

## A systematic comparison of morphology and seed proteins of early- and late-flowering forms of *Medicago scutellata*

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Received June 29, 1992

SMALL, E., BAUCHAN, G. R., SALTER, R., BROOKES, B., and AURICHT, G. C. 1993. A systematic comparison of morphology and seed proteins of early- and late-flowering forms of *Medicago scutellata*. *Can. J. Bot.* **71**: 183–192.

Two hundred and thirty-nine germ plasm accessions of *Medicago scutellata* grown under greenhouse conditions exhibited a strongly bimodal distribution of flowering time. Numerical taxonomic analysis showed that the early- and late-flowering forms are substantially different morphologically. The early-flowering form is smaller and less vigorous, has fewer flowers in the inflorescence, and fewer serrations on the leaf blades and stipules than the late-flowering form. Analysis of seed proteins using polyacrylamide gel electrophoresis identified 11 seed proteins that can be used to distinguish the two forms and putative hybrids. Although both forms appear widespread in the circum-Mediterranean indigenous distribution area, only about one-third of the accessions represented the early-flowering form, which seemed to predominate only on isolated Mediterranean islands. The two forms may merit infraspecific taxonomic recognition.

**Key words:** *Medicago scutellata*, Fabaceae, taxonomy, classification, systematics, seed proteins, electrophoresis, flowering time.

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Deux cent trente neuf accessions germoplasmiques du *Medicago scutellata* cultivées en serre ont montré une distribution fortement bimodale du temps de floraison. L'analyse par taxonomie numérique montre que les formes à floraison hâtive et à floraison tardive ont des morphologies passablement distinctes. La forme à floraison hâtive est petite et moins vigoureuse, possède moins de fleurs dans l'inflorescence et des limbes foliaires et des stipules moins dentelés que la forme à floraison tardive. L'analyse des protéines des graines par électrophorèse sur gel de polyacrylamide permet d'identifier 11 protéines séminales qui peuvent être utilisées pour distinguer les deux formes ainsi que les présumés hybrides. Bien que les deux formes semblent largement répandues dans la région indigène du pourtour méditerranéen, seulement environ un tiers des accessions représentent la forme à floraison hâtive, laquelle semble prédominer seulement sur des îles isolées de la Méditerranée. Les deux formes pourraient mériter une reconnaissance taxonomique au niveau infra-spécifique.

**Mots clés :** *Medicago scutellata*, Fabaceae, taxonomie, classification, systématique, protéines séminales, électrophorèse, moment de la floraison.

[Traduit par la rédaction]

### Introduction

Bauchan and Elgin (1984), in a study of chromosomal variation of 72 accessions of *Medicago scutellata* (L.) Mill., observed three previously unreported variants: nonvigorous plants that flowered about 30 days after planting and had one flower per inflorescence; vigorous plants that flowered about 60 days after planting and had three flowers per inflorescence; and intermediate plants. Preliminary studies by Bauchan et al. (1986) indicated that samples of the early- and late-flowering forms possessed different seed proteins and that hybrids of these forms have proteins from both parents. Such evidence of differentiation is of interest because as Heyn (1963) reported,

"*M. scutellata* (L.) Mill. is one of the least variable annual species of *Medicago* and, in spite of its wide distribution, no varieties have been described." Lesins and Lesins (1979) agreed that *M. scutellata* exhibits limited variation. Erac and Tokluoglu (1983) reported a diploid ( $2n = 16$ ) accession of *M. scutellata* that might seem to merit taxonomic attention, but this report was proven erroneous (G. R. Bauchan, unpublished data). All accessions counted to date have  $2n = 30$  (Bauchan and Elgin 1984; Mariani and Falistocco 1991).

The goals of the present study are to examine morphological variation in a large sample of *M. scutellata* grown under standard conditions to reduce environmental variation, particularly attempting to clarify the early- and late-flowering variants reported earlier, and to examine the relationships between morphology and seed proteins in a selected sample of accessions representing the early- and late-flowering forms

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TABLE 1. Characters examined and mode of assessment of character states

<b>Stem</b> (of one representative plant typical of the accession)	
1.	Length (base to end of longest branch; mm)
2.	Number of branches on basal 20 cm of main stem
<b>Leaf</b> (one representative typical leaf from middle of plant; terminal leaflet, where applicable)	
3.	Length leaflet blade (mm)
4.	Width leaflet blade (mm)
5.	Widest point of leaflet (1, distal fifth; 2, 3, 4; 5, proximal fifth)
6.	Number of serrations on one side of leaflet
7.	Distribution of leaflet serrations (1, distal fifth; 2, 3, 4; 5, entire margin)
8.	Number of abaxial leaflet simple trichomes/mm <sup>2</sup>
9.	Number of abaxial leaflet gland-tipped trichomes/mm <sup>2</sup>
10.	Stipule length (mm)
11.	Number of serrations on one side of stipule
<b>Inflorescence</b> (one typical inflorescence, one typical flower where applicable)	
12.	Length of peduncle (mm)
13.	Number of flowers per peduncle
14.	Percentage peduncle is of subtending leaf petiole
15.	Length inflorescence axis is prolonged as awn beyond most distal pedicel (mm)
16.	Flower (standard petal) length (mm)
17.	Calyx length (mm)
18.	Calyx tooth (longest) as percentage of calyx length
19.	Calyx length as percentage of corolla length
<b>Fruit</b> (based on one representative, typical fruit)	
20.	Diameter at widest coil (mm)
21.	Number of fruit coils
<b>Flowering time</b>	
22.	Number of weeks from planting to initial anthesis in at least one plant of the accession

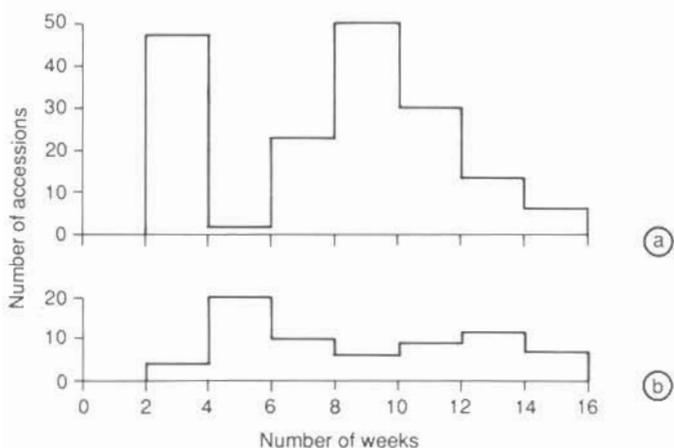


FIG. 1. Frequency histograms showing biweekly numbers of accessions reaching anthesis in (a) the Australian collection of 171 and (b) the USDA collection of 68.

and putative hybrids. Clarifying intraspecific variation of this species is desirable since *M. scutellata* is economically important as a source of pasture cultivars in Australia (Mackay 1981) and is potentially useful as a source of germ plasm for alfalfa improvement (Quiros and Bauchan 1988).

#### Materials and methods

Five seeds of each of 171 accessions from the Australian *Medicago* Genetic Resource Centre, Department of Agriculture, South Australia (Appendix 1) were planted on March 7, 1989, each in a 12.7-cm pot of loam soil, and were grown to maturity in a greenhouse in Ottawa. Supplementary lighting to maintain 16 h of light was

provided. The minimum temperature was 21°C, and the maximum was 25°C. The time in weeks from planting to first observation of anthesis was recorded for each accession. Herbarium vouchers of specimens with mature fruits and flowers were prepared for study and are deposited in DAO. Labels on these vouchers present information on original sites of collection, and much of this information is also available in Plant Introduction Review (Canberra). Australian germ plasm accession numbers and corresponding codes for the herbarium vouchers at DAO are given in Appendix 1.

Each accession was considered an operational taxonomic unit (OTU) for examination by numerical taxonomic methods. The most typical plant of the five available for each accession was chosen for all measurements except flowering time. Characters were measured as described in Table 1. Characters were mostly continuous or meristic but included three ratios (Nos. 14, 18, and 19). While character ratios are advisedly avoided (Sokal and Rohlf 1969, pp. 17–19), these three were adopted because they have been used frequently by taxonomists of *Medicago*, particularly for the *M. scutellata* complex (e.g., Heyn 1963).

Agglomerative cluster analysis of the collected data (22 characters of 171 OTUs) was conducted using a dissimilarity coefficient based on the Gower (1971) similarity coefficient for mixed data such as were available, with scaling by range. Similarity ( $s$ ) was converted to dissimilarity ( $d$ ) by setting

$$[1] \quad d = (1 - s^2)^{1/2}$$

The unweighted pair-group method using arithmetic averages (average linkage) was adopted because it has become the most widely employed clustering method and in our personal experience, produces reasonable classifications with more fidelity than other methods.

Based on the above measure of dissimilarity, ordination by principal coordinates analysis was carried out after Gower (1966a, 1966b). The algorithms used for both cluster and principal coordinates analyses were prepared by L. P. Lefkovitch and are in Program S045 of the library of the Statistical Research Section of

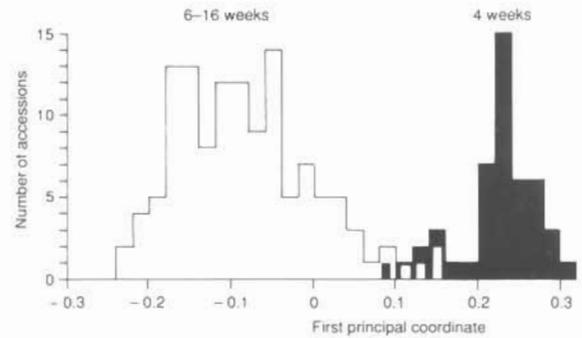
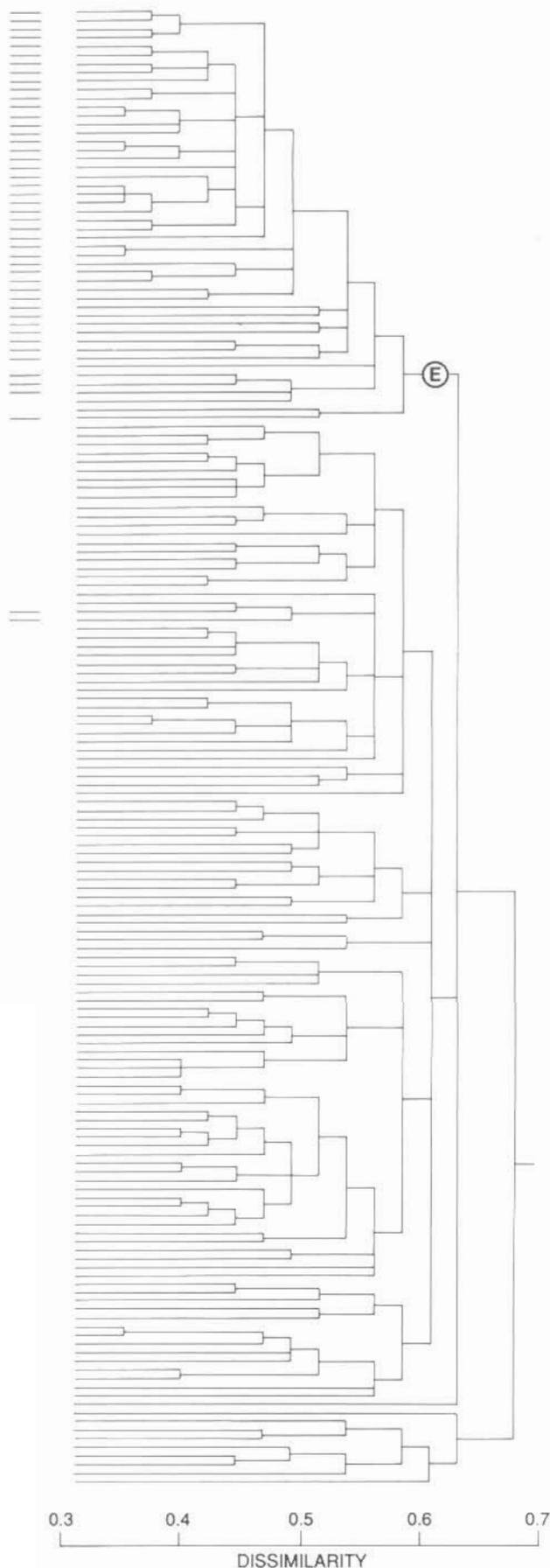


FIG. 3. Histogram showing frequency of 124 late-flowering (6–16 weeks) and 47 early-flowering (4 weeks) OTUs of *Medicago scutellata* in relation to intervals of 0.02 of first principal coordinate.

TABLE 2. Correlation coefficients of first three principal coordinates (accounting for 13.8, 7.1, and 5.1% of the variation, respectively) with morphological characters and weeks to flowering

Character	PC 1	PC 2	PC 3
1. Length branch	-0.76*	-0.08	0.31*
2. Number branches	-0.39*	0.32*	0.09
3. Length leaflet	-0.20	-0.56*	-0.01
4. Width leaflet	-0.43*	-0.53*	0.07
5. Widest point leaflet	0.07	-0.34*	0.14
6. No. leaflet serrations	-0.66*	-0.18	-0.18
7. Distribution serrations	0.19	-0.34*	-0.45*
8. No. simple trichomes	-0.26	0.18	0.02
9. No. glandular trichomes	-0.17	0.54*	0.04
10. Stipule length	-0.42*	0.34*	-0.38*
11. No. stipule serrations	-0.70*	0.44*	-0.18
12. Length peduncle	-0.23	0.02	-0.68*
13. No. flowers per peduncle	-0.74*	0.00	-0.16
14. Percent peduncle of petiole	-0.44*	0.09	-0.38*
15. Length floral awn	0.12	0.05	-0.53*
16. Flower length	0.14	-0.60*	-0.03
17. Calyx length	-0.28*	-0.51*	-0.05
18. Percent calyx tooth of calyx	-0.17	-0.32*	0.23
19. Percent calyx of corolla	-0.45*	-0.08	0.02
20. Fruit diameter	-0.04	-0.31*	-0.18
21. Number fruit coils	-0.28*	-0.41*	-0.23
22. Weeks to flowers	-0.77*	-0.02	0.26

NOTE: See Table 1 for fuller interpretation of characters; all correlations based on  $n = 171$  OTUs from Australian collection. \*, coefficients significant at  $P = 0.05$ , using sequential Bonferroni correction as discussed in text.

Research Program Services of Agriculture Canada in Ottawa. Pearson product-moment correlation coefficients were found between each of the 22 characters and the first three principal coordinates, using the SAS system (SAS Institute Inc. 1985), to assess the relative contributions of the former to the latter. Significance at  $P = 0.05$  was found, correcting the bias in considering the 66 different combinations as independent tests by the sequential Bonferroni test, as recommended by Rice (1989).

Early-flowering plants (47 accessions that first flowered during the 4th week after sowing) and late-flowering plants (124 accessions that first flowered between the 6th and 16th weeks after sowing) were compared employing  $Q$ -technique canonical variate analysis (multiple discriminant analysis in which the prior probabilities of the

FIG. 2. Dendrogram resulting from average linkage (UPGMA) clustering of 171 OTUs of *Medicago scutellata*. At left of dendrogram note lines showing positions of early-flowering OTUs. Note cluster designated E, containing most of the early-flowering OTUs.

TABLE 3. Comparison of means and variances of early-flowering and late-flowering forms of *Medicago scutellata*, and importance of the morphological characters (in combination with higher ranked characters) in separating the groups, based on Australian collection

Character	Early-flowering (n = 47)		Late-flowering (n = 124)		Descending order of importance
	$\bar{x}$	$\sigma^2$	$\bar{x}$	$\sigma^2$	
1. Length branch*	457	9673	1041	49508	1
2. Number branches*	3.87	2.461	5.02	3.926	11
3. Length leaflet	21.1	12.86	22.0	12.91	9
4. Width leaflet*	10.6	6.450	13.1	9.718	8
5. Widest point leaflet	2.57	0.380	2.54	0.315	16
6. No. leaflet serrations*	10.7	3.291	14.4	8.625	3
7. Distribution serrations	4.30	0.3876	4.16	0.3152	14
8. No. simple trichomes	5.81	2.984	6.51	5.211	7
9. No. glandular trichomes	3.83	2.753	4.08	3.701	12
10. Stipule length*	8.53	3.269	9.65	5.621	21
11. No. stipule serrations*	4.15	3.347	8.93	16.30	4
12. Length peduncle	15.9	12.16	16.3	24.10	6
13. No. flowers per peduncle*	1.00	0.000	2.09	0.6506	2
14. Percent peduncle is of petiole*	75.0	332.1	96.1	1336	18
15. Length floral awn	8.65	6.092	7.71	6.675	13
16. Flower length	6.04	0.3221	5.89	0.5902	17
17. Calyx length*	4.13	0.3340	4.57	0.5683	20
18. Percent calyx tooth is of calyx	52.6	115.4	56.3	94.55	19
19. Percent calyx is of corolla*	68.1	99.29	77.0	75.85	5
20. Fruit diameter	12.3	2.386	12.4	2.315	15
21. Number fruit coils*	6.64	0.2468	6.95	0.4038	10
22. Weeks to flowers*	4.00	0.000	10.0	4.876	

NOTE: See Table 1 for fuller description of characters. \*, means significantly different at  $P = 0.05$  (using sequential Bonferroni correction, as discussed in text).

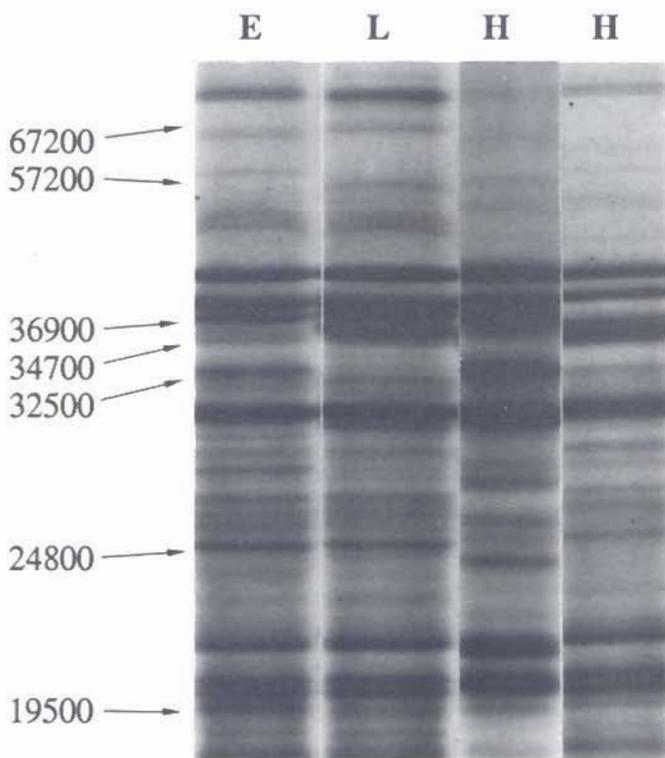


FIG. 4. Seed protein electrophoretic banding patterns. The numbers on the left are molecular weights, and accession numbers are at the bottom. E, early-flowering; L, late-flowering; H, putative hybrid.

TABLE 4. Seed proteins for three accessions exemplifying early-flowering (1764), late-flowering (1766), and putative hybrid (1828) forms of *Medicago scutellata* (cf. Fig. 4 and text)

Band No.	Mol. wt.	1764	1828	1766
1	67 200	-	+	+
2	65 900	+	+	-
3	62 300	-	-	-
4	59 200	+	+	-
5	58 500	-	-	-
6	57 200	-	+	+
7	36 900	+	+	+
8	34 700	+	+	+
9	32 500	+	+	+
10	28 400	+	+	+
11	19 500	+	+	+

NOTE: The hybrid shows all proteins of parents. +, present; -, not detected.

groups are equal and not proportional to the sample sizes). The defining character (weeks to flowering) was omitted so that the resulting classificatory analysis would be on morphology alone. Means for the 22 characters of the two groups were compared by univariate analysis of variance. As with the set of correlation coefficients, the bias in considering these as independent tests was corrected by the sequential Bonferroni test after Rice (1989). A forward stepwise procedure was used to rank the order of importance of characters as discriminators after discounting correlation with higher ranked variables (see Dixon

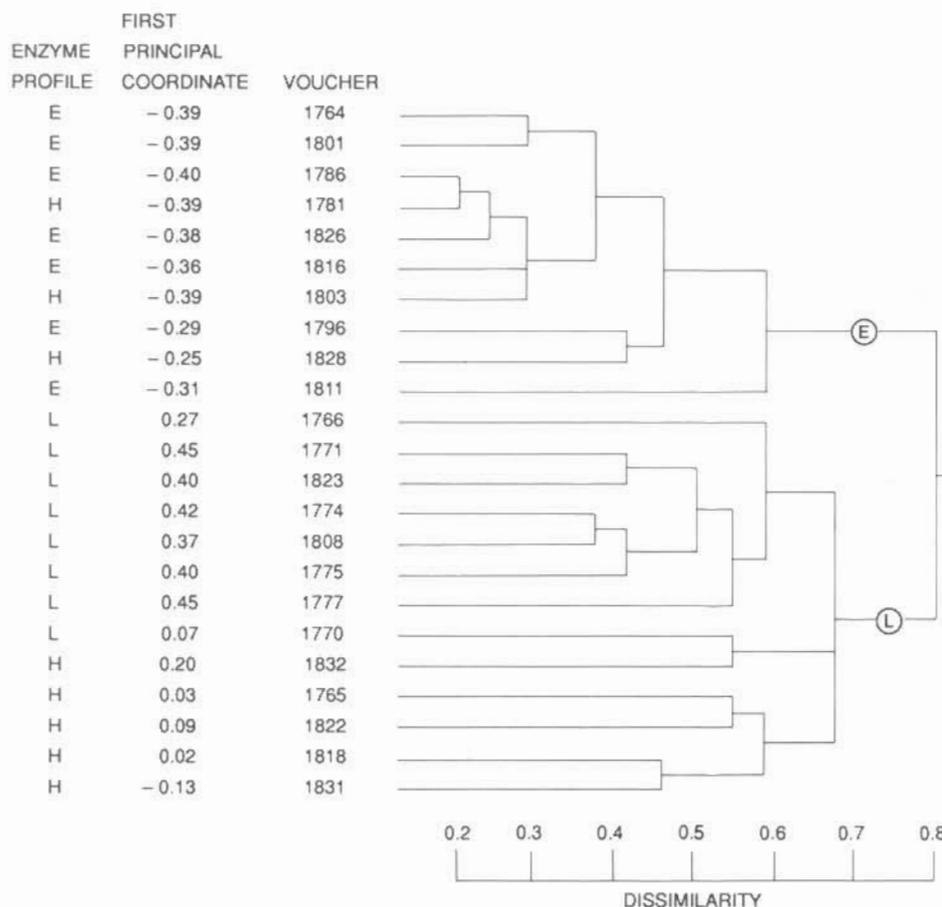


FIG. 5. Dendrogram resulting from average linkage (UPGMA) clustering based on five morphological characters and flowering time for 23 OTUs of *Medicago scutellata* analysed electrophoretically for seed proteins (electrophoretic profiles: E, early; H, hybrid; L, late). At left of dendrogram note enzyme profile, first principal coordinate (accounting for 42.3% of the variation), and voucher (Appendix 2). Note that the two main clusters, labelled E and L, separate the early and late accessions, respectively, and that the first principal coordinate separates the two clusters. Accessions with hybrid electrophoretic profiles occur in both groups.

1965 for description of sequential *F*-test, in discussion of stepwise discrimination analysis). These procedures were carried out using Program S015, prepared by B. Thompson, in the library of the Statistical Research Section of Research Program Services of Agriculture Canada in Ottawa.

Five seeds of each of 68 accessions of *M. scutellata* from the USDA germ plasm collection were sown on 24 May 1990, under similar conditions and in the same greenhouse as the Australian accessions. It would have been desirable to grow these along with the Australian collections grown earlier, but they were unavailable. As before, time from planting to first observation of anthesis was recorded, and herbarium specimens were made at maturity. Information on the sources of these collections is recorded on the herbarium vouchers at DAO, and much of the information is also available in the GRIN computer network (USDA 1989). USDA accession numbers and DAO voucher herbarium codes are given in Appendix 2. Because the United States collection was considerably smaller than the Australian collection grown earlier, was not grown at the same time, and (as noted below) flowered later, it was decided not to combine the collected data. Instead, the data from the USDA collection were used as a test of consistency of the relationship between morphology and flowering time as revealed by the data for the Australian collection. The analysis of the Australian collection indicated the characters that best separated the early- and late-flowering groups; the five best characters (Table 3) were used to identify the accessions in the U.S. collection to early-flowering or late-flowering morphological groups. That is, each of the 68 accessions was scored for the five characters

and evaluated on the basis of average similarity as to which group it was morphologically closest. Then the extent to which the OTUs respectively assigned to these morphological groups were actually early- or late-flowering was examined.

Seed proteins from 23 accessions (Fig. 5) from the USDA collection were analysed. These accessions were deliberately chosen on the basis of morphology to represent approximately equal numbers of early-flowering, late-flowering, and putative hybrid accessions (Fig. 5), although it should be emphasized that apparent putative hybrids were uncommon. The accessions represent the range of morphological and geographical variation (Appendix 2), but the choice was carried out well before the numerical taxonomic analyses conducted here, on the basis of preliminary impression. All seeds analysed in this study were produced in the greenhouse in Beltsville, Md. at the same time. Single seeds from each accession were ground in a mortar and pestle and weighed after grinding. For every 1 mg of pulverized seed 0.03 mL of extraction buffer was added. The extraction buffer contained 60 mM Tris (hydroxymethyl) aminoethane, 3.6 mM disodium ethylenediamine tetraacetic acid (EDTA), 3.4 mM phenyl methyl sulfonyl fluoride (PMSF), 4% sodium dodecyl sulphate (SDS), 10%  $\beta$ -mercapto-ethanol, 40% glycerol, and 0.04% bromophenyl blue at pH 6.8. The samples were incubated at 40°C for 2.5 h in a shaking water bath, heated to 100°C for an additional 2 min, then centrifuged for 3 min in a microcentrifuge. Twenty-five microlitres of the supernatant was loaded into each lane of the gel. Gradient SDS - polyacrylamide gel electrophoresis (SDS - PAGE) was used to separate the proteins (Laemmli 1970) utilizing a Protein I electro-

TABLE 5. Sources of early-flowering and late-flowering accessions of the Australian collection (Aust.; Appendix 1), and of the USDA collection (USDA; Appendix 2)

Source	Early-flowering		Late-flowering	
	Aust.	USDA	Aust.	USDA
Algeria	1		3	
Argentina			1	
Australia	3	6	3	5
Crete	4		2	
Cyprus	14	4	1	
Czechoslovakia		1		
Denmark	1			
France			3	1
Germany	1	2		
Greece	2	2	8	1
Hungary	2	1		
Israel		3	17	1
Italy		1	11	4
Jordan			9	
Lebanon	1	1		
Malta	1			
Morocco			2	
Portugal			6	1
Russia	2		1	
Sicily	1		23	2
Spain	2		2	1
Sweden		1		
Syria		3	6	
Tunisia	2		10	
Turkey	2	1	10	3
Uruguay				2
U.S.A.	2	2	3	3
Unknown	6	10	3	6
Totals	47	38	124	30
Grand totals		85		154

phoresis system (BioRad Laboratories, Richmond, Calif.). The gels were 13 × 14 cm in size. The separation gel gradient was from 7.5 to 15% acrylamide with AcrylAide™ (FMC Corporation, Rockland, Maine) substituted for bis-acrylamide as the cross linker at pH 8.8 (Nochumson and Gibson 1983). The separation gels were made using a gradient former with a peristaltic pump attached to pump the gel between two plates of glass with one plate containing a clear plastic sheet of GelBond®. The stacking gel was a 4% acrylamide gel with bis-acrylamide used as the crosslinker at pH 6.8. Fifteen samples were placed in each gel with one lane containing a protein molecular weight standard (Sigma Chemical Co., St. Louis, Mo.). The electrode buffer was 3 mM Tris, 2.9 mM glycine, and 1% SDS at pH 8.3. Electrophoresis was performed on two gels at a time at 60 mA constant current (E-C Apparatus Corporation, St. Petersburg, Fla.) for 4.5 h. Following electrophoresis, the gel, secured to the GelBond® sheet, was stained overnight with 0.2% Coomassie brilliant blue R-250 (BioRad Laboratories, Richmond, Calif.), 45% methanol, and 10% glacial acetic acid. The gels were destained for a minimum of 12 h in a solution of 45% methanol and 10% glacial acetic acid. After destaining, the gels were placed in a preserving solution of 7% glacial acetic acid and 5% glycerol for at least 2 h and then dried in an incubator at 50°C for 24 h. Fourteen seeds from each accession were evaluated for uniformity within each accession, using one gel per accession. Four additional gels were made with each accession represented only once. The gels provided a permanent transparent record, with the blue protein bands occurring in the lanes. Gels were analysed using an Ultrascan XL laser densitometer with GelScan XL software (Pharmacia/LKB Biotechnology, Inc., Piscataway, N.J.). The GelScan XL software was used to determine the molecular

weight of each protein using the protein standards as a reference, and the relative percentage of each protein was determined by calculating the area under the scanned curve using the Gaussian fit method. Each sample lane was scanned and the average molecular weight and the relative protein percentage were determined for each protein.

Numerical taxonomic analysis was carried out on selected morphological data for the 23 accessions from the USDA collection analysed electrophoretically. Based on flowering time and the set of five morphological characters best discriminating the early- and late-flowering groups of the Australian collection, cluster and principal coordinate analyses were conducted as described above for the Australian collection. The intention of these analyses was to examine the concordance of morphology, flowering time, and chemistry.

Illustrations were prepared of the early- and late-flowering forms (Figs. 6, 7), and the applicable nomenclature was examined.

### Results and discussion

The Australian and United States collections of wild *M. scutellata* both appeared to be bimodal with respect to flowering time when grown in a greenhouse in Ottawa (Fig. 1). The modes for the USDA collection are about 3 weeks later than for the Australian collection, presumably because of the different times of planting and possibly other uncontrolled factors. Vernalization and photoperiod greatly affect flowering time in some annual species of *Medicago* but not in *M. scutellata*, which has been found to be almost insensitive to both factors (Clarkson and Russell 1975). *Medicago scutellata* is very resistant to alteration of flowering time by water stress compared with other species of *Medicago* (Clarkson and Russell 1976).

Cluster analysis of the 171 Australian accessions, based on 21 morphological characters and flowering time, resulted in a fairly well defined cluster of 48 accessions (Fig. 2), which included 45 of the 47 early-flowering accessions (i.e., those flowering in the 4th week following planting). This analysis clearly suggests correlated morphological and flowering-date differentiation in *M. scutellata*.

Examination of the first principal coordinates based on these same data produced a bimodal, more or less binormal distribution in which the 47 early-flowering accessions separated off from all but 6 of the 124 accessions that flowered later than 4 weeks (Fig. 3). The relative contributions of the 21 morphological characters and flowering time to the first principal coordinate are indicated by the correlation coefficients in Table 2. A mixture of morphological characters (notably length of longest branch, number of marginal serrations on the leaflet and also on the stipule, and number of flowers on the peduncle) and flowering time substantially determined the first principal coordinate. While size characters such as length of longest branch might be correlated with flowering time simply because longer maturation allows more growth, clearly there are characters distinguishing early- and late-flowering plants that are not so simply explained. Once again, this information strongly suggests taxonomically significant correlated morphological and flowering-date separation within *M. scutellata*.

The second and third principal coordinates of the data for the Australian collection were also examined for possible taxonomic structure. Note in Table 2 that the second principal coordinate seems to reflect absolute size characters (particularly the highest correlations, with characters 3, 4, 16, and 17), whereas the highest correlations with the third principal coordinate (for characters 12 and 15) are inflorescence characters. In neither case were we able to discern systematically

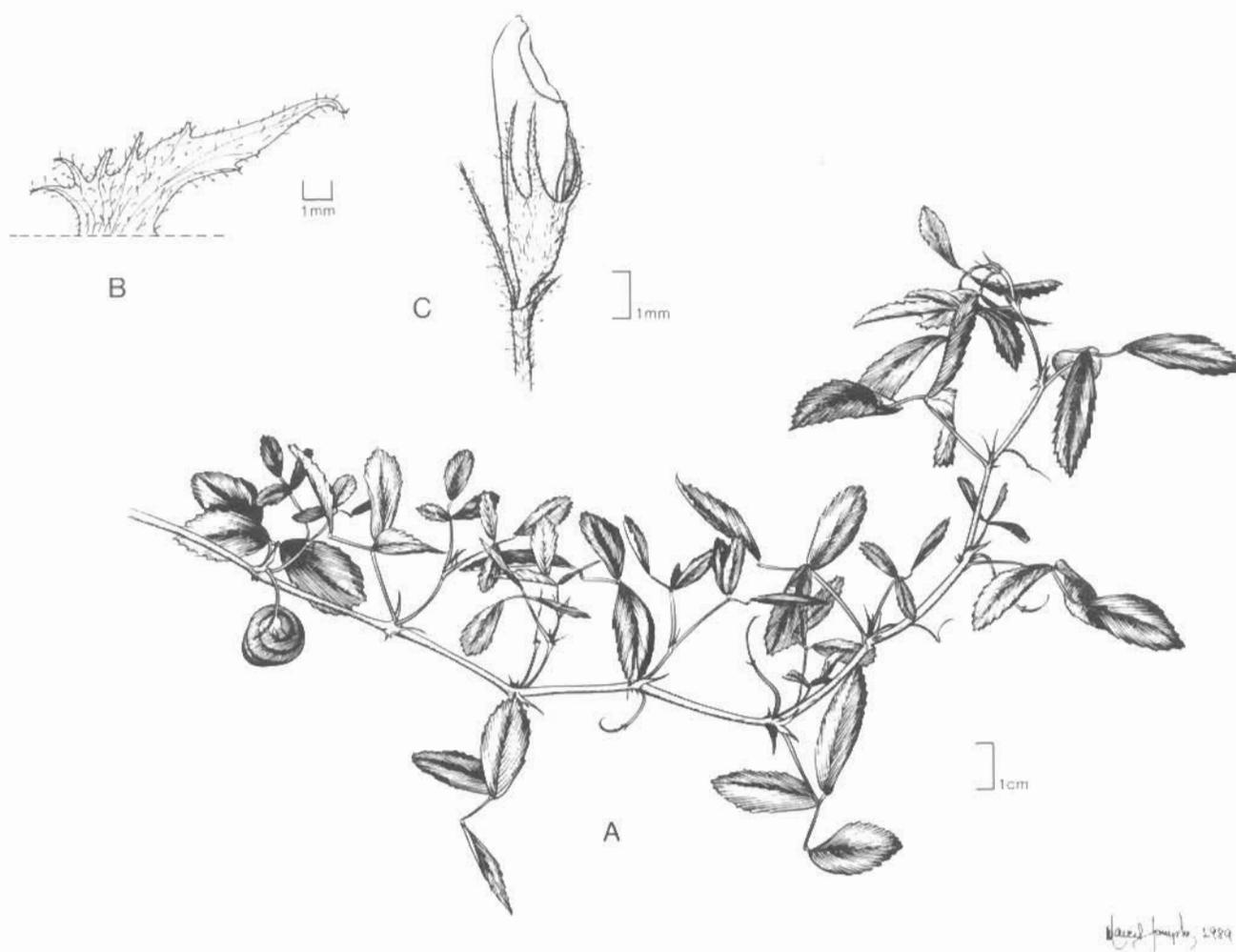


FIG. 6. Illustrations of early-flowering form of *Medicago scutellata* (based on greenhouse-grown plant voucher E. Small M1728 at DAO). (A) Branch. (B) Stipule. (C) Inflorescence.

important variation. The relationships of flowering time and the second and third principal coordinates were also examined as had been done for the first principal coordinate; the second and third principal coordinates showed no indication of relationship with flowering time (also note in Table 2 that the correlation coefficients with flowering time are not significant).

Classificatory discriminant analysis based on the 21 morphological characters examined in the Australian collection almost perfectly separated the early-flowering and late-flowering groups: all 47 early-flowering accessions (that had been assigned a priori by flowering during the 4th week) were morphologically identified as members of the early-flowering group, and 122 of the 124 accessions that flowered later than 4 weeks were morphologically assigned correctly to their respective group. The order of combined discriminating value (i.e., discounting correlation with higher ranked variables) is shown in Table 3. The most discriminating (in combination) five characters respectively were the following: length of longest branch (more than twice as long in the late-flowering group); number of flowers per peduncle (invariably 1 in the early-flowering group and averaging 2.09 in the late-flowering group); number of serrations on one side of the leaflet (average 10.7 in the early-flowering group, 14.4 in the late-flowering group); number of stipule serrations (averaging 4.15 in the early-flowering group, 8.93 in the late-flowering

group); and the calyx to corolla lengths as a percentage (averaging 68.1% in the early-flowering group, 77.0% in the late-flowering group). The means of 12 of the 22 characters examined differed significantly by one-way analyses of variance between the early- and late-flowering groups (Table 3).

The finding of correlation between morphology and flowering time, obtained above for the Australian collection, was then tested for consistency by reference to the USDA collection. Use of the most discriminating set (for the Australian collection) of five morphological characters to classify the 68 accessions in the American collection resulted in 38 of the collections being assigned to the early-flowering group as defined morphologically (Appendix 2). When the flowering times of these accessions were subsequently examined, the set of 38 proved to constitute all of those that flowered before 10 weeks (Fig. 1b), while those that flowered from 10 to 16 weeks corresponded to the accessions identified morphologically as members of the late-flowering group. Therefore, under our experimental conditions of cultivation, early- and late-flowering plants constitute consistently distinguishable morphological groupings.

Finally, a selected group of the American collection representing the early- and late-flowering groups, and some putative hybrids, was examined simultaneously for seed proteins, flowering time, and morphology. Utilizing electro-

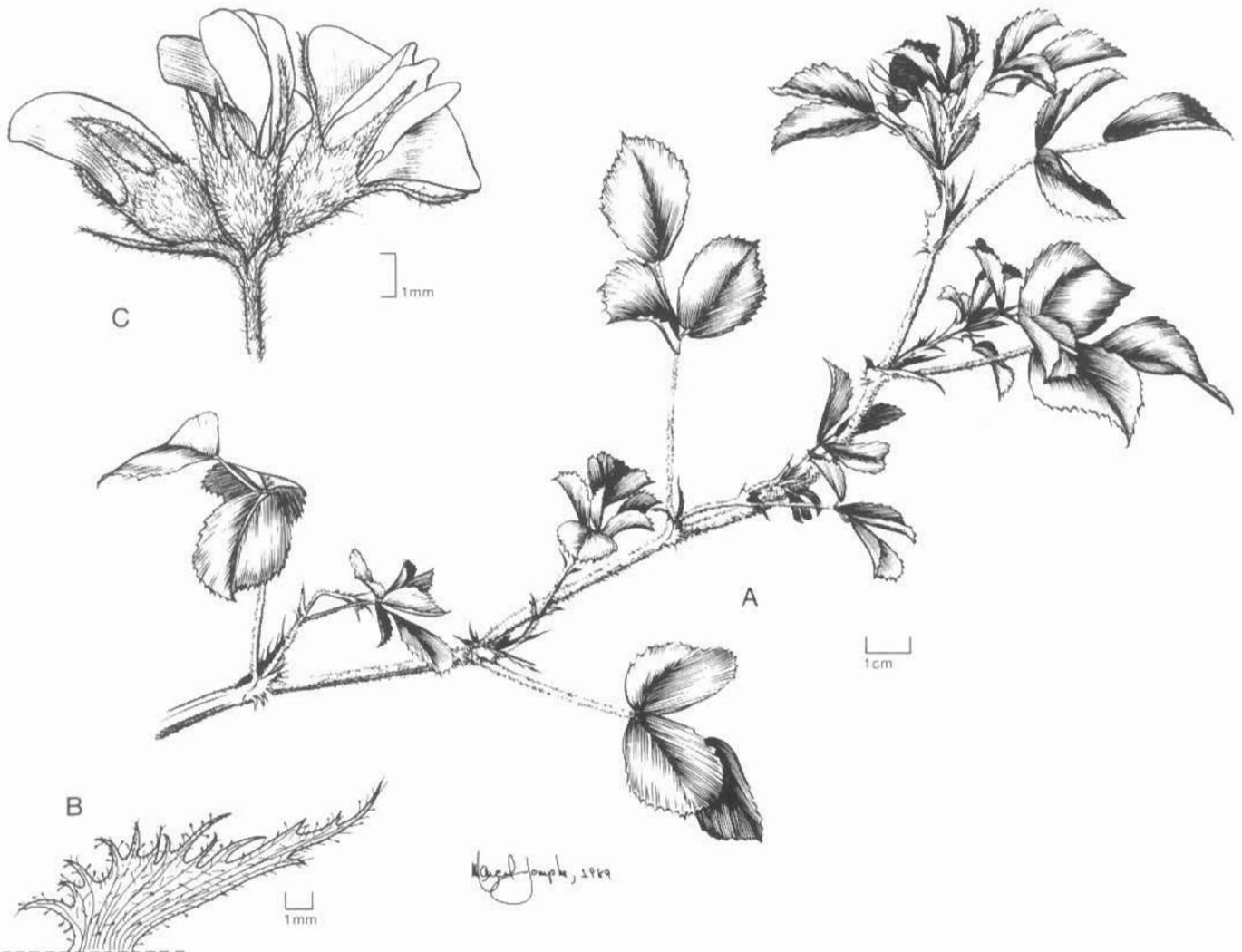


FIG. 7. Illustrations of late-flowering form of *Medicago scutellata* (based on greenhouse-grown plant voucher E. Small M1647 at DAO). (A) Branch. (B) Stipule. (C) Inflorescence.

phoresis on 23 of the American accessions, 11 seed protein bands could be used to distinguish the early- and late-flowering plants, as well as putative hybrids (Fig. 4). All of the seeds from the same accession had identical banding patterns. The seven early-flowering accessions had the same banding pattern; the eight late-flowering accessions were also homogeneous but showed a different pattern. Different high molecular weight (MW) (between 67 200 and 57 200) seed proteins were unique to each group. For the late-flowering accessions these were 67 200 and 57 200 MW protein, and for the early-flowering accessions 65 900 and 59 200 MW proteins. These could also be used to identify putative hybrid accessions, as illustrated for three accessions in Table 4. Some of the eight putative hybrid accessions had a combination of those proteins found in the early- and late-flowering accessions (e.g., accession 1828 had four: 67 200, 65 900, 59 200 and 57 200 MW proteins). All of the putative hybrid accessions that were analysed appeared to be hybrid as seen by the presence of more than two high molecular weight proteins in the region. Accession 1765 has unique seed proteins (62 300 and 58 500 MW). This accession was also unique in lacking a seed protein with a molecular weight of 36 900 MW that occurred in all of the other accessions analysed.

There were quantitative differences in the remainder of the bands that tended to distinguish the early- and late-flowering accessions. Consistent trends included the following: for the 34 700 MW protein, more in the late-flowering accessions than in the early; for 32 500 MW protein, more in the early-flowering accessions than in the late; for 24 800 MW protein, comparable amounts in both the early- and late-flowering accessions; and for 19 500 MW protein, about five times the amount in the early-flowering accessions than in the late. The electrophoretic samples were too limited for statistical analysis, but as noted below, the chemical differences parallel morphological differences to a very high degree.

Cluster and principal coordinates analysis of morphology of the 23 accessions of the USDA germ plasm collection for which electrophoretic analyses of seed proteins were available confirmed the congruence of morphology, chemistry, and flowering time (Fig. 5). The accessions chemically identified as early flowering all fell into the early-flowering cluster defined morphologically, and the accessions chemically identified as late flowering all fell into the late-flowering cluster defined morphologically (Fig. 5). The disposition of putative hybrids was less clear. Note that of the eight accessions identified electrophoretically as hybrids (Fig. 5), three fell into the

early-flowering group, and five fell into the late-flowering group. Since the early- and late-flowering groups can be experimentally hybridized (Bauchan et al. 1986), a range of introgressed hybrids could be expected in nature, with various degrees of similarity to the two basic groups.

Country of origin of the accessions studied is summarized, insofar as information was available, in Table 5. It needs to be kept in mind that *M. scutellata* is indigenous to Mediterranean circle countries; of the accessions listed in Table 5, those identified as originating from Argentina, Australia, Czechoslovakia, Denmark, Germany, Hungary, Sweden, Uruguay, U.S.A., and probably Russia are either acquisitions from botanical gardens, or introductions, probably naturalized. Eighty-five (35.6%) of the 239 accessions studied are identified here as belonging to the early-flowering group. Both groups seem widely distributed, with the late-flowering group generally occurring in greater frequency. This may reflect competitive superiority of the much more vigorous (at least in biomass and seed productivity) late-flowering form. The predominant occurrence of the early-flowering group in Crete and Cyprus, and in Malta (from which only one collection was available) is noteworthy. This may indicate a tendency for the early-flowering form to occupy relatively small and isolated Mediterranean islands and may reflect habitat specialization. The early- and late-flowering forms of *M. scutellata* both occur in many countries, and since the two groups can be hybridized experimentally, and plants of some accessions seem to combine characteristics of both groups, hybridization between the two groups and gene exchange are possible in nature.

One of the accessions examined (USDA collection 469098) was 'Robinson', the principal cultivar of *M. scutellata*, which originated from a ruderal South Australian population (Mackay 1981). This accession was identified as a member of the early-flowering group (Appendix 2), which seems correct, as Crawford (1985) noted the exceptional earliness of *M. scutellata* 'Robinson' compared with all 12 other annual species of *Medicago* grown at Parafield, South Australia. It is interesting to note that this early-flowering cultivar is relatively less vigorous in comparison to the late-flowering group under greenhouse conditions. Apparently under the conditions 'Robinson' is typically cultivated in Australia its short life cycle is important and its performance is not inferior. Indeed, "'Robinson' has produced the highest seed yields of all annual medic cultivars tested in experiments in South Australia between 1966 and 1969" (Mackay 1981).

The early- and late-flowering groups of *M. scutellata* (Figs. 6, 7) possibly merit infraspecific taxonomic recognition. For choice of future nomenclature it may be noted that Heyn (1959) lectotypified the name *M. polymorpha* var. *scutellata* L., hence *M. scutellata* (L.) Mill., by Fig. 3 in Morison Pl. Hist. Oxon. 2: Tab. 15, 1680, whereas Burt (1964) argued that the lectotype should be the description in J. Bauh. Hist. Pl. 2: 384, 1650. In either case the lectotype seems to be the late-flowering form.

However, additional investigation is required. Subsequent to this study, the senior author examined the large collection of *M. scutellata* at HUI, and the morphological characters that served to discriminate the two groups grown in the greenhouse were generally not adequate for discrimination of most plants collected in nature, which were more variable than the greenhouse plants. It is also desirable to clarify the extent of hybridity in nature and the geography of the groups, and for

this isozyme analysis could be especially helpful.

#### Acknowledgments

We thank L. Goertzen and B. Siefring for excellent technical assistance, M. Jomphe for preparing Figs. 6 and 7, and S. Warwick for critical comments.

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## Appendix 1

Accession numbers and herbarium voucher codes (E. Small M-series in DAO, shown in parentheses) of Australian collection of *Medicago scutellata*.

Early-flowering (4 weeks)	GREECE: 2228 (1604), 4963 (1654), 11560 (1677), 22570 (1722), 22571 (1723), 22572 (1724), 22940 (1726), 25387 (1757)
UNKNOWN ORIGIN: 3421 (1626), 3448 (1628), 18637 (1708), 18639 (1710), 18641 (1712), 18643 (1714)	ISRAEL: 715 (1589), 2670 (1606), 2671 (1607), 2672 (1608), 2673 (1609), 2675 (1611), 3106 (1621), 3109 (1622), 3110 (1623), 4283 (1641), 4332 (1642), 4333 (1643), 4334 (1644), 5070 (1655), 18134 (1701), 18136 (1702), 18645 (1716)
ALGERIA: 11482 (1676)	ITALY: 664 (1587), 2223 (1603), 2686 (1620), 3509 (1631), 3510 (1632), 3512 (1633), 3513 (1634), 3514 (1635), 3774 (1638), 4608 (1651), 22573 (1725)
AUSTRALIA: 388 (1581), 1868 (1597), 4417 (1649)	JORDAN: 14145 (1681), 14146 (1682), 14147 (1683), 14149 (1684), 14150 (1685), 14151 (1686), 18066 (1698), 18074 (1699), 18085 (1700)
CRETE: 655 (1583), 2110 (1600), 2115 (1601), 17547 (1697)	MOROCCO: 24308 (1754), 24309 (1755)
CYPRUS: 387 (1580), 1596 (1593), 1801 (1594), 2678 (1612), 2679 (1613), 2680 (1614), 2681 (1615), 2682 (1616), 18646 (1717), 23748 (1727), 23749 (1728), 23750 (1729), 23751 (1730), 23752 (1731)	PORTUGAL: 392 (1582), 1806 (1595), 2683 (1617), 2685 (1619), 3507 (1629), 4336 (1645)
DENMARK: 1317 (1591)	RUSSIA: 4418 (1650)
GERMANY: 5615 (1656)	SICILY: 2056 (1598), 4609 (1652), 8042 (1664), 24088 (1732), 24089 (1733), 24090 (1734), 24092 (1736), 24093 (1737), 24095 (1738), 24096 (1739), 24097 (1740), 24099 (1742), 24100 (1743), 24101 (1744), 24102 (1745), 24103 (1746), 24104 (1747), 24105 (1748), 24106 (1749), 24107 (1750), 24108 (1751), 24109 (1752), 24110 (1753)
GREECE: 17544 (1695), 17545 (1696)	SPAIN: 1859 (1596), 2669 (1605)
HUNGARY: 3508 (1630), 3572 (1637)	SYRIA: 14154 (1687), 14156 (1688), 14157 (1689), 15037 (1690), 22320 (1720), 22321 (1721)
LEBANON: 3515 (1636)	TUNISIA: 4062 (1640), 5936 (1657), 5937 (1658), 5938 (1659), 5939 (1660), 5940 (1661), 11477 (1671), 18516 (1705), 18517 (1706), 18518 (1707)
MALTA: 18640 (1711)	TURKEY: 658 (1584), 659 (1585), 661 (1586), 2674 (1610), 3803 (1639), 4339 (1647), 10263 (1666), 10264 (1667), 11932 (1679), 11933 (1680)
RUSSIA: 1339 (1592), 5962 (1662)	U.S.A.: 3382 (1625), 17380 (1692), 17382 (1694)
SICILY: 24098 (1741)	
SPAIN: 1313 (1590), 18644 (1715)	
TUNISIA: 18360 (1703), 18439 (1704)	
TURKEY: 4338 (1646), 7621 (1663)	
U.S.A.: 17379 (1691), 17381 (1693)	
Late-flowering (6–16 weeks)	
UNKNOWN ORIGIN: 3443 (1627), 4669 (1653), 18642 (1713)	
ALGERIA: 11478 (1672), 11479 (1673), 11480 (1674)	
ARGENTINA: 10262 (1665)	
AUSTRALIA: 3381 (1624), 4416 (1648), 20057 (1719)	
CRETE: 2082 (1599), 2130 (1602)	
CYPRUS: 18645 (1718)	
FRANCE: 10469 (1668), 10472 (1669), 10683 (1670)	

## Appendix 2

Accession numbers and herbarium voucher codes (E. Small M-series in DAO, shown in parentheses) of the USDA collection of *Medicago scutellata*. E, H, and L designate early-flowering, putative hybrid, and late-flowering accessions, respectively.

Early-flowering (4–9 weeks)	TURKEY: 487403 (1821)
UNKNOWN ORIGIN: 143869 (1794), 143870 (1795), 217447 (1767), 189569 (1772), 197807 (1780), 197809 (1782), 197814 (1787), 197818 (1791), 197820 (1778), 487390 (1810)	U.S.A.: 197808 (1781) [H], 307446 (1764) [E]
AUSTRALIA: not available (1803) [H], not available (1804), 469098 (1807) (cv. Robinson), 197810 (1783), 197813 (1786) [E], 197815 (1788)	Late-flowering (10–16 weeks)
CYPRUS: 197821 (1779), 365965 (1798), 368963 (1796) [E], 368964 (1797)	UNKNOWN ORIGIN: not available (1832) [H], 117370 (1793), 120044 (1765) [H], 197811 (1784), 308830 (1768), 308831 (1769)
CZECHOSLOVAKIA: 487389 (1809)	AUSTRALIA: not available (1805), not available (1806), 189570 (1773), 189571 (1774) [L], 197356 (1776)
GERMANY: 487394 (1812), 487395 (1813)	FRANCE: 197819 (1792)
GREECE: not available (1827), 487408 (1826) [E]	GREECE: 487404 (1822) [H]
HUNGARY: 487396 (1814)	ISRAEL: 197355 (1775) [L]
ISRAEL: 368966 (1799), 368967 (1800), 368968 (1801) [E]	ITALY: 487405 (1823) [L], 487406 (1824), 487410 (1829), 487411 (1830)
ITALY: 487407 (1825)	PORTUGAL: 487388 (1808) [L]
LEBANON: 487409 (1828) [H]	SICILY: 487401 (1819), 487402 (1820)
SWEDEN: 487392 (1811) [E]	SPAIN: 487412 (1831) [H]
SYRIA: 487397 (1815), 487398 (1816) [E], 487399 (1817)	TURKEY: 170552 (1771) [L], 295606 (1802), 487400 (1818) [H]
	URUGUAY: 161415 (1770) [L], 307447 (1766) [L]
	U.S.A.: 197354 (1777) [L], 197816 (1789), 197817 (1790)