Analysis of Genetic Distance among Nine *Medicago* Species by Using DNA Polymorphisms

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Summary

The relationship of 9 *Medicago* species belonging to 4 subgenera was analyzed by using restriction fragment length polymorphism (RFLP) methodologies. We analyzed 85-132 RFLP band patterns, which were caused by chloroplast DNA variations, to estimate the genetic distance among the species. These species were clustered together at around 0.1 to 0.4 level of distance for marker, indicating that the *Medicago* species have a large genetic similarity. The most similar species were *M. littoralis* and *M. truncatula* and the most dissimilar one was *M. lupulina*. The cluster analysis based on RFLP band patterns differentiated 9 species in another 4 groups: a) *M. littoralis*, *M. truncatula*, *M. scutellata* and *M. sativa*; b) *M. orbicularis* and *M. radiata*; c) *M. rigidula* and *M. minima*; d) *M. lupulina*. Comparing the previous classification of *Medicago* species with that in this study, there are two discrepancies; *M. lupulina* is the most distant species from others and *M. sativa* (subgenus *Medicago*) was closely clustered with *M. scutellata*, *M. littoralis* and *M. truncatula* classified in subgenus *Spirocarpos*.

Key Words : *Medicago*, alfalfa, RFLP, phylogenetic study.

Introduction

The genus *Medicago* (tribe Trifolieae, family Fabaceae), as currently defined (Lesins and Lesins 1979), is presented by about 55 annual and perennial, diploid and tetraploid species with basic chromosome numbers 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 crops. Since some of *Medicago* species are resistant to insect and disease (Ridland and Berg 1981, Barbetti 1987), the phylogenetic study of *Medicago* species should 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 agroecological resources.

Cytology, isozyme and RFLP studies of some *Medicago* species, specially of alfalfa, are well documented (Simon and Simon 1965, Damerval 1983, Rose et al. 1986, Kiss et al. 1993). However, a comparative study of genetic variation among *Medicago* species has rarely 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 DNA markers.

In the present study, a comparative study has been undertaken by using DNA polymorphism to estimate the genetic distance of 9 species belonging to 4 subgenera of the genus *Medicago*. Since Southern transcaucasian area is known to be origin of alfalfa (Ford-Lloyd and Jackson 1986), six species native to north-west of Iran are included.

Materials and Methods

Plant materials

Nine *Medicago* species used in this study are shown in Table 1. The classification corresponds to that proposed by Lesins and Lesins (1979).

RFLP analysis

Total DNAs were isolated from bulked young leaves of at least 4 plants of each species as described by Honda and Hirai (1990). Total DNAs were reextracted by phenol-chloroform, precipitated by ethanol and digested with EcoRI, BamHI, HindIII and PstI restriction en-

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

Name	Section	Subgenus	Origin
<i>M. orbicularis</i>	Orbicularia	Orbiculares	Azerb. Iran
<i>M. radiata</i>	Orbicularia	Hymenocarpos	Azerb. Iran
<i>M. minima</i>	Spirocarpos	Leptospirae	Azerb. Iran
<i>M. rigidula</i>	Spirocarpos	Pachyspirae	Azerb. Iran
<i>M. littoralis</i>	Spirocarpos	Pachyspirae	Australia
<i>M. truncatula</i>	Spirocarpos	Pachyspirae	Australia
<i>M. scutellata</i>	Spirocarpos	Rotatae	Australia
<i>M. lupulina</i>	Lupularia	—	Azerb. Iran
<i>M. sativa</i>	Medicago	Falcago	Azerb. Iran

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donucleases according to supplier instructions. DNA fragments were separated by 0.8 % agarose gels (20 V for 16 h), 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 (4 h) and hybridization (16 h) were performed at 65-68 °C by using the following probes: BamHI-1, BamHI-3, BamHI-7 and BamHI-8 fragments of rice chloroplast DNA (Hirai et al. 1985). 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

RFLP band patterns possessing 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 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

Total DNAs were prepared from green leaves of 9 *Medicago* species. They were digested with restriction enzymes and were separated by agarose gel electrophoresis. Southern hybridization was carried out with DNA fragments of rice chloroplast DNA as probes. To clarify how the markers were assessed, we have chosen certain blot photographs as shown in Figure 1. The number of these polymorphisms could be increased using other restriction enzymes and probes. Finally, we analyzed 85-132 RFLP band patterns for 9 *Medicago* species. Resemblance matrices for RFLP band patterns are summarized in Table 2. The genetic distances between the *Medicago* species analyzed in this study were smaller than that in genus *Medicago* based on traditionally used characters (Small 1981). But they were larger than distances in genus *Lycopersicon* based on RFLP data, using nuclear DNA fragments as probes

Fig. 1. Examples of RFLP bands in 9 *Medicago* species.

A. BamHI-7 probe, EcoRI digest; B. BamHI-3 probe, EcoRI digest; C. BamHI-1 probe, HindIII digest.

Table 2. Genetic distance between 9 *Medicago* species for RFLP band patterns

sat.	orb.	rad.	min.	rig.	lit.	tru.	scu.	lup.	
—	241	308	398	271	222	218	172	436	<i>M. sativa</i>
	—	308	414	361	313	353	346	451	<i>M. orbicularis</i>
		—	425	367	330	368	333	467	<i>M. radiata</i>
			—	233	364	395	398	368	<i>M. minima</i>
				—	293	286	316	346	<i>M. rigidula</i>
					—	129	63	495	<i>M. littoralis</i>
						—	227	479	<i>M. truncatula</i>
							—	451	<i>M. scutellata</i>
								—	<i>M. lupulina</i>
$\bar{X}=0.337$									
Distance×1000

Fig. 2. Dendrogram of 9 *Medicago* species based on 85-132 RFLP bands.

(Miller and Tanksley 1990).

From the cluster analysis based on RFLP band patterns (Fig. 2), 9 *Medicago* species were clustered together at around 0.1 to 0.4 level of distance for marker, indicating that the *Medicago* species have a large genetic similarity. And the most similar species were *M. littoralis* and *M. truncatula* and the most dissimilar one was *M. lupulina*. Further, the cluster analysis differentiated 9 species in another 4 groups: a) *M. littoralis*, *M. truncatula*, *M. scutellata* and *M. sativa*; b) *M. orbicularis* and *M. radiata*; c) *M. rigidula* and *M. minima*; d) *M. lupulina*. Dissimilarity level between group a and b was 0.34 and that between c and d was 0.37. Five species of *M. littoralis*, *M. truncatula*, *M. scutellata*, *M. rigidula* and *M. minima* were classified in subgenus *Spirocarpos* by Lesins and Lesins (1979), however, they were separately clustered into two groups. This indicates that these two groups were phylogenetically distant. The origin of *M. rigidula* and *M. minima* (group c, Iran) is distant from that of *M. littoralis*, *M. truncatula*, and *M. scutellata* (group a, Australia). This supports the genetic distance between these two groups.

Comparing the previous classification of *Medicago* species with that in this study, it is possible to note two discrepancies; *M. lupulina* was the most distant species from others and *M. sativa* was closely clustered with *M. scutellata*, *M. littoralis* and *M. truncatula*. *M. sativa* and the other three species were classified in different subgenus, *Medicago* and *Spirocarpos*, respectively (Lesins and Lesins 1979). Besides the origin of *M. sativa* was the Near East (Ford-Lloyd and Jackson 1986) and that of another three species was Australia. For the *Medicago* species in this study, it indicates that *M. sativa* is related more closely with the species in subgenus *Spirocar-*

pos whose origin is Australia than those in another subgenus whose origin is the Near East. Further investigations should be followed by using more *Medicago* species whose origin is the Near East.

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Literature Cited

- Agarwal, K. and P. K. Gupta (1983) Cytological studies in Genus *Medicago*. Linn. Cytologia 48 : 781-793.
- Barbetti, M. J. (1987) Effect of temperature and humidity on disease caused by *Phoma medicaginis*, resistance in some *Medicago* cultivars and the incidence of seed-borne inoculum. Aust. J. Exp. Agric. 27 : 851-856.
- Damerval, C. (1983) Comparaison de six especes de luzernes annuelles a l'aide de caracteres biometriques et enzymatiques. Agronomie 3 : 971-982.
- Ford-Lloyd, B. and M. Jackson (1986) Crop evolution and diversity. In "Plant genetic resources", Edward Arnold (Publishers) Ltd., London, 10-36.
- Hirai, A., T. Ishibashi, A. Morikami, N. Iwatsuki, K. Shinozaki and M. Sugiura (1985) Rice chloroplast DNA : a physical map and the location of the genes for the large subunit of ribulose 1,5-bisphosphate carboxylase and 32 kD photosystem II reaction center protein. Theor. Appl. Genet. 70 : 117-122.
- Honda, H. and A. Hirai (1990) A simple and efficient method for identification of hybrids using nonradioactive rDNA as

- probe. Jpn. J. Breeding 40 : 339-348.
- Kiss, G. B., G. Csanadi, K. Kalman, P. Kalo and L. Okresz (1993) Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. Mol. Gen. Genet. 238 : 129-137.
- Lesins, K. A. and I. Lesins (1979) In "Genus *Medicago* (Leguminosae)." Dr. W. Junk bv. Publishers. London UK, 31.
- Miller, J. C. and S. D. Tanksley (1990) PFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. Theor. Appl. Genet. 80 : 437-448.
- Puckridge, D. W. and R. J. French (1983) The annual legume pasture in cerealley farming systems of Southern Australia : a review. Agric. Ecosystems Environ. 9 : 229-236.
- Quiros, F. Q. (1983) Alfalfa, luzerne (*Medicago sativa*). In "Isozymes in plant genetics and breeding, B." S. D. Tanksley, T. G. and Orton (eds) Elsevier, 253.
- Ridland, P. M. and G. N. Berg (1981) Seedling resistance to pea aphid of lucerne, annual medic and clover species in Victoria. Aust. J. Exp. Agric. Anim. Husb. 21 : 506-511.
- Romesburg, H. C. (1990) In "Cluster analysis for researchers." Robert E. Krieger publishing co. Florida, U. S. A., 144-150.
- Rose, R. J., L. B. Johnson and R. J. Kemble (1986) Restriction endonuclease studies on chloroplast and mitochondrial DNAs of alfalfa protoclonal. Plant Mol. Biol. 6 : 331-338.
- Simon, J.P. and A. Simon (1965) Relationships in annual species of *Medicago*; Number and morphology of chromosomes. Aus. J. Agric. Res. 16 : 37-50.
- Sneath, P. H. A. and P. R. Sokal (1973) In "Numerical taxonomy." W. H. Freeman and co, San Francisco, U. S. A., 230-234.
- Small, E. (1981) A numerical analysis of major groupings in *Medicago* employing traditionally used characters. Can. J. Bot. 59 : 1553-1577.
- Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 : 503-517.