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ASSESSMENT OF RELOCATION OF *TRIGONELLA CYLINDRACEAE* L. AND *T. POLYCERATIA* (L.) TRAUTV. TO GENUS *MEDICAGO* AS INFERRED BY RAPD AND RFLP ANALYSES

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Abstract

The taxonomic relocation of both *Trigonella cylindraceae* and *T. polyceratia* either to *Trigonella* or *Medicago* was assessed based on two molecular markers, RAPD and RFLP. Through RAPD analysis, 109 bands were generated by ten random primers with an average 11 products/primer. The maximum number of RAPDs (76 bands) associated with the highest percentage of polymorphism (43.3%) was recorded for *T. polyceratia*, while the minimum was observed in *T. foenum-graecum* (63 bands and 32.3%). RFLP analysis of 18S rDNA was performed by using five restriction endonucleases (EcoRI, HindIII, BamHI, HinfI and TagI). The restriction endonuclease HinfI and TagI produced two and three fragments respectively, and no variation was observed in these sites among the studied species. However, another three restriction endonuclease (EcoRI, HindIII & BamHI) were undigested. The present study suggested to reallocate of both *T. cylindraceae* and *T. polyceratia* to *Medicago* and certified the value of RAPD analysis for the taxonomic intention.

Introduction

The legume family is the third largest family of angiosperms with approximately 730 genera and over 19400 species worldwide. On the other hand, 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). The genus *Trigonella* is one of the largest genera (close to 135 species) of the tribe Trifolieae, subtribe Trigonellinae in the subfamily Papilionoideae. This subtribe grouped three genera *Medicago*, *Melilotus* and *Trifolium* with *Trigonella* (Bena, 2001; Dangi *et al.*, 2004).

Many taxonomical studies have been carried out on ascertaining the relationships and the delimitation between *Medicago* and *Trigonella*. These studies tried to solve the problem by floral, seed and pollen morphology or biochemical analyses (Small, 1986; 1989; Classen *et al*., 1982; Small *et al.*, 1981; Bena, 2001; Ahmed & Marzouk, 2002; Marzouk, 2006). The main taxonomic problem arises from those species known as medicagoid *Trigonella*, whose exhibit flower and seed similarities with individuals of *Medicago*, especially those characters related to the explosive tripping pollination mechanism (Small *et al.*, 1987). Consequently, some authors transferred these medicagoid *Trigonella* species to genus *Medicago* as Small *et al.* (1987); Boulos (1999) and Bena (2001). Others retained them within *Trigonella* and indicated the close similarity between them in morphological characters and flavonoids (Taeckholm, 1974; Kawashty *et al.*, 1998). Despite the abundance of information based on molecular markers in phylogenetic and taxonomic relationships within *Medicago* species (Brummer *et al.*, 1995; Mariani *et al.*, 1996; Bena *et al.*, 1998), the prime delimitation between *Medicago* and *Trigonella* is supported mainly by morphological features.

A recent study on *Trigonella* species in Egypt showed that both *T. cylindraceae* and *T. polyceratia* have some morphological, including characteristic explosive tripping pollination mechanism, and protein electrophoretic characters similar to those of the genus *Medicago* (Ahmed & Marzouk, 2002; Marzouk, 2006). Although the study of Bena (2001) supported the transferration of *T. polyceratia* to *Medicago* by using molecular sequencing, it was not concerned with the other species *T. cylindraceae*.

The specific objective of the present study is to scrutinize the utility of both RAPD and RFLP markers in determining whether to retain *T. polyceratia* and *T. cylindraceae* under *Trigonella* or transfer them to *Medicago*.

Materials and Methods

Plant material: Seeds of *Trigonella polyceratia* and *T. cylindraceae*, beside *T. foenum-graecum* and *Medicago sativa* as two outgroups were obtained from ICARDA (International Center for Agricultural Research in the Dry Areas) at Aleppo, Syria and from NPGS (National Plant Germplasm System) at Washington University, USA (Table 1). Prior to analysis, each seed was selected individually after inspection under the dissecting microscope. Voucher seed specimens were deposited at Alexandria University herbarium (ALEX).

RAPD analysis: Fifteen plants of each accession were grown in pots for DNA isolation. Two grams of young leaf tissue were harvested for each plant and frozen in liquid nitrogen for DNA extraction. Plant DNA was extracted using wizard Genomic DNA purification kit (Promega, Comp. USA) and equal amount of DNA from each of the fifteen plants was pooled together for each accession. Pooled samples from different accessions for each species were subjected to RAPD examination. RAPD analysis was performed using arbitrary decamer primers procured from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, England HP79NA) (Table 2). The amplification reaction mixture (25 µl) contained 10 mM Tris-HCI (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 1% triton-100, 100 mM of each dNTPs, 15 pmoles of primer, 50 ng genomic DNA, and 1.5 µ of Taq DNA polymerase. Amplification was carried out for 45 cycles, each consisting of a denaturing step of 1 minute at

94°C, followed by annealing step of 1 minute at 36°C and an extension step of 2 minutes at 72°C. The last cycle was followed by 7 minutes of extension at 72°C. Amplified products were electrophoresed on 1.6% agarose gel using 1.0 × TAE buffer (10 mM Tris HCl and 1 mM EDTA pH 8.0) and visualized by ethidium bromide staining for 30 minutes and then destained in deionized water for 10 minutes. Amplification products were compared with 1000 bp DNA ladder (Amersham Pharmacia Biotech. Inc., USA). The patterns were photographed and analyzed by Total Lab Program (www.Totallab.com).

Table 1. Origin, source and accession number of the studied species. ICARDA: International Center for Agricultural Research in the Dry areas, NPGS: National Plant Germplasm System (USA).

Species	Origin	Source	Accession number
Medicago sativa L.	Turkey	ICARDA	IG 101521
Trigonella polyceratia (L.) Trautv	Turkey	NPGS	PI 369154
	Morocco	ICARDA	IG 58812
	Spain	ICARDA	IG 58826
Trigonella cylindraceae Desv	Turkey	ICARDA	IG 110385
	Syria	ICARDA	IG 16202
Trigonella foenum-graecum L.	Turkey	NPGS	PI 173136
	Morocco	ICARDA	IG 115598
	Algeria	ICARDA	IG 16510
	Egypt	ICARDA	IG 16856

Table 2. Sequences of ten random primers selected in the present study.

Primer	% GC	Nucleotide sequence 5' to 3'
OPG-05/0.88	60	CTGAGACGGA
OPN-11/2.22	60	ATCCGAGTGT
OPV-08/1.05	70	GGACGGCGTT
OPS-19/1.34	60	GAGTCAGCAG
OPK-01/0.86	60	CATTCGAGCC
OPS-06/0.33	70	CAGGGTAGTG
OPZ-12/0.47	60	TCAACGGGAC
OPT-07/0.54	70	GGCAGGCTGT
OPM-11/1.24	60	GTCCACTGTG
OPK-11/1.02	60	AATGCCCCAG

RFLP analysis: DNA was extracted from seeds ground in liquid nitrogen using wizard Genomic DNA purification kit (Promega, Comp. USA). The partial ribosomal 18S rDNA was amplified using the primers NS1(5′-GTA GTC ATA TGC TTG TCT C-3′) and NS2 (5′-GGC TGC TGG CAC CAG ACT TGC-3′) (White *et al*., 1990) (Fig. 2). The PCR program was: initial denaturation at 95ºC for 15 minutes; 35 cycles of 94ºC for 30 seconds followed by annealing at 55°C for 1 minutes and extension at 72°C for 2 minutes., the final extension step at 72ºC for 10 minutes. The reaction mixture was analyzed by electrophoresis on 2% agarose in 1x Tris-Borate EDTA buffer, stained with ethidium bromide to be photographed by using gel documentation system. PCR products approximately 550bp of the 18S rDNA gene were digested using different restriction enzymes *(Tag*I, *Eco*RI, *Hind*III, *Hin*fI, *Bam*HI). The reaction components were 5µl of the PCR product, 2µl of each respective enzyme buffer, 12µl water, and 1 unit of enzyme. Restriction reactions were then incubated at 37°C for 4 hours except *Tag*I which was incubated at 65°C for 2 hours. The digested DNA was separated on 2.0% (w/v) agarose in 0.5x TBE buffer, the gel was stained. The band size was compared with suitable DNA molecular weight marker and photographed using gel documentation system.

Data analysis: The obviously reproducible RAPDs were scored and entered as binary characters (1 for presence and 0 for absence). Genetic similarity was calculated based on of Nei's similarity coefficient using the NTSYS-pc program (Rolf, 2002). The similarity matrix was subjected to cluster analysis by the Unweighted Pair Group Method with Arithmetic averages (UPGMA). The percentage of polymorphism was estimated as the proportion of polymorphic bands with the total number of bands.

Results

The ten random primers that were selected for analysis generated 109 RAPDs, with an average 11 products/primer and size ranged from 100 - 1000 bp (Fig. 1). The total number of bands, fingerprinting bands and the percentage of polymorphism for each species are summarized in Table 3.

Cluster analysis indicated the segregation of *T. foenum-graecum* from the three other studied species at 0.46 coefficient (Fig. 4). Successively, at 0.358 coefficient, *M. sativa* was isolated from both *T. cylindracea* and *T. polyceratia.*

The maximum of both RAPD bands (76 bands) and average percentage of polymorphism (43.3%) were generated with *T. polyceratia*. Contrary, the minimum recorded for *T. foenum-graecum*, 63 bands with 32.3% as an average of polymorphism. The RAPD products for *M. sativa* were 65 bands with 33.6% as an average of polymorphism. While the products generated with *T.*

cylindracea were 70 bands with 38.7% as an average of polymorphism (Table 3). For fingerprinting bands, the maximum estimated with the primer OPK-11/1.02 in *T. polyceratia* (five bands), followed by the primers OPG-05/0.88 and OPM-11/1.24 in *T. foenum-graecum* (four bands), pursued by the primer OPN-11/2.22 in *T. cylindracea* (three bands). However, the fingerprinting bands for *M. sativa* were not associated with specific primer. The number of shared bands among various studied species indicated the maximum number of common bands between *T. cylindracea* and *M. sativa* (eight bands), while there was not any common band detected between *T. cylindracea* and *T. foenum-graecum* (Table 4).

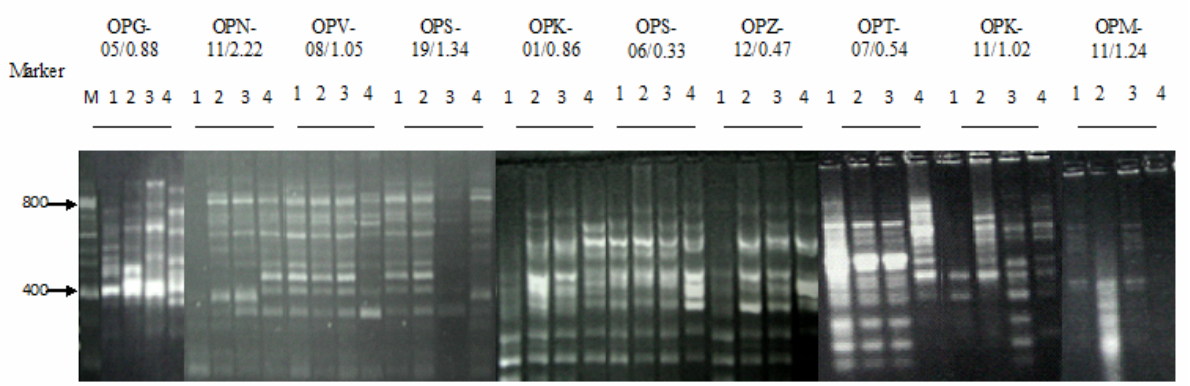

Fig. 1. RAPD profiles of 1, *Megicago sativa*; 2, *Trigonella polyceratia*; 3, *Trigonella cylindracea*; 4, *Trigonella foenum-graecum* obtained from the use of 10 primers.

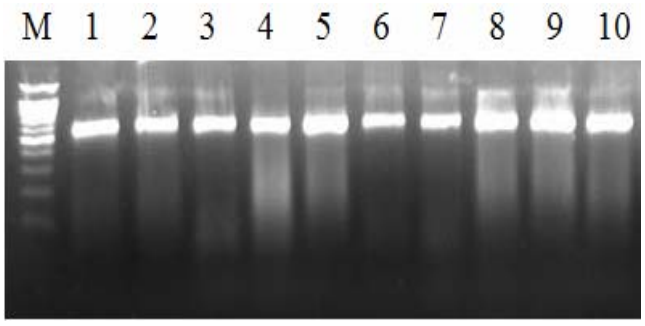

Fig. 2. The 18S rDNA was amplified using the primers NS1 and NS2. 1, *Medicago sativa* (Turkey); 2, 3 and 4 *Trigonella polyceratia* (Turkey, Moracoo and Spain respectively); 5 and 6 *Trigonella cylindraceae* (Turkey and Syria respectively) and 7,8,9 and 10 *Trigonella foenum-graecum* (Turkey, Moracoo, Algeria and Egypt respectively).

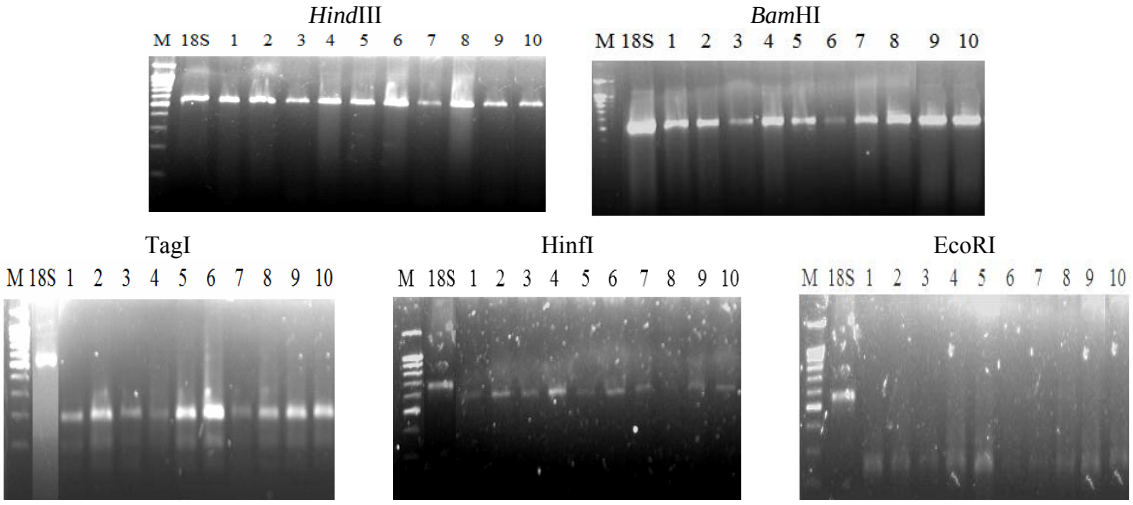

Fig. 3. Agarose gel of restriction fragments of PCR-amplified 18S rDNA with primers NS1 and NS2 and digested by 5 restriction enzymes (*Hind*III, *Bam*HI, *Tag*I, *Hin*fI and *Eco*RI). 1, *Medicago sativa* (Turkey); 2, 3 and 4 *Trigonella polyceratia* (Turkey, Moracoo and Spain respectively); 5 and 6 *Trigonella cylindraceae* (Turkey and Syria respectively) and 7,8,9 and 10 *Trigonella foenum-graecum* (Turkey, Moracoo, Algeria and Egypt respectively).

Restriction fragment length polymorphism (RFLP) analysis of 18S rDNA (≈550bp) from the species studied were analyzed by digestion with the five different restriction endonucleases, three of them (*Eco*RI, *Hind*III& *Bam*HI) did not generate any fragments (Fig. 3). The restriction endonuclease *Tag*I generated three fragments with an average 50, 200 and 300 bp. The RFLP pattern of the restriction enzyme *Tag*I was the same length in the species studied. The restriction endonuclease *Hin*fI generated two fragments with an average 250 and 300 bp and no variation was observed in that site among the species studied.

Table 3. The total number of bands (T.N.B), fingerprinting bands (F.B.) and the percentage of polymorphism (%P.) for studied species.

Primers	*Medicago sativa*			*Trigonella polyceratia*			*Trigonella cylindracea*			*Trigonella foenum graecum*		
	T.N.B	F.B.	% P.	T.N.B	F.B.	% P.	T.N.B	F.P.B.	% P.	T.N.B	F.B.	% P.
Primer 1 (OPG-05/0.88)	4	0	25	4	0	25	7	2	50	5	4	33
Primer 2 (OPN-11/2.22)	2	0	18	8	0	73	7	3	64	5	0	45
Primer 3 (OPV-08/1.05)	10	0	20	9	0	10	10	0	20	9	0	10
Primer 4 (OPS-19/1.34)	10	1	82	8	0	64	8	1	64	1	0	0
Primer 5 (OPK-01/0.86)	7	1	18	10	0	45	8	0	27	9	0	36
Primer 6 (OPS-06/0.33)	8	0	22	7	0	11	9	0	33	6	0	0
Primer 7 (OPZ-12/0.47)	4	1	40	7	0	70	4	0	40	9	2	90
Primer 8 (OPT-07/0.54)	14	0	64	10	0	36	10	0	36	9	0	29
Primer 9 (OPM-11/1.24)	3	1	25	6	1	50	5	0	42	7	4	58
Primer 10 (OPK-11/1.02)	3	0	22	7	5	67	2	0	11	3	1	22
Mean			33.6			43.3			38.7			32.3
Total	65			76			70			63		

Table 4. The number of common bands among studied species in matrix form (the number indicated between brackets).

	Megicago sativa	*Trigonella polyceratia*	*Trigonella cylindracea*
Medicago sativa			
Trigonella polyceratia	OPN-11/2.22 (2) OPS-19/1.34 (2) OPK-11/1.02 (1)		
Trigonella cylindracea	OPG-05/0.88 (1) OPS-19/1.34 (1) OPS-06/0.33 (2) OPT-07/0.54 (4)	OPG-05/0.88 (1) OPN-11/2.22 (1) OPK-01/0.86 (1) OPM-11/1.24 (3) OPK-11/1.02 (1)	
Trigonella foenum-graecum	OPK-11/1.02 (1)	OPN-11/2.22 (2) OPK-01/0.86 (1)	-----

Discussion

The acquired data in this study proved the significance of RAPD in the discrimination among studied species, which is in concordance with the results of Harris (1999); Wu *et al.* (1999); Onarici & Sumer (2003); Dangi *et al.* (2004). RAPD products are usually dominant markers and are inherited in simple Mendelian fashion, therefore, verified as to be taxonomically and evolutionary useful at all taxonomic levels, especially at the intra- and interspecific levels (Demeke & Adames, 1994; Nkongolo *et al.,* 2002).

On the other hand, RFLP data for studied species points out a major congruence among the bands digested with five different restriction enzymes, which suggests the absence of length mutation, but does not confirm the absence of site mutation. Many studies demonstrated no significant variation of 18S rDNA sites using different restriction enzymes as Hayasaki *et al*. (1997; 2001).

The current results indicated that both *T. cylindracea* and *T. polyceratia are* linked with *M. sativa* more than with *T. foenum-graecum.* Although, Kawashty *et al*. (1998) analyzed the flavonoid profile of *T. cylindracea* and *T. polyceratia* and reported the close similarity between them and *T. foenum-graecum* and he suggested that these two species retained in *Trigonella*. Conversely, the transposition of *T. cylindracea* and *T. polyceratia* to *Medicago was* confirmed through morphological, anatomical and protein electrophoresis by Marzouk (2006) and Ahmed and Marzouk (2002). Correspondingly, Bena (2001) reported that *T. polyceratia* was jointed more confidently with *Medicago* rather than with *Trigonella* through the sequencing of two ribosomal transcribed spacers (ITS1 and ITS2) and a part of external

transcribed spacer (ETS). The present study also showed that *T. polyceratia* expressed the highest value of mean polymorphic loci (43.3%) followed by *T. cylindracea* (38.7%) then *M. sativa* (33.6%), while *T. foenum-graecum* enclosed the lowest value of mean polymorphic loci (32.3%). That probably may be attributed to the high ability for outcrossing associated with medicagoid corolla and tripping pollination mechanism characteristic for *Medicago* (Small *et al.*, 1987; Ahmed & Marzouk, 2002). The present study showed that *T. cylindracea* and *T. polyceratia*, which have been classified for a long time in *Trigonella*, joined with *Medicago* rather than *Trigonella*, which is supported by the number of both significant primers and common bands shared among different studied species. *Medicago sativa* shared with *T. cylindracea* and *T. polyceratia* with eight bands (from four primers) and five bands (from three primers) respectively. Meanwhile, *T. cylindracea* and *T. polyceratia* revealed seven common bands extracted from five primers. However, *T. foenum-graecum* demonstrated only three common bands, from two primers, with *T. polyceratia* and without any common ones with *T. cylindracea*.

This present study clarified the enigmatic taxonomic position of *T. cylindraceae* and *T. polyceratia* by the proposition of taxonomic transferation of both to genus *Medicago* and validated that RAPD techniques is sensitive and powerful for this taxonomic purpose.

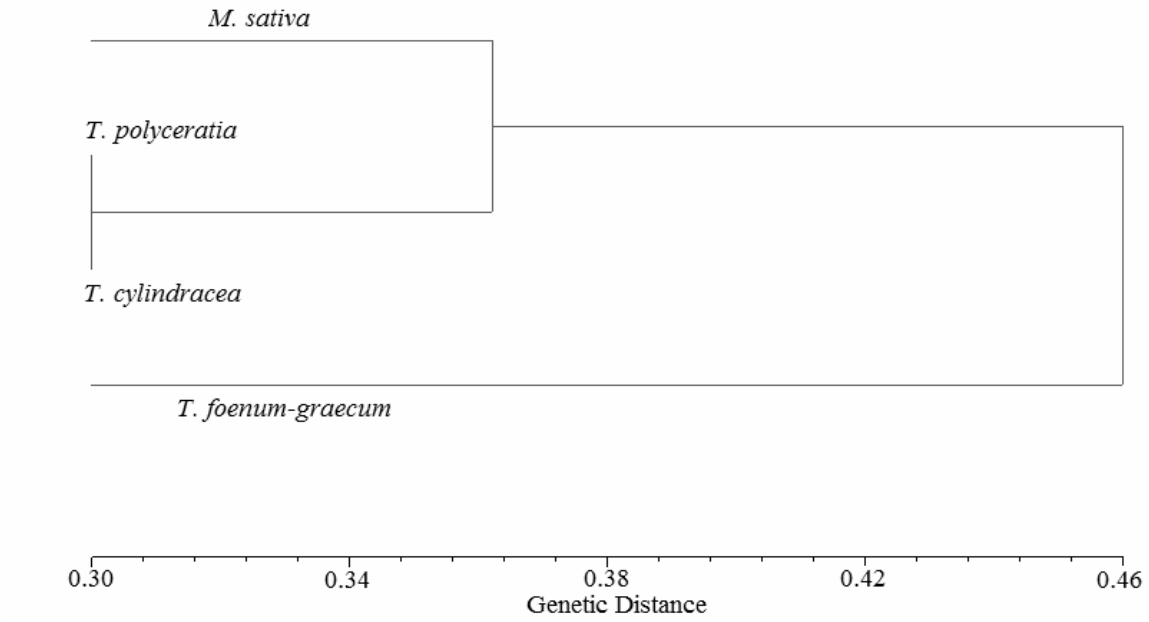

Fig. 4. Dendrogram based on genetic distance computed from RAPDs using algorithm of Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in the four studied species.

References

Ahmed, M.F. and Marzouk, R.I. 2002. A numerical study on the genus *Trigonella* L. (Leguminosae) in Egypt. Proc.2nd *Int.Conf.Biol.Sci.(ICBS) Fac.Sci*., Tanta University, 2: 189-222.

Bena, G. 2001. Molecular phylogeny supports the morphologically based taxonomic transfer of the "medicagoid" *Trigonella* species to the genus *Medicago* L. *Pl. Syst. Ecol.,* 229: 217-236.

Bena, G., M.F. Jubier, J. Olivieri, and B. Lejeune.1998. Ribosomal external and internal transcribed spacers: combined use in the phylogenetic analysis of *Medicago* (Leguminosae). *J. Mol. Evol*., 46: 299- 306.

Boulos, L. (Ed.) 1999. *Flora of Egypt*. volume 1 (Azollaceae to Oxalidaceae). Al-Hadara Publishing, Cairo, Egypt.

Brummer, E.C., J.H. Bouton and G. Kochert.1995. Analysis of annual *Medicago* species using RAPD markers. *Genome*, 38: 362- 367.

Classen, D., C. Nozzolillo and E. Small.1982. A phenolic-taxometric study of *Medicago* (leguminosae). *Can. J.Bot.,* 60: 2477-2495.

Dangi, R.S., M.D. Lagu, L.B. Choudhary, P.K. Ranjekar and V.S. Gupta. 2004. Assessment of genetic diversity in *Trigonella foenum-graecum* and *Trigonella caerulea* using ISSR and RAPD markers. *BMC Plant Biology*, 4:13.

Demeke, T. and R.P. Adames. 1994. The use of PCR – RAPD analysis in plant taxonomy and evolution. *In*: Griffin, H. and A.M. Griffin (Eds.) *PCR technology: current innovations*. CRC press, Inc., Boca Raton, Florida.

Harris, S.A. 1999. RAPDs in systematic-a useful methodology? *In*: Hollingsworth, P.M., R.M. Batrman and R.J. Gornall (Eds.) *Molecular Systematics, Plant and Evolution*. Taylor and Francis, London, pp. 221-228.

Hayasaki, M., T. Morikawa and J.M. Leggett.1997. RFLP analysis of Hypericum perforatum L. somaclones and their progenies. *Euphytica,* 95: 229-235.

Hayasaki, M., T. Morikawa and J.M. Leggett. 2001. Intraspecific variation of 18S-5.8S-26S rDNA sites revealed by FISH and RFLP in wild oat, Avena agadiriana. *Genes and Genet. Syst*., 76: 9-14.

Kawashty, S.A., M.F. Abdalla, E.M. Gamal El Din and N,A,M. Saleh.1998. The chemosystematics of Egyptian Trigonella species. *Biochem. Sys. Ecol*., 26: 851-856.

Mariani, A., F. Pupilli and O. Calderini. 1996. Cytological and molecular analysis of annual species of the genus Medicago. *Can. J. Bot.,* 74: 299-307.

Marzouk, R.I. 2006. Seed Protein analyses as a support to the transfer of *Trigonella cylindracea* Desv. and T. polyceratia (L.) Trautv. To genus Medicago L. *Taeckholmia*, 26: 17-33.

Nkongolo, K.K., P. Michael and W.S. Gratton. 2002. Identification and characterization of RAPD markers

inferring genetic relationships among Pine species. *Genome*, 45: 51-58.

Onarici, S.G. and S. Sümer. 2003. Protein and DNA in systematic biology. *Turk. J. Biol.,* 27: 47-55.

Rolf, F.J. 2002. NTSYS-Pc: Reference Manual. Exeter publishing Ltd. New York.

Small, E. 1989. Polythetic generic separation in tribe Trifolieae subtribe Trigonellinae (Leguminosae). *Can.J.Bot.*, 67:1480-1492.

Small, E., C.W. Crompton and B. Brookes. 1981. The taxonomic value of floral characters in tribe Trigonelleae (Leguminosae) with special reference to Medicago. *Can.J.Bot.*, 59: 1578-1598.

Small, E., P. Lassen and B.S. Brookes 1987. An expanded circumscription of Medicago (Leguminosae, Trifolieae) based on explosive flower tripping. *Willdenowia*, 16: 415-437.

Small, E. 1986. Pollen-ovule patterns in tribe Trifolieae (Leguminosae). *Plant Syst. Evol*., 160: 195-205.

Taeckhom, V. (Ed.) 1974. *Student's Flora of Egypt* (2ed.), Corporation Printing Company, Beirut.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990 Amplifcation and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M.A., D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.) *PCR protocols*. A guide to methods and applications. San Diego, CA, USA: Academic Press.

Wojciechowski, M.F., M. Lavin and M. I. Sanderson. 2004. A phylogeny of legumes (Leguminosae) based on analysis of the plastid matK gene resolves many well-supported subclades within the family. *Amer. J. Bot.,* 91:1846-1862.

Wu, J., K.V. Krutovskii and S.H. Strauss. 1999. Nuclear DNA diversity, population differentiation, and phylogenetic relationships in the California closed-cone pines based on RAPD and allozyme markers. *Genome,* 42: 893-908.

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