Plant Tissue Cult. & Biotech. **23**(1): 59-66, 2013 (June)

PTC&B

Evaluation of Genetic Relationship between *Trigonella-Melilotus* complex Using CCMP Markers

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Key words: CCMP, DNA fingerprinting, Genetic correlation, *Melilotus*, *Trigonella*

Abstract

To distinguish the taxa at the specific and varietal levels a range of DNA fingerprinting techniques are being employed. The objective of the current study was to establish the genetic correlation between the *Trigonella-Melilotus* complex i.e. *M. indica*, *M. alba* and *Trigonella polyceratia* using ten Consensus Chloroplast Microsatellite Primers (CCMPs). The polymor-phism between the two genera indicated that they are intimately related and symbolize novel incongruity. Owing to less significant level of genetic variation, detected through CCMP, the differences between the two genera could be accredited to gene mutation or inconsequential chromosomal alterations.

Introduction

The legume family is the third largest family of angiosperms with approximately 730 genera and over 19400 species distributed world-wide. The Papilionoideae has received the most attention, because it is the largest and most widespread of the three legume subfamilies with an estimated 476 genera and 13860 species (Wojciechowski et al. 2004). For instance, the level of coumarin in some species of *Trigonella* is nearly same as that found in most species of *Melilotus.* Many species of *Trigonella* are attacked by the sweetclover (Melilotus) weevil, *Sitona cylindricollis* Fahr.

Linnaeus (1753) classified *Melilotus* as one group under *Trifolium*. Seringe (1825) placed the two genera *Melilotus* and *Trigonella* in a special section known as Grammocarpus of genus *Trigonella.* Bhattacharyya (1958) concluded that *Trigonella* and *Melilotus* might be treated as two subgenera. Various DNA fingerprinting techniques have been used to differentiate the taxa at the specific and varietals levels. These include RAPD and ISSR (Abdel 2009, Bussell et al. 2005). Both furnish very good taxonomic markers to determine the pattern of changes occurring in DNA. Further chloroplast specific micro satellites are used

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to assess the maternal and paternal plastid inheritance (Cato and Richardson 1996, Basha and Sujatha 2009, Karnawat et al. 2013), evaluation of interspecific polymorphism and the detection of hybridization and introgression and phylogeny of plant population.

Melilotus, known as Melilot or Sweet-clover, belongs to tribe Trifolieae and is a genus of the family Fabaceae. It comprises about 20 species originally from Europe and Asia, it is now distributed world-wide mainly in the temperate and subtropical regions of Europe, Asia and North Africa (Polhill and Raven 1981). According to Sharma and Tiagi (1979) two species occur in Jaipur (Rajasthan) and these are *Melilotus alba* and *M. indica.* The two species are distinguishable on the basis of length of inflorescence at fruiting stage and color of corolla.

Exclusive of the interference of environmental conditions, molecular markers can quickly and precisely genetic differences which are more dependable than morphological or biochemical methods. To explore chloroplast variation, universal primers targeted to mononucleotide repeats occurring in chloroplast genome is a useful tool. Moreover, chloroplast specific microsatellites have been used to assess maternal and paternal plastid inheritance (Cato and Richardson 1996) and appraisal of interspecific polymorphism.

In addition to assessing genetic relationship among the species, CCMPs are used to evaluate the organelle specific primer polymorphism (Hora and Malik 2012), identification of maternal and paternal parents and phylogeny of plant populations (Karnawat et al. 2013).

The objective of the present study was to evaluate the effectiveness of CCMP marker in determining the genetic relationships between the two genera *Melilotus* and *Trigonella*.

Materials and Methods

Fresh, young leaves were sampled from the plants of the two genera collected from the gram fields adjacent to Jaipur National University, Jaipur, and main campus in the month of February, 2012 and were recognized morphologically. These leaves were used for ensuing molecular biology studies.

DNA extraction: DNA isolation was performed on five grams of leaf tissue ground in liquid nitrogen. Total genomic DNA was extracted individually from younger leaves of species following the standard CTAB method with minor modifications by the addition of 2% sodium meta bisulphate (Doyle and Doyle 1990). DNA concentrations were determined electrophoretically versus known amount of λ DNA as standards. For PCR, DNA samples were adjusted to a concentration of 5 ng/μl.

CCMP primer analysis: A set of ten CCMP primers specific to chloroplast genomes of dicotyledonous angiosperms (Weising and Gardner 1999) were used for the characterization of organellar genome of two genera i.e., *Trigonella* and *Melilotus.* The PCR amplifications were carried out in a 10 µl reaction mixture containing 5 ng of genomic DNA, 1x PCR buffer (10 mM Tris pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 0.4 µM each of forward and reverse primers, 150 µM of each dNTPs and 0.6 Units of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in GeneAmp 9700 thermal cycler (Perkin Elmer Applied Biosystems) with initial denaturation at 94ºC for 4 min; 35 cycles of denaturation at 92ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 45 s; and a final extension at 72ºC for 5 min. The amplified PCR products were resolved by electrophoresis on 4% agarose (Bangalore Genei, India) gel and visualized by ethidium bromide staining. Banding pattern was recorded under ultraviolet light and documented in Alpha Innotech Fluorchem gel documentation system. As the amplification products that resulted from organelle specific markers were run on agarose gels.

Data analysis: The reproducible bands were scored and entered as binary traits ("1" for presence and "0" for absence). Genetic similarity was computed based on jaccard's similarity coefficient using NTSYS-Pc program (Rolf 2002). Further the similarity matrix was subjected to cluster analysis by the UPGMA. The percentage of polymorphism was calculated as the proportion of polymorphic bands with the total number of bands.

Results and Discussion

Chloroplast specific microsatellites are used to assess the maternal and paternal plastid inheritance (Cato and Richardson 1996), evaluation of interspecific polymorphism and the detection of hybridization and introgression and phylogeny of plant population. For details one may refer to Basha and Sujatha (2009). With a view to assessing genetic relationships among the two species, to evaluate the organelle specific primer polymorphism, and identification of maternal and consensus chloroplast microsatellite primers were used. Ten primers specific to chloroplast genome gave amplification products. All the CCMP primers except ccmp 8 and 9 yielded a single, discrete PCR product.

Allele sizes of amplification products (bp) from two different genera generated by consensus chloroplast microsatellite primers (CCMP1 to 10) are set in Table 1. Very diminutive difference was observed in allele size of the two different genera with CCMP1, 3 and 5. The two different genera appear to be genetically similar. The largest allele size of amplification product generated by CCMP was with CCMP10 (190 bp) and the smallest is with CCMP 2 (70 bp). All

other primers gave amplification product, which was comparable with that of *Argemone* and tobacco species.

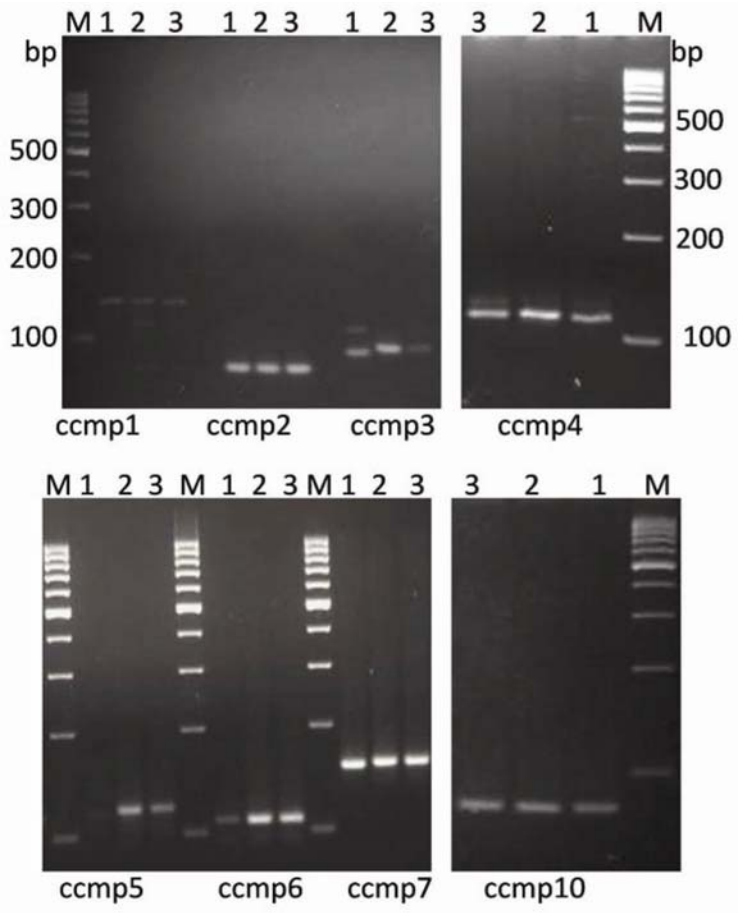

Fig. 1. PCR profiles of the *M. indica*, *M. alba* and *Trigonella polyceratia* generated by different CCMP primers. M - Marker 1. *Melilotus incdica*, 2. *Melilotus alba* and 3. *Trigonella polyceratia.*

Melilotus is so closely related to *Trigonella* that it is extremely difficult to separate the two genera. On the basis of cytological data and from the point of view of external morphology, Bhattacharyya concluded that *Melilotus* and *Trigonella* were very closely related that the two genera might be treated as two subgenera under one genus.

Cluster analysis indicated the segregation of *T. polyceratia* from the other two *Melilotus* species at 0.45 (Fig. 2, Table 2). Further at 0.847 *M. parviflora* and *M. alba* are nearly similar. Apparently, the two genera appear to be isolated.

Heyn (1966) reported the presence of intermediate species between the three genera *Medicago, Trigonella* and *Melilotus*. Therefore, some species of *Medicago viz. M. ruthenica* and *M. polycarpa* are considered by most botanists to be intermediate between *Trigonella* and *Medicago.* In fact, *Medicago* supported a monophyletic group distinct from that formed by *Melilotus,* which is included within *Trigonella* and *Trifolium* basal to the remainder of *Trifolieae*. The present investigations bring out the significance of ccmp in discriminating among the disputed taxa, and are in accordance with the studies of Basha and Mulpuri (2009).

Table 1. Allele size of amplification products generated by consensus chloroplast microsatellite primers (CCMP1 to 10).

Species	Size of amplification product (bp)							
primer	Ccmp1	Ccmp2	Ccmp3	Ccmp4	Ccmp5	Ccmp6	Ccmp7	Ccmp10
T. polyceratia	140	70	80	130	135	120	160	190
			102					
M. parviflora	129	70	82	130	137	120	160	190
	140							
M. alba	140	70	82	130	137	120	160	190
A. Mexicana	139	240	112	150	100	95	150	115
N. tabacum	139	189	112	126	121	103	133	103

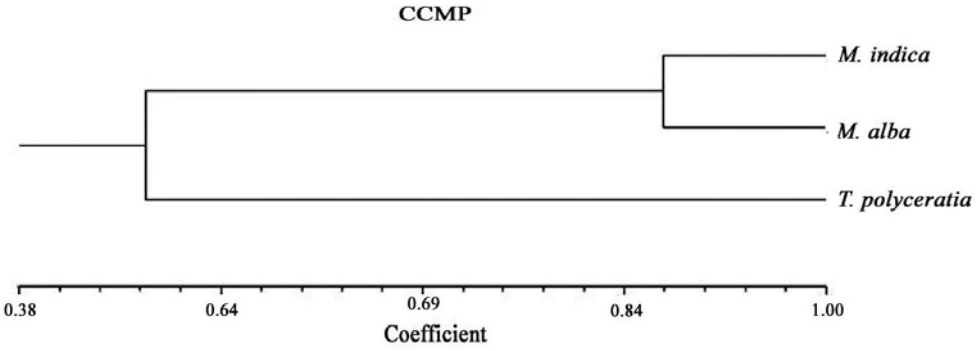

Fig. 2. Dendrogram based on genetic distance computed from UPGMA in the three species.

The present analysis indicates that *T. polyceratia* is linked with *Melilotus.* Marzauk and El-Bakataushi (2011) based on RAPD analysis suggested that *T. polyceratia* is more closely related to *Medicago sativa* than *T. foenum graecum*. Interestingly, Kawashty et al. (1998) inferred close affinity between *T. foenum graecum* and *T.polyceratea* on the basis of flavonoid profiles. According to these workers *T. polyceratia* needs to be retained in the genus *Trigonella.* Marzouk

(2006) and Ahmad and Marzauk (2002) confirmed inclusion of *T. polyceratia* under *Medicago* on the basis of their protein profiles, morphology and anatomical data. Earlier Bena (2001) suggested inclusion of *T. polyceratia* under *Medicago* based on the sequence of two ribosomal transcribed spaces (ITS1, ITS2) and external transcribed spaces (ETS).

Table 2. Jaccard's similarity coefficient.

	M. indica	*M. alba*	*T. polyceratia*
M. indica	1.00		
M. alba	0.87	1.00	
T. polyceratia	0.45	0.50	1.00

Chloroplast specific microsatellites are used to assess the maternal and paternal plastid inheritance (Cato and Richardson 1996), evaluation of interspecific polymorphism and the detection of hybridization and introgression and phylogeny of plant population. With a view to assessing genetic relationships among the two species, to evaluate the organelle specific primer polymorphism, and identification of maternal and consensus chloroplast microsatellite primers were used.

Present investigations have attempted to examine the level of genetic variation within *Trigonella-Melilotus* complex. The two marker systems have been employed to evaluate genetic diversity and to seek deep insight on the phylogenetic relationship of two genera. The data from the present study should be seen as a basis for future researches with a view to defining the level of genetic relationship. Present investigation has also given significant indicators in unravelling genotype relationship and this may further help in developing and planning breeding program.

Present molecular studies unequivocally aid in the establishment of genetic relationships between *Trigonella, Melilots* and *Medicago* and clarify their inscrutable genetic relationships. Present authors propose that the genus *Trigonella* belongs to tribe Trifolieae, subtribe Trigonellinae of the subfamily Papilionoideae. This subtribe should also include three other related genera e.g. *Medicago*, *Melilotus* and *Trifolium* along with *Trigonella* as proposed by Bena (2001) and Dangi et al. (2004).

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