KJPR Korean J.Plant.Res. 10(3) 250~257(1997)

Analysis of Phylogenetic Relationships among *Medicago* Species by Proteins Banding Patterns and RFLP Markers

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ABSTRACT.

The relationship of nine *Medicago* species belonging to four subgenera were analyzed by using SDS-PAGE and restriction fragment length polymorphism (RELP) methodologies. Sixty-eight bands of alcohol and salt soluble proteins and 85-133 RFLP markers were used to estimate the genetic distance among the species. These species were clustered together at around 0.1 to 0.4 level of distance for both kind of markers, indicating that *Medicago* species have a large genetic similarity. A combined cluster diagram, at a dissimilarity level of 0.3, differentiated nine species in four groups: group 1, *M. littoralis, M. truncatula, M. scutellata* and *M. rigidula; group 2, M. sativa* ; group 3, *M. lupulina* ; group 4, *M. orbicularis, M. radiata* and *M. minima*. All of them, but except for *M. minima*, corresponded to the existing four subgenera of the genus *Medicago* classified by Lesins and Lesins (1979). The most similar species were *M. littoralis* and *M. trucatula* and the most dissimilar one was *M. lupulina*. In separate cluster diagrams based on RFLP and protein markers, some differences were observed. In the case of RFLP or DNA markers, *M. sativa* (alfalfa) was distantly clustered with other *Medicago* species. But in the case of protein markers, *M. sativa* was closely clustered with *M. scutellata, M. littoralis* and *M. truncatula*.

Key words: Alfalfa, RFLP, Chloroplast DNA, Protein marker, Polymorphism, Genetic distance.

INTRODUCTION

The genus *Medicago* (tribe Trifoliae, family Fabaceae), as currently defined (Lesins and Lesins, 1979), is presented by about 55 annual and perennial, diploid and tetraploid species with basic chromosome numders of X=8 (or X=7). The annuals are predominantly autogamous, while the perennials are allogamous (Quiros, 1983). *Medicago* species are considered to have the subtropical and temperate regions of north hemisphere of the old world as their dispersion center (Agarwal and Gupta, 1983). Alfalfa (*Medicago sativa*) is one of the most important cultivated forage crop. Since some of *Medicago* species are insect and disease resistant (Ridland and Berg, 1981; Barbetti , 1987), the phylogenetic study of *Medicago* species shduld be important for breeding of alfalfa. In addition, their ability of fixing atmospheric nitrogen and their usefulness in ley-farming (Puckridge and French, 1983) make them important as agroecological resources.

Cytology, isozyme and RFLP studies of some *Medicago* species, especially of alfalfa, are well documented (Simon and Simon, 1965; Damerval, 1983; Rose et al., 1986; Palmer et al., 1987; Kiss et al., 1993). However, a comparative study of genetic variation among of these species has not been carried out. Small (1981) studied 55 *Medicago* species using 75 traditionally used characters and found some discrepancies with regard to the existing major grouping of the species. Hence, it is desirable to increase our knowledge of genetic distance of these species by employing not only traditionally used characters, but equally qualitative characters such as protein and DNA markers.

In the present study, a comparative study has been undertaken by using protein and DNA polymorphism to estimate the genetic distance of nine species belonging

Table 1. List and characteristics of *Medicago* species used in this study

Species	Subgenus	Section	Origin
M. Orbicularis	Obicularia	Orbiculares	Azerb. Iran
M. radiata	〃	Hymenocarpos	〃 〃
M. minima	Spirocarpos	Leptospirae	〃 〃
M. rigidula	〃	Pachyspirae	〃 〃
M. littoralis	〃	〃	Australia
M. truncatula	〃	〃	〃
M. scutellata	〃	Rotatae	〃
M. lupulina	Lupularia	-	Azerb. Iran
M. sativa	Medicago	Falcago	〃 〃

to four subgenera of the genus *Medicago*.

MATERIALS AND METHODS

Nine *Medicago* species used in this study are shown in Table 1. The classification corresponds to that proposed by Lesins and Lesins (1979). Salt soluble proteins (SSP) were extracted from mature leaves by grinding 0.5g fresh foliage in 1 ml of 50 mM Tris-HCl (pH 7.5) and 0.5M NaCl at 4℃ for 30 min. The same proteins were also extracted from seeds by grinding 30 mg in 1ml of the above solution at 4℃ which was then frozen at -20℃ and thawed 3 times during 24h to disrupt tissue and release the proteins (Miller et al., 1972). Alcohol soluble proteins (APS) were extracted by grinding 1g of mature leaves or 40mg of seeds in 1ml of 55% 2-propanol, 2% 2-mercaptoethanol and 1mM PMSF (Lyznik and Tsai, 1989). The homogenates were kept at 80℃ for 1h. Centrifugations were performed at 12000rpm for 10min. Supernatants were used either immediately or stored at -20℃ (SSP) or -4℃ (ASP). Chloroplasts were isolated from bulk leaves using Saltz and Beckman method (1981). Twenty, 45 and 60% sucrose gradient centrifugation were carried out and chloroplast pellets at the final step were suspended in adequate volume of SSP extract solution. The protein were released after several freezing and thawing steps.

SDS-polyacrylamide gel cletrophoresis (SDS-PAGE): One dimensional SDS-PAGE was carried out according to Laemmli (1970). Twelve percent of resolving slab gels were used and formed between two glass plates (16 X 16 X 0.1cm). Samples were prepared for electrophoresis by adding 5 μl of 2-mercaptoethanol, and 3.75 μl of 0.002% bromophenol blue in 62.5mM Tris-HCl (pH 6.8), containing 10% glycerol and 2% SDS (Hames and Rickwood, 1985). For APS, these quantities were doubled. At least three individuals from each species, together and/or separately were analysed in each case. The extracted proteins were stained with silver nitrate, fallowing the method suggested by Switzer et al. (1979).

RFLP and DNA hybridization: Total DNA were isolated from bulked young leaves of at least four plants of each species as described by Honda and Hirai (1990). Total DNAs were reextracted by phenol-chloroform, precipitated by ethanol and digested with *EcoR*I, *BamH*I, *Hind*III and *Pst*I restriction endonucleases according to supplier instructions. DNA fragmemts were separated by 0.8% agarose gels (20V for 16h), stained with ethidium bromide and transferred onto nylon membrane by Southern blotting (Southern, 1975). DNA inserts were bound to nylon membrane by UV cross-linking for 3-5 min. Prehybridization (4h) and hybridization (16h) were performed at 65-68℃ by using the following probes: *BamH*I-1, *BamH*I-3, *BamH*I-7 and *BamH*I-8. These probes consisted of four chloroplast DNA fragments obtained from rice, kindly donated by Dr. Hirai (The University of Tokyo, Japan). The sizes of them are 19.2, 9.0, 5.0 and 4.0 kb, respectively. Southern hybridization was carried out using a Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim, Germany).

Analysis of data: Protein and RFLP band patterns posessing unambiguous resolutions were coded by 0 or 1 depending on their absence or presence in each species. The resemblance matrices were calculated directly from data matrices, using the ratio of " number of 1-0 matches"/ "total number of bands" as an index of genetic distance, which corresponds to simple matching coefficient in the form of dissimilarity (Romesburg, 1990). In this method "absence" contributed

Table 2. Scores of 68 protein markers for 9 *Medicago* species.

organ	Rfx 100	Salt soluble proteins								
		sativa	*orbicularis*	*radiata*	*minima*	*rigidula*	*littoralis*	*truncatula*	*scutellata*	*lupulina*
leaf	5	1	0	1	1	1	1	1	1	1
	6	1	1	1	1	1	1	1	1	1
	12	1	1	1	1	1	1	1	1	1
	16	1	1	1	1	1	1	1	1	1
	28	1	1	1	1	1	1	1	1	1
	36	1	1	1	1	1	1	1	1	1
	41	1	1	1	1	1	1	1	1	1
	43	1	1	1	1	1	1	1	1	1
	46	1	1	1	1	1	1	1	1	1
	55	0	1	0	1	1	1	1	1	0
	59	0	0	0	0	0	1	1	0	0
	60	1	1	1	1	1	0	0	1	1
	87	1	0	0	0	0	0	0	0	0
	89	1	1	1	1	1	1	1	1	1
	91	1	0	0	0	0	0	0	0	0
	93	0	0	0	0	0	0	0	0	0
	94	1	0	0	0	0	1	1	1	0
	95	0	0	0	0	0	0	0	0	1
	96	1	1	1	1	1	0	0	1	0
seed	14	0	1	1	1	1	1	1	1	0
	15	1	0	0	0	1	0	1	0	1
	30	1	1	1	1	1	1	1	1	1
	33	0	0	0	1	1	0	0	0	0
	54	1	0	0	1	1	1	0	0	0
	56	0	0	1	1	0	0	0	1	1
	59	0	1	0	0	1	1	1	1	0
	66	1	1	0	0	1	1	1	1	1
	69	1	1	1	0	1	1	1	1	0
	74	0	1	0	1	1	1	1	1	1
	76	1	1	1	1	0	1	1	1	1
	83	1	1	1	1	1	1	1	1	1
	90	0	1	1	1	1	1	1	1	1
chloroplast	38	0	1	1	1	1	1	1	1	1
	40	1	0	1	1	0	0	0	0	0
	58	1	1	1	1	1	1	1	1	1
	61	1	1	1	1	1	1	1	1	1
	65	1	1	1	1	1	1	1	1	1
	66	0	0	0	0	0	1	1	1	0
	67	1	1	1	0	0	1	1	0	1
	70	0	0	0	0	0	0	0	0	1

organ	Rfx 100	Salt soluble proteins								
		sativa	*orbicularis*	*radiata*	*minima*	*rigidula*	*littoralis*	*truncatula*	*scutellata*	*lupulina*
leaf	5	1	0	1	1	1	1	1	1	1
	8	0	1	0	0	0	0	0	0	0
	9	1	0	0	0	1	1	1	1	1
	17	1	1	1	1	1	1	1	1	1
	18	0	0	0	1	0	0	0	0	0
	35	0	0	1	1	1	0	0	0	0
	36	1	1	0	1	1	1	1	1	1
	37	1	0	0	0	0	0	0	0	1
	38	0	1	1	1	1	0	1	1	1
	46	0	0	0	0	0	0	0	1	0
	50	0	0	1	0	0	0	0	0	0
	54	1	0	0	0	1	1	1	0	0
seed	21	0	0	1	1	1	0	0	1	1
	23	1	1	0	0	1	1	1	1	0
	24	1	0	0	1	0	0	0	0	0
	25	0	1	1	1	1	1	1	1	1
	26	1	0	0	0	0	0	0	0	0
	34	1	1	1	1	1	1	1	1	1
	36	0	0	0	0	1	1	1	1	0
	38	0	0	0	0	0	0	1	1	0
	39	1	1	1	1	0	0	0	0	0
	40	0	0	1	0	0	0	0	0	1
	49	1	1	1	0	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	0
	51	0	0	0	0	0	1	1	0	0
	52	1	1	1	1	1	0	0	1	1
	85	1	0	1	0	0	0	0	0	0
	90	0	1	1	1	1	1	1	1	1
	94	0	0	0	0	1	1	1	1	0

Table 3. Scores for 85-133 RFLP markers for 9 *Medicago* species(1-9)

probe	mol.size kb	EcoRI 1	2	3	4	5	6	7	8	9
BamHI-1	11.8	0	1	-	0	0	0	0	0	0
	9.8	1	0	-	0	0	1	1	1	0
	6.6	0	0	-	0	0	0	0	0	1
	6.4	0	0	-	1	0	0	0	1	0
	5.8	0	0	-	0	0	0	0	0	1
	4.2	0	0	-	1	1	0	1	0	0
	4.0	1	1	-	1	1	1	0	1	0
	3.8	0	0	-	0	0	0	0	0	1
	2.7	0	0	-	1	0	0	1	0	0
	2.2	0	0	-	0	0	0	0	0	1
	2.1	1	0	-	0	0	1	0	1	0
	2.0	0	0	-	0	0	0	1	0	0
	1.4	1	1	-	1	1	1	1	1	1
BamHI-3	14.0	0	1	0	0	0	0	0	0	0
	10.8	0	0	0	0	0	1	1	1	0
	7.0	0	0	0	1	1	0	0	0	0
	6.8	0	0	0	0	0	0	0	1	0
	5.6	0	0	0	0	0	1	0	0	0
	5.3	0	0	0	0	0	1	0	0	1
	4.9	1	1	1	0	0	0	1	1	0
	4.8	0	0	0	1	1	0	0	0	0
	2.7	0	0	0	1	1	0	0	0	0
	2.5	0	0	1	0	0	0	0	0	0
	2.3	1	1	0	0	0	1	1	1	1
BamHI-7	7.0	0	0	0	1	1	0	0	0	1
	5.6	1	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	1
	5.4	0	1	0	0	0	0	0	0	0
	3.8	0	0	0	0	1	1	1	0	0
	3.5	0	1	0	0	0	0	0	0	0
	3.2	0	0	1	0	0	0	0	0	0
	2.7	0	0	0	0	1	0	0	0	0
	2.4	0	1	0	0	0	0	0	0	0
	2.2	1	1	1	1	1	1	1	1	1
	2.0	1	1	1	1	1	1	1	1	1
	1.9	0	1	0	0	0	0	0	0	0
BamHI-8	3.6	1	0	0	0	0	0	-	0	0
	3.2	0	0	0	0	0	1	-	0	0
	3.0	0	0	1	0	0	0	-	0	0
	1.7	0	0	0	0	0	0	-	0	1
	1.6	0	0	0	0	0	0	-	1	0
	1.3	1	1	1	0	0	0	-	0	0
	1.2	0	0	0	1	1	1	-	1	1
	1.1	1	1	1	1	1	1	-	1	1

probe	mol.size kb	Hind Ⅲ 1	2	3	4	5	6	7	8	9
BamHI-1	13.0	0	1	0	1	0	0	0	0	1
	12.0	0	1	0	1	0	0	0	0	1
	11.0	0	0	0	1	0	0	0	0	1
	9.0	0	1	0	1	0	1	0	0	0
	7.5	1	1	1	0	1	1	1	1	0
	6.5	0	1	0	1	0	0	0	0	1
	5.6	1	1	0	1	1	0	0	0	1
	5.4	0	0	0	0	0	1	1	0	0
	4.7	0	0	1	0	0	0	0	0	0
	4.6	1	1	0	0	0	1	1	0	0
	4.5	0	0	1	1	1	0	0	0	0
	4.2	0	0	0	0	0	0	0	0	1
	4.0	0	0	0	0	0	0	0	1	0
	3.2	0	0	0	0	0	0	0	1	0
	2.8	0	1	1	0	0	1	1	0	0
BamHI-3	23.3	1	1	1	0	0	-	0	0	0
	23.2	0	0	0	0	1	-	0	1	1
	23.4	0	0	0	1	0	-	0	0	0
	10.5	1	0	0	0	0	-	1	1	0
	9.6	0	1	1	0	0	-	0	0	0
	8.8	0	0	1	0	0	-	0	0	0
	8.0	1	1	0	0	0	-	0	0	0
	7.5	0	0	0	1	1	-	1	0	0
	7.3	0	0	0	1	1	-	0	0	1
	6.8	0	1	0	0	0	-	1	0	0
	6.6	0	0	1	1	0	-	0	1	1
	5.0	0	0	0	1	1	-	1	0	0
	1.6	1	1	1	1	1	-	0	1	1
BamHI-7	13.4	0	0	0	1	0	-	0	0	0
	12.0	0	0	0	1	0	-	0	0	0
	11.5	1	1	1	1	1	-	0	1	0
	10.0	0	0	0	0	0	-	0	0	1
	8.0	1	1	0	1	1	-	1	1	1
	7.5	0	0	0	1	0	-	0	0	0
	7.0	0	0	1	0	0	-	0	0	0
	4.8	0	1	0	0	0	-	0	0	0
	3.8	0	0	1	0	0	-	0	0	0
	3.7	0	1	0	0	0	-	0	0	0
	2.2	1	1	0	0	1	-	1	1	0
	1.9	0	0	0	1	0	-	0	0	1
	1.7	0	0	0	1	0	-	0	0	0
	1.0	1	1	1	1	1	-	1	1	1

probe	mol.size kb	Bam HI 1	2	3	4	5	6	7	8	9
BamHI-1	9.0	0	0	0	1	1	0	0	0	0
	7.0	0	1	0	0	0	0	0	0	0
	6.8	0	0	1	0	0	0	0	0	0
	5.7	1	0	0	0	0	0	0	1	0
	5.6	0	0	0	0	0	1	1	0	0
	5.1	1	0	0	0	0	1	1	1	0
	4.7	1	1	1	1	1	0	1	1	1
	3.6	1	1	1	1	1	1	1	1	0
	3.3	0	0	0	0	0	0	0	0	1
BamHI-3	12.6	0	0	0	0	0	0	0	0	1
	11.0	1	1	0	1	1	1	1	1	0
	10.7	0	0	0	0	0	0	0	0	1
	7.0	0	0	0	0	0	0	1	1	0
	6.2	1	1	1	0	0	1	1	1	0
	5.4	0	0	0	0	0	0	0	1	0
	5.2	1	0	0	0	0	0	0	0	0
	5.0	0	0	0	0	0	0	0	0	1
	4.6	0	0	0	1	0	1	1	0	0
	3.8	0	0	0	0	1	0	0	0	1
	3.5	0	0	0	0	0	0	0	0	1
	3.2	1	0	0	1	1	1	1	0	0
	3.0	0	1	1	0	0	0	0	0	0
	2.8	0	1	1	0	0	0	0	0	0
BamHI-7	15.0	0	0	1	1	0	0	0	0	0
	7.0	0	0	0	0	0	0	0	0	1
	6.6	0	0	0	0	0	0	0	0	1
	6.4	1	1	0	0	1	1	1	0	0
	5.0	0	0	0	0	0	0	0	1	0
	4.2	0	0	0	0	0	0	0	1	0
	2.6	1	0	0	0	1	1	1	1	1
	1.0	1	1	1	1	1	1	1	1	1
BamHI-8	15.0	0	0	1	0	1	0	0	0	1
	14.0	1	0	0	1	0	0	1	1	0
	12.0	0	1	0	0	0	0	0	0	0
		Pst Ⅰ								
BamHI-1	12.0	0	0	0	1	1	0	-	0	1
	11.0	0	0	1	0	0	1	-	0	0
	10.0	1	1	1	0	1	1	-	1	0
	7.6	0	0	0	1	1	0	-	0	0
	5.8	1	1	0	0	1	0	-	1	0
	2.3	0	0	0	0	0	0	-	0	1
BamHI-3	17.0	1	1	1	1	1	-	1	1	1
	15.0	0	0	0	0	1	-	0	0	0
	8.5	0	0	0	1	0	-	0	0	0
	5.8	0	0	0	0	0	-	0	0	1
	5.5	1	0	1	0	1	-	1	1	0
	4.7	0	0	0	0	0	-	0	0	0

1=*M. sativa* 2=*M. orbicularis* 3=*M. radiata*
4=*M. Minima* 5=*M. rigidula* 6=*M. littoralis*
7=*M. truncatula* 8=*M. scutellata* 9=*M. lupulina*

equally to "presence" in the calculation of similarity (then of dissimilarity). Finally, the dendrograms were constructed by the clustering method of UPGMA(unweighted pair group method using arithmetic averages) described by Sneath and Sokal (1973).

RESULTS AND DISCUSSION

The scores of 68 band patterns of alcohol and salt solution proteins from leaves, seed and chloroplasts of nine *Medicago* species are given in Table 2. For simplicity, each band is represented by the proportion of its migration distance over migration of bromophenol blue(Rf). Also, the scores of 85-133 RFLP markers for nine *Medicago* species are given in Table 3. RFLP markers are represented by their molecular sizes using *Hind*III digested Lambda DNA as molecular weight marker. The number "1" indicates presence of the band and "0" indicates its absence. To clarify how markers were assessed, we have chosen certain gel or blot photographies (Fig. 1 and Fig. 2). It is clear that in spite of equal volumes of protein samples used in acrylamide gels or equal volumes of DNA samples in agarose gels, the staining intensity differences for some bands within or between species are evident. For simplicity, we did not quantify them in the present study and discarded only non-consistent or repeated protein banding patterns in different organs. For example, in the case of leaf salt soultion proteins, the highly active and monomorphic band of Rf=0.28, which is determined as chloroplast protein (absent in non green organs such as root or callus), is present in both leaf and chloroplast patterns. It is then scored only

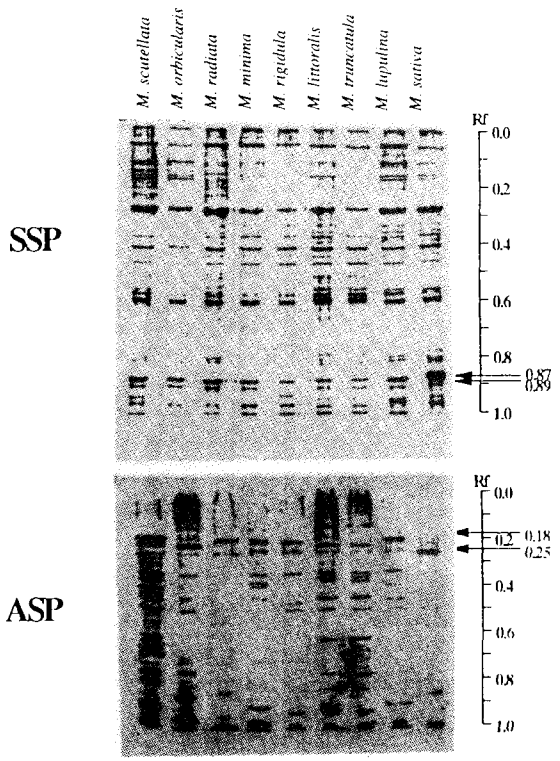

Fig. 1. Profiles of SDS-PAGE of salt soluble proteins(SSP) from leaf organ and alcohol soluble proteins(ASP) from seed in 9 *Medicago* species.

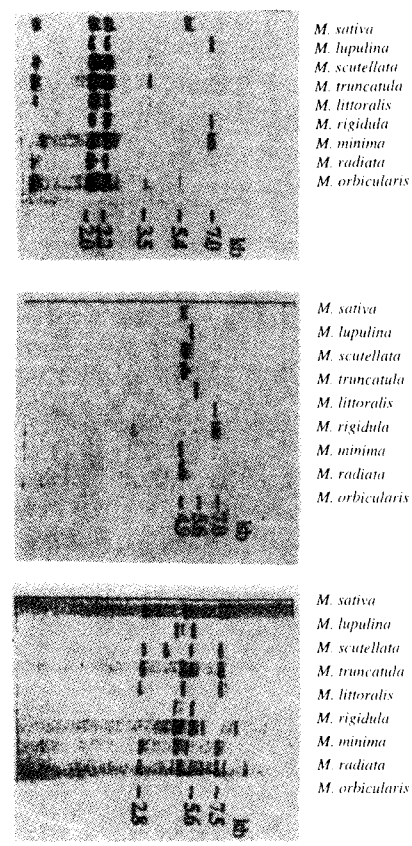

Fig. 2. Profiles of RFLP markers in 9 *Medicago* species. A, B; The genomic DNA was digested with *Eco*RI and hybridized with B7 and B3 as a probe, respectively. C; The genomic DNA was digested with *Hind* III and hybridized with B1 as a probe.

once as other similar cases. Of particular interest here is that certain bands could be easily used by any researcher to identify the species or their hybrids. The laborious and expensive method of southern blotting exhibited more stable RFLP markers (Fig. 2). The number of these markers could be increased using other restriction enzymes and probes. However, considering the relative simplicity of SDS-PAGE markers (Fig. 1), it makes very useful, especially because it may probably exhibits larger genetic variation than RFLP, which reflects only chloroplast DNA variations in the present study. Two case of protein markers were interesting (Fig. 1): 1) Leaf salt soluble protein of Rf =0.87 was present in all samples of perennial and purple flowered *M. sativa* and absent for other annual and yellow flowered species. The adjacent band of Rf=0.89 was present in all species. In orther to find a possible biological significance of this polymorphism, other perennial and purple flowered species should be studied. 2) Seed alcohol soluble proteins having Rf of 0.18-0.25 are also interesting because one of the bands had a good resolution which differentiated the species easily. In this study, we have taken in consideration the poorly stained bands existing at the Rf between 0.18-0.25 for all gels. They probably have smaller weight than well stained bands which may have a larger amount of proteins. Resemblance matrices for protein, RFLP and combination of both markers are summarized in Table 4. The average values of genetic distances for the two kinds of markers have good concordance. The magnitude of genetic distances between the *Medicago* species analyzed in this study are smaller than that in genus *Medicago* based on traditionally used characters (Small, 1981). But they are larger than distances in genus *Lycopersicon* based on RFLP data, using nuclear DNA fragments as probes (Miller and Tanksley, 1990). Combined genetic distances in Table 4 (PR) were calculated by accepting two weights for protein markers and one weight for RFLP markers, because protein markers reflect genetic variation in two organs. The cluster analysis based on protein and RFLP markers (Fig. 3) showed some deplacements of species. The

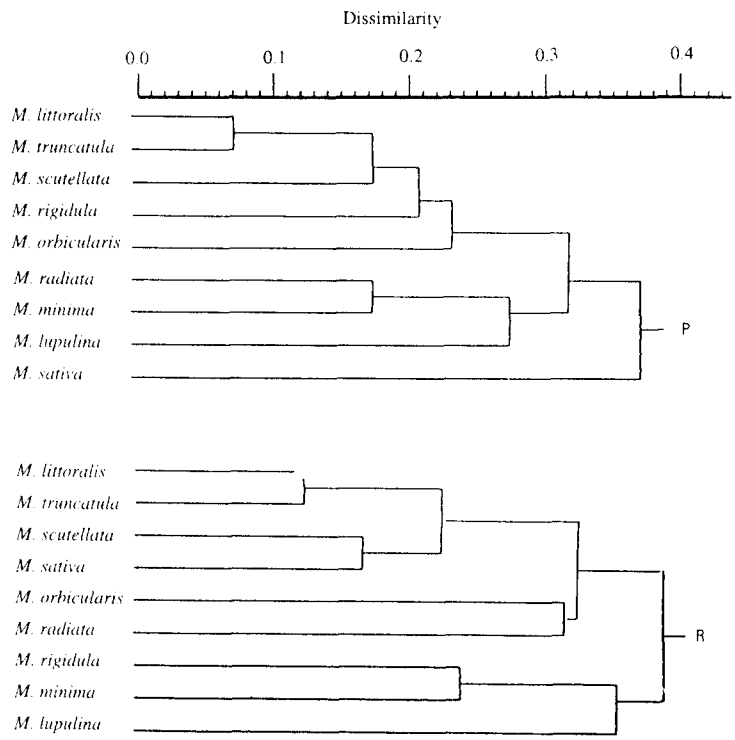

Fig. 3. Dendrograms among 9 *Medicago* species based on protein pattern(P) and RFLP markers(R).

Table 4. Genetic distances between 9 *Medicago* species for protein(P), RFLP(R) and combined markers(PR).

	sat.	orb.	rad.	min.	rig.	lit.	tru.	scu.	lup.	
P	-	343	358	403	403	373	403	418	358	*M. sativa*
R	-	241	308	398	271	222	218	172	436	
PR	-	309	341	401	359	323	341	336	384	
		-	224	239	224	239	239	194	284	*M. orbicularis*
		-	308	414	316	313	353	346	451	
		-	252	297	270	264	277	245	340	
			-	194	328	403	403	299	269	*M. radiata*
			-	425	367	330	368	333	467	
			-	271	341	379	391	310	335	
				-	254	388	418	284	313	*M. minima*
				-	233	364	395	398	368	
				-	247	380	410	322	331	
					-	224	224	179	328	*M. rigidula*
					-	293	286	316	346	
					-	274	244	225	334	
						-	60	194	373	*M. littoralis*
						-	129	263	495	
						-	83	217	414	
							-	164	343	*M. truncatula*
							-	227	479	
							-	185	388	
								-	299	*M. scutellata*
								-	451	
$\overline{X}p = 0.296$								-	350	
$\overline{X}r = 0.337$									-	*M. lupulina*
$\overline{X}pr = 0.310$									-	

Distance × 1000

most important of these deplacements is as follow: *M. sativa* which is distantly related to other species in terms of protein markers was closely clustered with *M. scutellata, M. littoralis* and *M. truncatula* in the case of RFLP markers, indicating the closer affinity of these four "fast growing" species for chloroplast DNA (data not shown). The combined

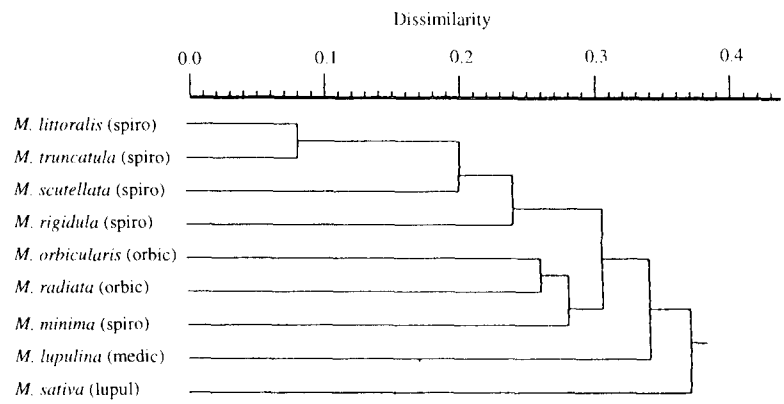

Fig. 4. Dendrogram among 9 *Medicago* species based on both protein and RFLP markers(see in Table 1).

cluster analysis (Fig. 4), at a dissimilarity level of 0.31, differentiated nine species in four groups: 1) *M. littoralis, M. truncatula, M. scutellata* and *M. rigidula* ; 2) *M. sativa* ; 3) *M. lupulina* ; 4) *M. orbicularis, M. radiata* and *M. minima*. All of them corresponded to the existing four subgenera of genus *Medicago* (Lesins and Lesins, 1979) except for *M. minima*, which is very distant from *Spirocarpos* subgenus (see Table 1), should be examined comprehensively. For the nine *Medicago* species in this study, there is a significant positive correlation (r=0.68 for 34df) between cophenetic values of protein and RFLP qualitative characters (Fig.4) and those of traditionally used characters measured by Small (1981). However, comparing the previous classification of *Medicago* species with that in this study, it is possible to note some discrepancies: *M. lupulina* is the most distant species from others and *M. minima* has been clustered in the group of *M. orbicularis* and *M. radiata*. Further investigation should be followed specially by using the RFLP analysis of nuclear DNA.

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