one species of *Lathyrus* from North America was sampled in our study, but analyses of plastid restriction site data indicate that species from North and South America do form a monophyletic group (Asmussen and Liston, 1998). The three species of *Vicia* from North America do not form a monophyletic group in our analyses and are found in two distinct clades, while the third species is part of a basal polytomy in Vicieae (Fig. 1).

In summary, results presented in this paper (Fig. 1) provide strong support for a monophyletic Vicieae that includes *Vicia*, *Lathyrus*, *Pisum* and *Lens*. *Vicia* is paraphyletic with regard to other genera in Vicieae, while *Lathyrus* and *Lens* are each monophyletic and *Lathyrus* plus *Pisum* form a monophyletic group. It will be useful to sample more species of *Vicia* to further test its hypothesised paraphyly. The monophyly of *Trifolium* is supported. A close relationship of *Trifolium* to the other genera in Trifolieae (*Ononis*, *Medicago*, *Trigonella* and *Melilotus*) is questioned, while the relationship of *Ononis* with those latter genera is confirmed. The delimitation of *Medicago* and *Trigonella sensu* Small and Jomphe (1989) is also well-supported, although *Trigonella* is paraphyletic with regard to *Melilotus*. Given the results of the

phylogenetic analyses presented here, loss of the non-protein amino acid canavanine may have occurred independently in one or two clades within Vicieae and in the cultivated chick pea, *Cicer arietinum*. It is possible that trifoliate leaves have evolved independently in this large group of papilionoid legumes: once in the lineage leading to the monotypic *Parochetus*, and at least three times within the vicioid clade; once within *Ononis*, independently in the common ancestor of subtribe Trigonellinae, and again in the lineage leading to *Trifolium*.

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