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BRIEF COMMUNICATION

Genetic diversity assessment in Greek *Medicago truncatula* genotypes using microsatellite markers

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Abstract

In this study we examined the genetic diversity and geographic scale of genotype distribution within the model legume species *Medicago truncatula* widely distributed in pasture and marginal agricultural lands in Greece and other Mediterranean countries. Thirty one *Medicago truncatula* and *Medicago littorialis* accessions were chosen on the basis of their geographical distributions and studied using 9 polymorphic simple sequence repeats (SSR) markers. The number of alleles per locus varied between 3 and 7. A total of 42 alleles were detected with a mean value of 4.66 alleles per locus. Geographic origin was not related with genotypic similarity among accessions. However, there were instances of close genetic relatedness between accessions from neighboring locations in a geographic compartment. In conclusion, the presented data revealed extensive *M. truncatula* genotype dispersal in Greece pointing to the significance of preserving local genetic resources in their natural environment.

Additional key words: molecular markers, phylogenetic analysis, simple sequence repeats.

Germplasm characterization is important for evaluation of genetic resources and utilization of valuable genotypes for breeding purposes. *Medicago truncatula*, a close relative of alfalfa, has gained much attention in plant research serving as a model species in legumes, like *Arabidopsis* in non-legume dicot plants. *M. truncatula* is also important as a forage legume crop especially in Australia. Genetic diversity within a species especially in locations where it is endemic and represent a center of origin or initial distribution must be evaluated and preserved (Mirali *et al.* 2007). Microsatellites or simple sequence repeats (SSRs) have become the preferred markers to examine genetic diversity in many studies (Dikshit *et al.* 2007, Joshi and Dhawan 2007) since they exhibit high levels of variability, ease and reliability of scoring, co-dominant inheritance and short lengths (Sunnock 2000). Polymorphic microsatellites have been isolated and characterized in *M. truncatula* (Baquerizo-Audiot *et al.* 2001; Choi *et al.* 2004, Mun *et al.* 2006). SSRs have been used to examine population polymorphisms revealing an unexpected large amount of intrapopulation polymorphism at specific locations in this self-fertilizing species (Bonnin *et al.* 2001). SSRs have also been used to evaluate genetic diversity in large germplasm collections maintained by the South Australian Research and Development Institute (SARDI) of the Australian Medicago Genetic Resources Center (Ellwood *et al.* 2006) and the Genetic Resources and Mediterranean *Medicago* Plant Breeding Laboratory (INRA, Montpellier, France) (Ronfort *et al.* 2006) revealing unusually high genotype dispersal that could be due to animal and trade-related movement. The only positive and significant associations between genetic similarity and geographic proximity were found for individuals originating from the same or neighbouring populations. However, no clear evidence for isolation by distance could be detected in analyses conducted in a wide spatial scale (Ronfort *et al.* 2006). Presently there are no studies aiming at *Medicago truncatula* germplasm characterization in the area of a single country, which

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Abbreviations: MEGA - molecular evolutionary genetics analysis; NJ - Neighbour joining; SSR - simple sequence repeats.
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could provide important information for *in situ* conservation and utilization of plant genetic resources. The aim of this study is to estimate the extent of *M. truncatula* genetic diversity and examine the relation of genetic diversity with geographic distance within this species in Greece. This could be useful for the development of strategies and methodologies for conservation of plant genetic resources and improvement of valuable *M. truncatula* germplasm utilization for breeding purposes.

Seventeen accessions of *Medicago truncatula* Gaertn. and four accessions of *Medicago littorialis* Rhode *ex* Hornem. collected from local populations on the basis of their geographical distributions, environmental descriptors of the site and pod characteristics were chosen from 5 geographic regions of Greece (Macedonia, Thessaly, Sterea Ellada, Peloponnesus and Crete). Nine cultivar accessions of *M. truncatula* from Australia as well as one cultivar accession of *M. truncatula* from France were chosen as control material. Seeds from each of the above accessions are maintained at the Genetic Resources and Mediterranean *Medicago* Plant Breeding Laboratory and were kindly provided by J.-M. Prosperi. Genomic DNA was extracted from 200 mg of fresh leaf material from single plants with the *Qiagen* (Chatsworth, USA) DNeasy plant mini kit according to manufacturer's instructions. Polymorphism was assayed on each DNA sample at nine microsatellite loci: MTSA5, MTSA6 and MTR52 that are dinucleotide repeat loci, MTR58 that is a dinucleotide and trinucleotide imperfect locus, MTR58C that is a dinucleotide imperfect locus, MT660538 that is a dinucleotide imperfect repeat locus, and MT660252, MAA660749 and MT660456, which are trinucleotide repeat loci (Baquerizo-Audiot *et al.* 2001). Amplification reactions were performed in a final volume of 0.05 cm^3 in the presence of 50 ng template DNA, 10 pmol of each primer, 0.2 mM of each dNTP, 1 U Taq polymerase (*New England Biolabs*, Ipswich, USA), and 1× Taq polymerase buffer supplied by the manufacturer but with final concentration of $MgCl_2$ adjusted to 2 mM. PCR was carried out using the *PTC 200* thermocycler (*MJ Research*, Waltham, USA). After 4 min at 94 °C, 35 cycles were performed with 30 s at 94 °C, 45 s at a temperature between 50 - 60 °C depending on the primer pair as suggested (Baquerizo-Audiot *et al.* 2001), 1 min at 72 °C, and a final step of 5 min at 72 °C. 0.005 cm^3 of each amplified product was mixed with 0.0005 cm^3 of 10× loading buffer (98 % formamide, 10 mM EDTA, pH 8, 1 % xylene cyanol, 1 % bromophenol blue) and were loaded onto 6 % *MetaPhor®* agarose gel (*Cambrex*, Berkshire, UK) in 1× TAE buffer, stained with ethidium bromide, and visualized by UV radiation. Amplifications were performed at least twice with high reproducibility. Results were also confirmed on polyacrylamide gels. For each primer the number of bands and the differences in size of band products were recorded from the examination of ethidium bromide stained gels. The sizes of the bands were estimated using the *UVIDoc* version *99.04* software (*UVI*, Cambridge, UK).

The 9 microsatellite markers detected a total of 35 alleles with a mean value of N_A = 4.66 alleles per locus or 0.15 alleles per locus per individual. All loci were polymorphic. All accessions could be distinguished on the basis of SSR polymorphisms of the 9 loci and no identical genotypes were observed. The number of alleles ranged from 3 to 7 (Table 1). The largest number of alleles per locus occurred for locus MAA660456 (7 alleles per locus). Loci MTSA5 and MAA660538 had 6 alleles, loci MTR52, MTR58 had 5 alleles, locus MTSA6 had 4 alleles and the less polymorphic loci were MTR58C, MAA660252 and MAA660749 with 3 alleles. Similar results were recorded in the Aude population study where the same set of SSR markers was employed with an average N_A = 5.6 alleles per locus but a low proportion of 0.018 alleles per locus per individual (Bonnin *et al.* 2001). Numbers of alleles of the same magnitude (between 1 and 11 per locus) with average 5.8 alleles per locus were recorded for the most frequent alleles in the INRA-Montpellier collection when rare alleles with frequency below 0.05 were removed (Ronfort *et al.* 2006). However, much higher number of alleles were detected in the full range of alleles in the INRA-Montpellier collection (3 - 53 alleles per locus) with an average N_A = 20.7 alleles per locus or 0.06 alleles per locus per individual (Ronfort *et al.* 2006) and the SARDI collection (16 - 42 alleles per locus) with N_A = 24.83 alleles per locus or 0.126 alleles per locus per individual (Ellwood *et al.* 2006).

Similarity estimates were curried out using the *Microsatellite Analyzer* version *4.00* software (Dieringer and Schlotterer 2003) to calculate Nei's D_A genetic distance and allele frequencies. The D_A distance matrix was employed to the *MEGA 3.1* software (Kumar *et al.* 2004) in order to construct a NJ tree (Saitou and Nei 1987). NJ has been shown to be the most accurate method to obtain the best topology using microsatellites as genetic markers (Takezaki and Nei 1996). The NJ dendrogram based on D_A distance (Fig. 1) showed that the 31 accessions of *M. truncatula* and *M. littorialis* formed two major branches. The first branch consisted of 21 accessions and all of them were *M. truncatula*. Accessions in this branch could be separated into two groups (Fig. 1). Group I was poly-morphic and contained different accessions and cultivars from Greece, Australia and France. Group II contained only 4 Australian cultivars. In the first major branch the maximum similarity observed between the *M. truncatula* accessions GRE020 (Peloponnesus) and GRC052 (Thessaly). The second branch contained 6 *M. truncatula* and 4 *M. littorialis* accessions. However, Harbinger is a cultivar that has been registered in Australia as *M. littoralis* (see http://www.pi.csiro.au/ahpc/legumes/pdf/harbinger.pdf and references therein) and it is ambiguous if it should be assigned as *M. truncatula*. This branch has several interesting features. All *M. littoralis* genotypes clustered there. Half of the Peloponnesus genotypes belonged in this branch and 2 were *M. truncatula* while

Table 1. Primers, allele size observed, number of alleles observed in 31 accessions and frequency of each allele for a specific locus. Dash indicates no amplification.

Locus	Alleles per locus	Allele size [bp]	Alleles observed	Allele frequency
TSA5	6	210	8	0.2857
		230	3	0.1071
		235	2	0.0714
		240	11	0.3929
		245	2	0.0714
		250	2	0.0714
		-	3	
MTSA6	4	130	4	0.1818
		140	11	0.5000
		145	2	0.0909
		155	5	0.2273
MTR52	5	55	2	0.0870
		60	7	0.3043
		67	8	0.3478
		70	4	0.1739
		83	2	0.0870
		-	9	
MTR58	5	140	6	0.1935
		150	2	0.0323
		160	12	0.3871
		170	4	0.1290
		180	5	0.2581
		-	8	
MTPG58C	3	120	7	0.2333
		130	2	0.0667
		140	21	0.7000
		-	1	
MAA660252	3	105	2	0.0645
		110	27	0.8710
		120	2	0.0645
MAA6600456	7	90	3	0.1034
		95	4	0.1379
		100	10	0.3448
		110	1	0.0345
		115	3	0.1034
		120	7	0.2414
		130	1	0.0345
		-	2	
MAA660538	6	130	8	0.2667
		160	1	0.0333
		195	2	0.0667
		210	4	0.1333
		220	1	0.0333
MAA660749	3	230	14	0.4667
		-	1	
		230	1	0.0333
		240	15	0.5000
		250	14	0.4667
		-	1	

3 were M. *littoralis*. Only two accessions from other compartments of Greece belong to this branch but none is *M. truncatula*. The rest genotypes in this branch are 3 cultivars from Australia. The highest similarity between genotypes in this branch was recorded among *M. truncatula* GRC043 and *M. littoralis* GRC024B, both from Peloponnesus. It is notable that several Australian cultivars formed a separate group in the dendrogram, as well as that *M. littoralis* accessions were in the same branch, indicating robustness of the analysis. Additionally this study revealed similarities between cultivars from Australia, and genotypes of Greece. *M. truncatula* is native in the Mediterranean basin but not in Australia. All Australian cultivars have originated from Mediterranean populations, although they are commonly cultivated extensively in Australia. The observed similarities might indicate that some Australian cultivars bear a proportion of Greek *M. truncatula* germplasm.

The D_A distance dendrogram revealed no characteristic pattern of organization in relation to the geographic location of the accessions. Spatial genetic structure was also investigated by testing for isolation by distance (IBD). Geographic distances between pairs of the

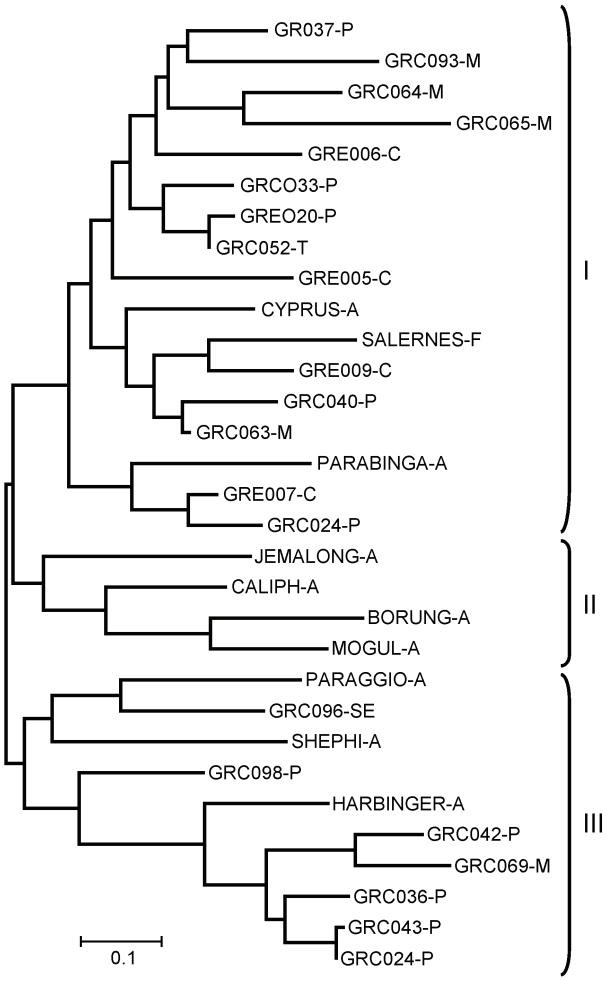

Fig. 1. Neighbor-joining dendrogram based on Nei's D_A genetic distances between 31 *Medicago truncatula* and *M. littoralis* accessions based on nine polymorphic nuclear microsatellite loci. Letters after genotype labels indicate accessions from Macedonia (M), Thessaly (T), Sterea Ellada (SE), Peloponnesus (P), Crete (C), France (F) and Australia (A). The scale indicates percentage similarities among genotypes.

Greek accessions were calculated from linear distances between exact positions derived from latitude and longitude information for each collection site. A Mantel test with 1000 random permutations was performed between the pair wise genetic distance D_A, and the natural logarithm of geographic distance. The analyses were performed using the *Isolation By Distance Web Service* (http://ibdws.sdsu.edu/~ibdws/) (Jensen *et al.* 2005) using indicators to differentiate accessions belonging to the same geographic compartment. Linear regression analysis between pair wise genetic distance and geographic distance did not show a clear trend or relationship (r = -0.06, P = 0.773). In the NJ analysis, there is some evidence of genotypic similarity between accessions originated from neighboring locations especially in Peloponnesus (Fig. 1) but there are also a considerable number of accessions showing genotypic relatedness although coming from places all over Greece. Unusual genotype dispersal was also found in the SARDI study that provided evidence of geographic clustering in North Africa, but no clear association between genotype and origin throughout the Mediterranean basin. In the INRA-Montpellier study, geographic-genetic correlation was found only for genotypes of the same or neighboring populations. In both studies, unusual genotype dispersal was recorded in the origin of some accessions which were geographically and physically isolated (Ellwood *et al.* 2006, Ronfort *et al.* 2006), something that was also evident in this work. A likely explanation for this phenomenon may be seed transport and dispersal through human migration, commercial trade and animal movement.

In conclusion, this investigation revealed high level of *Medicago truncatula* dispersal and genetic diversity in Greece pointing to the need for *in situ* preservation of the genetic resources found in this country.

References

Baquerizo-Audiot, E., Desplanqe, A.B., Prosperi, J.M., Santoni, S.: Characterization of microsatellite loci in the diploid legume *Medicago truncatula* (barrel medic). - Mol. Ecol. Notes **1**: 1-3, 2001.

Bonnin, I., Ronfort, J., Wozniak, F., Olivieri, I.: Spatial effects and rare outcrossing events in *Medicago truncatula* (Fabaceae). - Mol. Ecol. **10**: 1371-1383, 2001.

Choi, H.K., Mun, J.H., Kim, D.J., Zhu, H., Baek, J.M., Mudge, J., Roe, B., Ellis, N., Doyle, J., Kiss, G.B., Young, N.D., Cook, D.R.: Estimating genome conservation between crop and model legume species. - Proc. nat. Acad. Sci. USA **101**: 15289-15294, 2004.

Dieringer, D., Schlotterer, C.: Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. - Mol. Ecol. Notes **3**: 167-169, 2003.

Dikshit, H.K., Jhang, T., Singh, N.K., Koundal, K.R., Bansal, K.C., Chandra, N., Tickoo, J.L., Sharma, T.R.: Genetic differentiation of *Vigna* species by RAPD, URP and SSR markers. - Biol. Plant. **51**: 451-457, 2007.

Ellwood, S.R., D'Souza, N.K., Kamphuis, L.G., Burgess, T.I., Nair, R.M., Oliver, R.P.: SSR analysis of the *Medicago truncatula* SARDI core collection reveals substantial diversity and unusual genotype dispersal throughout the Mediterranean basin. - Theor. appl. Genet.: 1-7, 2006.

Jensen, J.L., Bohonak, A.J., Kelley, S.T.: Isolation by distance, web service. - BMC Genet. **6**: 13, 2005.

Joshi, P., Dhawan, V.: Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. - Biol. Plant. **51**: 22-26, 2007.

Kumar, S., Tamura, K., Nei, M.: MEGA3: Integrated software for molecular evolutionary genetics. Analysis and sequence alignment. - Brief Bioinf. **5**: 150-163, 2004.

Mirali, N., El-Khouri, S., Rizq, F.: Genetic diversity and relationships in some *Vicia* species as determined by SDS-PAGE of seed proteins. - Biol. Plant. **53**: 660-666, 2007.

Mun, J., Kim, D.J., Choi, H.K., Gish, J., Debelle, F., Mudge, J., Denny, R., Endre, G., Saurat, O., Dudez, A.M., Kiss, G., Roe, B., Young, N., Cook, D.: Distribution of microsatellites in the genome of *Medicago truncatula*: a resource of genetic markers that integrate genetic and physical maps. - Genetics **172**: 2541-2555, 2006.

Ronfort, J., Bataillon, T., Santoni, S., Delalande, M., David, J.L., Prosperi, J.M.: Microsatellite diversity and broad scale geographic structure in a model legume: building a set of nested core collection for studying naturally occurring variation in *Medicago truncatula*. - BMC Plant Biol. **6**: 28, 2006.

Saitou, N., Nei, M.: The neighbor-joining method: a new method for reconstructing phylogenetic trees. - Mol. Biol. Evol. **4**: 406-425, 1987.

Sunnock, P.: Efficient genetic markers for population biology. - Trends Ecol. Evol. **12**: 199-203, 2000.

Takezaki, N., Nei, M.: Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. - Genetics **144**: 389-399, 1996.