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Seed protein analyses as a support to the transfer of *Trigonella cylindracea* Desv. and *T. polyceratia* (L.) Trautv. to genus *Medicago* L.

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The present study has been planned to investigate the extent to which seed proteins and peptide mapping may be used as evidences to discuss the position of *T. cylindracea* Desv. and *T. Polyceratia* (L.) Trautv. To achieve this objective two outgroups (reference species): *Trigonella foenum-graecum* L. and *Medicago sativa* L., represent the type species for each genus were used. Each species was considered as an operational taxonomic unit (OTU) for the purpose of classification and ordination. The bands from 1D-SDS-PAGE or the spots from 2D-peptide mapping were scored as 0 representing absence and 1 representing presence, and employed in the analysis by using NTSYS-pc computer and PAST programs. Cluster analysis and PCA using the protein data from the two different techniques demonstrated that the similarity between *T. polyceratia* and *M. sativa* is more than that to *T. foenum-graecum*. The present paper accepts the transfer of *T. polyceratia* to *Medicago* and recommends further studies for *T. cylindracea*.

Key words: Peptide mapping, Seed proteins, *T. cylindracea*, *T. polyceratia*.

Introduction

The vindication for the transfer of *Trigonella polyceratia* to *Medicago* as "*M. polyceratia*" based on some fine floral structures termed medicagoid characters related to androecia, and those associated with the explosive mode of pollination "the tripping mechanism" (Jurzysta *et al.*, 1988; Small *et al.*, 1987 and 1990; Small & Fawzy, 1991; Boulos, 1999; Ahmed & Marzouk, 2002). Also the analyses of nrDNA internal transcribed spacer

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region (nrDNA ITS) and external transcribed spacer region (ETS) data supported the inclusion of medicagoid *Trigonella* in the genus *Medicago* (Bena, 2001). Meanwhile, Steele and Wojciechowski (2003) included the medicagoid *Trigonella* in section Bucerates (where *T. polyceratia* belonged) with the other species of *Medicago* based on sequences of the plastid gene *matK*. However, Small (1989) and Boulos (1999) kept *Trigonella cylindracea* within genus *Trigonella* L. On the other hand, Ahmed and Marzouk (2002) recommended the transfer of *T. cylindracea* to *Medicago* due to the presence of many anatomical and medicagoid floral characters. Both *T. polyceratia* and *T. cylindracea* exhibited the same triangular outline and anatomical details of the petiole as well as the wax deposits on leaflet hairs. Moreover, they also distinguished by the presence of the thumb and pocket structure, the keel petals are not prominently fused over the top of the staminal column, the conical apex of the staminal column, the standard petal with more than three vein clusters and the same cylindrical pod shape (Ahmed & Marzouk, 2002). Baum (1968) reported that the existence of the medicagoid corolla and androecium in both *Medicago* and *Trigonella*, warrant consideration for inference of their relationship, while the presence of the apparatus designed for tripping is a clear-cut criterion which exists only in *Medicago*.

The present study is planned to investigate the extent to which seed proteins and peptide mapping may be used to transpose *T. cylindracea* and verify that of *T. polyceratia* to *Medicago.*

Materials and Methods

Two seed sources were used for protein extraction: the first from ICARDA (International Center for Agricultural Research in the Dry Areas) at Aleppo, Syria; while the second from NPGS (National Plant Germplasm System) at Washington University, USA (Table1). To achieve the objective of this study two outgroups (reference species): *Trigonella foenum-graecum* L. and *Medicago sativa* L., which represent the type species for *Trigonella* and *Medicago* respectively, were used. Each species (*T. cylindracea* or *T. polyceratia* and the two reference species) was represented by a bulked seed sample from individuals of different accessions. Prior to analysis, each seed was selected individually after inspection under the dissecting microscope. Seeds containing obvious defects or those relatively small were rejected. Voucher seed specimens were deposited at the Alexandria University herbarium (ALEX).

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

Species	Origin	source	Accession number
Trigonella foenum-graecum L.	Syria	ICARDA	IG 110470
	Egypt	NPGS	PI 572539
	Morocco	NPGS	PI 613630
	Greece	NPGS	PI 199264
	Turkey	NPGS	PI 173136
Trigonella polyceratia (L.) Trautv.	Turkey	NPGS	PI 369154
	Spain	NPGS	PI 244328
	Morocco	NPGS	PI 517186
Trigonella cylindracea Desv.	Syria	ICARDA	IG 16171
	Turkey	ICARDA	IG 110385
Medicago sativa L.	Egypt	ICARDA	IG 101729
	Greece	ICARDA	IG 101060
	Syria	ICARDA	IG 101745
	Turkey	ICARDA	IG 101521
	Lebanon	ICARDA	IG 101605

A typical protocol consists of the separation of proteins by one dimensional polyacrylamide gel electrophoresis (1D-PAGE), enzymatic digestion and the determination of two dimensional peptide fingerprint for each species (2D-peptide fingerprinting) (Hames & Rickwood, 1990). Here, for 1D-PAGE the discontinuous SDS (Sodium Dodecyle Sulphate) system of Laemmli (1970) was used with some modifications as described by Hames & Rickwood (1990). One gram of seeds was macerated with sample buffer (2% SDS, 10% glycerol, 0.002% bromophenol blue, 5% β-mercaptoethanol), mixed with an equal volume of buffer of 0.5M Tris-HCl buffer (pH 6.8), submitted to heat treatment for 5 minutes in a boiling water bath, and then cooled to room temperature before centrifuged at 10000g for 5 minutes. Finally, the supernatants (containing 45µg protein) were loaded

onto 12.5% resolving gel using Mini-PROTEAN II cell (Bio-Rad) at 75 V through the stacking gel followed by 125 V to the end of the electrophoresis (2 hrs). The resultant gel was stained using commasei blue R250 (0.1% in methanol: glacial acetic acid: water in ratio 3: 1: 6).

For 2D-peptide fingerprinting, the method of Cleveland *et al*. (1977) was carried out. The bulked proteins of each species were dissolved in 0.05M Tris-HCl (pH 6.8) at concentration 0.25% (2.5mg protein sample/ml buffer). A volume of 100µl of each protein solution was mixed with 5, 10, 15 and 20µl of trypsin solution (20µl / ml) consecutively, placed in a water bath at 37ºC for 0, 1 and 2 hours. Each solution was heated to boiling for 3 minutes to stop enzymatic activity. Then 50µl of sample buffer (0.0625M Tris-HCl, pH 6.8, 10% glycerol, 0.002% bromophenol blue) was subjected to 12.5% first dimension alkaline native-PAGE at 120 V for 2 hours and stained with commasei blue G250 (Reinheckel *et al.,* 1995). The lanes of the alkaline native-PAGE were separated by SDS-PAGE in the second dimension. The lane of each species sample was cut from the native gel, dipped into a 1% mercaptoethanol solution for few seconds and placed on a glass plate at usual position of stacking gel. After covering with the second glass plat, 15 % separating gel was added and the rest of the vertical SDS-gel was polymerized. Three hours later at 200 V, the gels were stained with commasei blue R250 to visualize the positions of peptide "spot" (Grandier-Vazeille & Guerin, 1996).

A standard low protein marker (Sigma) composed of Bovine serum albumin 67KDa, Oval albumin 45KDa, Glyceraldhyde3phosphate 36KDa, Carbonic anhydrase 30KDa, Trypsinogen 24KDa, Trypsin inhibitor 20KDa and α-Lactalbumin 14.4KDa was used according to the method described in Hoefer Protein Electrophoresis Application Guide (1994).

Each species was considered as an operational taxonomic unit (OTU) for the purpose of classification and ordination. The bands (1D-PAGE) or the spots (2D-peptide mapping) were scored as 0 representing absence and 1 representing presence, and employed in the analysis by using NTSYS-pc computer program (Rohlf, 1990) and PAST program (Hammer & Harper, 2001). For the first program, the agglomerative cluster analysis was conducted using the average Manhattan distance coefficient for mixed data set (Sneath & Sokal, 1973). The dissimilarity matrix was analyzed by the unweighed pair group method with arithmetic average linkage (UPGMA). For the second program, the average Euclidean coefficient was used, and the similarity matrix was analyzed by single linkage.

Ordination of the set of OTU's (set of species) was achieved by means of principle component analysis (PCA) and by the construction of the minimum spanning tree as described by Hammer & Harper (2001).

Results

The electrophoretic patterns of total seed proteins using 1D-SDS-PAGE for the four studied species are presented in Plate1. A total of 20 peptide bands were recorded and distributed as 8, 10, 9 and 9 bands for *Trigonella foenum-graecum, T. cylindracea, T. polyceratia* and *Medicago sativa* respectively. The molecular weight of bands ranged from 67 to 14.4 KDa in *T. foenum-graecum* and *M. Sativa*, from 77.6 to 14.4 KDa in *T. cylindracea* and from 82.9 to 14.4 KDa in *T. polyceratia*. According to the obtained results, species-specific bands were recorded as a maximum number in *T. cylindracea*, which possessed 5 bands (77.6, 42, 39, 32 and 20 KDa), and as a minimum number in *T. polyceratia* and *M. sativa* that possessed 2 bands each (82.9, 41 KDa and 45, 34 KDa respectively). With respect to *T. foenum-graecum*, 3 species-specific bands were recognized at 43, 22.5 and 18 KDa.

In the first step of peptide mapping, the appropriate digestion of proteins in *T. foenum-graecum* and *M. sativa* extracts was achieved by the activity of 20 µl trypsin for 2 hours. However, in the cases of *T. cylindracea* and *T. polyceratia* the addition of 5µl enzyme for one hour was sufficient. The first dimension of alkaline native-PAGE is presented in Plate 2, and the peptide mapping (fingerprints) of the four inspected species in Plate 3a, b, c and d. The highest proportion of species-specific peptides was recorded in *T. polyceratia*, where 4 peptides with M.wt. of 33.3, 31.9, 20 and 17.4 KDa are recorded. On the other hand, the lowest proportion of species-specific peptides was found in *M. sativa* where two bands with M.wt. of 32.7 and 15.2 KDa. In 2D peptide mapping of *T. foenum-graceum* and *T. cylindracea* recorded a total of 15 and 12 spots respectively, both are distinguished by moderate proportion of species-specific peptides. The species-specific peptides of *T. foenum-graecum* were detected at M.wt. of 63.9, 42.2, 23.3, 16.3, 15 and 12.6 KDa, and those of *T. cylindracea* at M.wt. of 58.5, 43.2, 26.5 and 24.3 KDa.

The cluster analysis, by two different programs (NTSYS-pc and PAST) using two types of protein separation techniques (1D-SDS-PAGE and 2D-

peptide mapping), either separately or pooled together, discriminated the four studied species (Fig. 1).

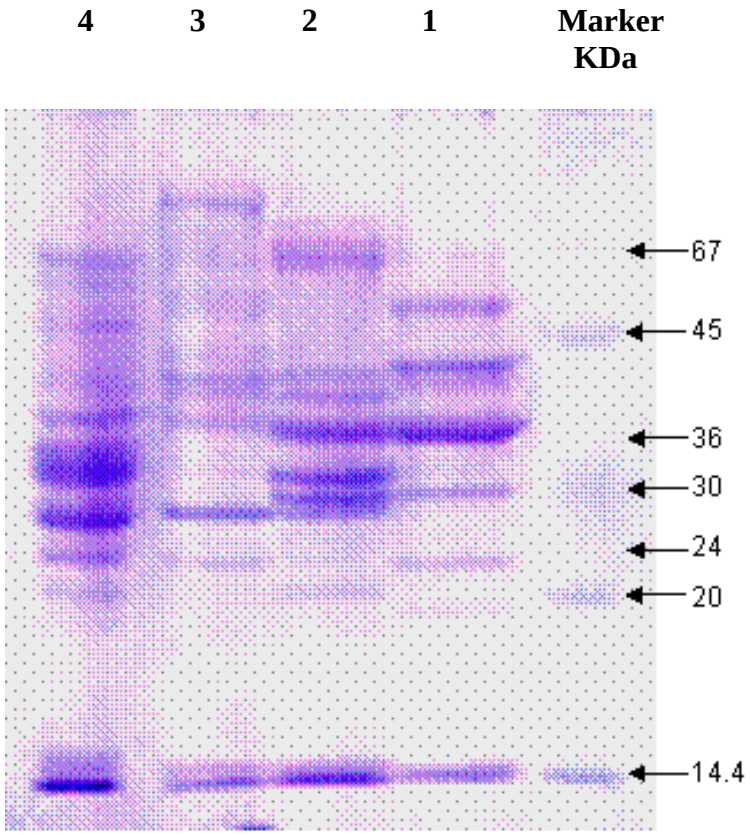

Plate1. The electrophoretic patterns of seed proteins (1D-SDS-PAGE) of the studied species (1, *T. foenum-graecum*; 2, *T. cylindracea*; 3, *T. polyceratia* and 4, *M. sativa*).

Trigonella foenum-graecum is clearly delimited from the other species. This taxonomic tree shows that *T. polyceratia* and *M. sativa* are possibly more related to each other than to *T. cylindracea*, which appeared distinct.

The results of principal component analysis and the minimum spanning trees are shown in relation to the four groups (Fig. 2a and b), which are not highly coincident with those of cluster analysis. Moreover, the principal component analysis for both techniques of protein separation, separately or pooled, indicated that the first two dimensions accounted for approximately 82% of variance (Table 2).

Discussion

It has been reported by Baum (1968) that the generic demarcation between *Medicago* and *Trigonella*, based on traditional taxonomic works relied on fruit attributes is highly debatable by previous authors. Small *et al.* (1981) indicated that *Medicago* could be distinguished from *Trigonella* on the basis of combinations of some floral attributes (as: interlocking wing and keel petals, relatively less apical fusion of the keel petals, relatively well-developed wing petal horn, staminal tubes conical at the apex and with a standard petal having more than three clusters of veins). Moreover, they suggested that *Trigonella* section Bucerates to be quite distinctive from the remaining *Trigonella* species. Baum (1968) suggested the detachment of the three sections, Bucerates, Lunatae and Isthmocarpae, which form a part of the 14 Širjaev's sections of *Trigonella*, on the account of some floral criteria more specified to *Medicago* than to *Trigonella*. The present study verifies the point of view that *Trigonella polyceratia*, section Bucerates may be treated as *Medicago polyceratia*. Ingham (1981) by chemotaxonomic investigations and Small *et al.* (1990) by seed characters supported the transfer of section Bucerates to *Medicago*. Recently, Ahmed and Marzouk (2002) added more weight for the segregation of *T. polyceratia* from *Trigonella* based on many anatomical and floral characters (as: petiole anatomy, wax deposits on leaflet hairs and floral characters accompanied by tripping mechanism).

Notwithstanding, Ahmed & Marzouk (2002) accepted the transposition of *T. cylindracea* under *Medicago*, based on shared anatomical and floral characters. Small *et al.* (1981, 1990) and Boulos (1999) kept *T. cylindracea* under genus *Trigonella*. The present investigation does not support the transfer of *T. cylindracea* to *Medicago* until further studies are made.

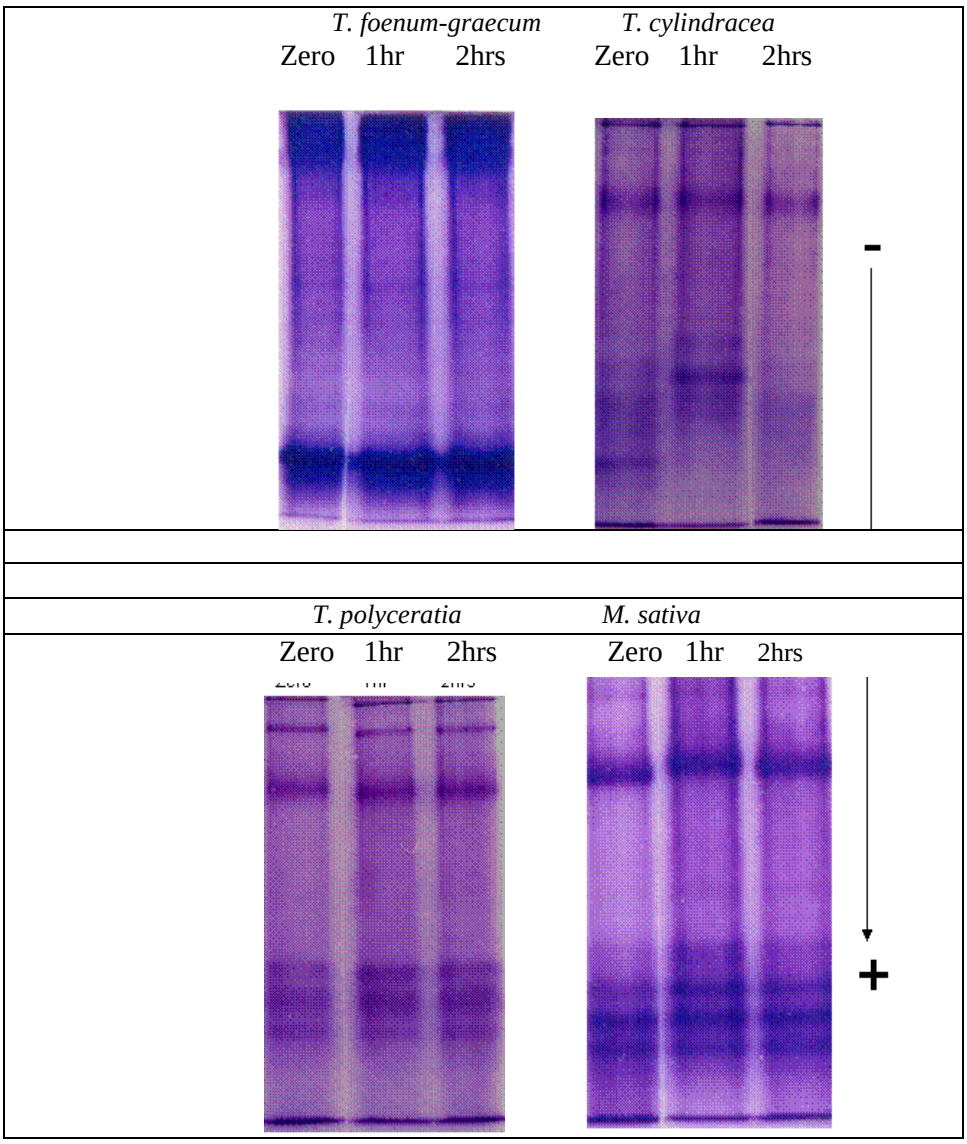

Plate 2. Alkaline native-PAGE of trypsin treated proteins extracted from the studied species. Zero, 1hr and 2hrs are samples of trypsin digested proteins at Zero, 1hr and 2hrs respectively.

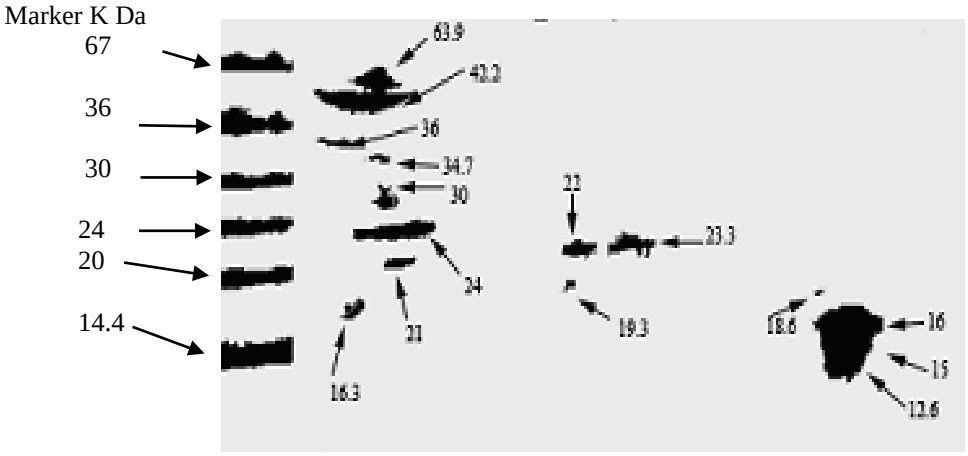

Plate 3a. 2D-Peptide map for alkaline native-PAGE of 20 µl trypsin treated proteins extracted and incubated for 2hrs from *T .foenum-graceum*.

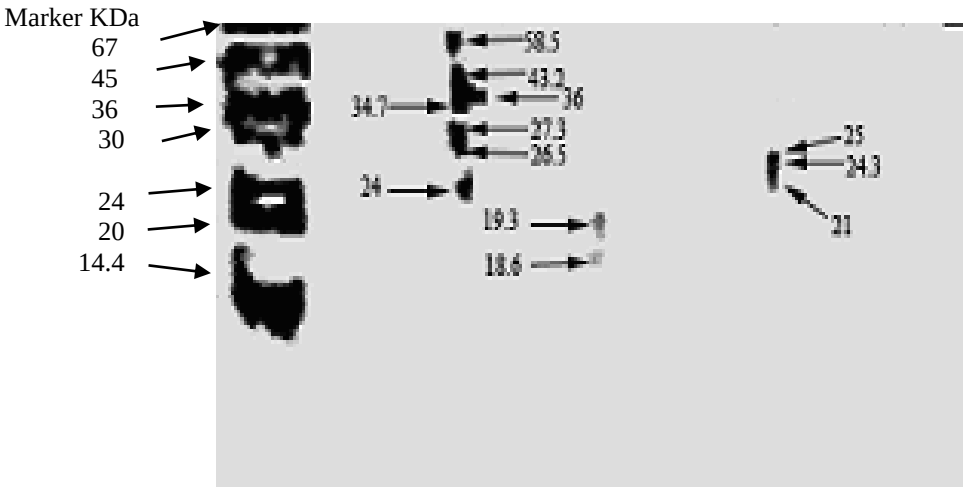

Plate 3b. 2D-Peptide map for alkaline native-PAGE of 5 µl trypsin treated proteins extracted and incubated for 1hr from *T. cylindracea*.

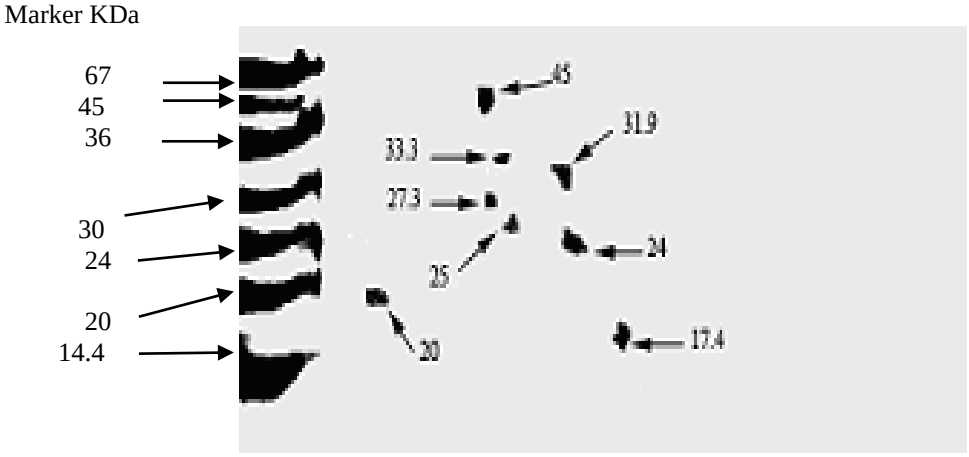

Plate 3c. 2D-Peptide map for alkaline native-PAGE of 5 µl trypsin treated proteins extracted and incubated for 2hrs from *T. polyceratia*.

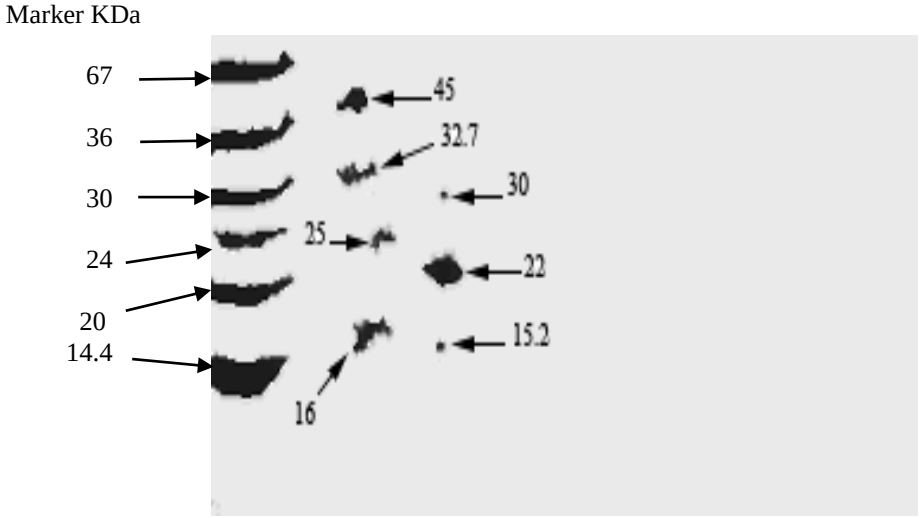

Plate 3d. 2D-Peptide map for alkaline native-PAGE of 20 µl trypsin treated proteins extracted and incubated for 2hrs from *M. sativa*.

Table 2a. Percentage variation accounted for along first two dimensions of PCA using 1D-SDS-PAGE

	Value	%Variance
Eigenvalue 1	2	42.8571
Eigenvalue 2	1.8047	38.673

Table 2b. Percentage variation accounted for along first two dimensions of PCA using 2D-peptide mapping

	Value	%Variance
Eigenvalue 1	3.67555	46.922
Eigenvalue 2	2.7401	34.981

Table 2c. Percentage variation accounted for along first two dimensions of PCA using both 1D-SDS-PAGE and 2D-peptide mapping

	Value	%Variance
Eigenvalue 1	5.67799	46.9903
Eigenvalue 2	3.7933	31.392

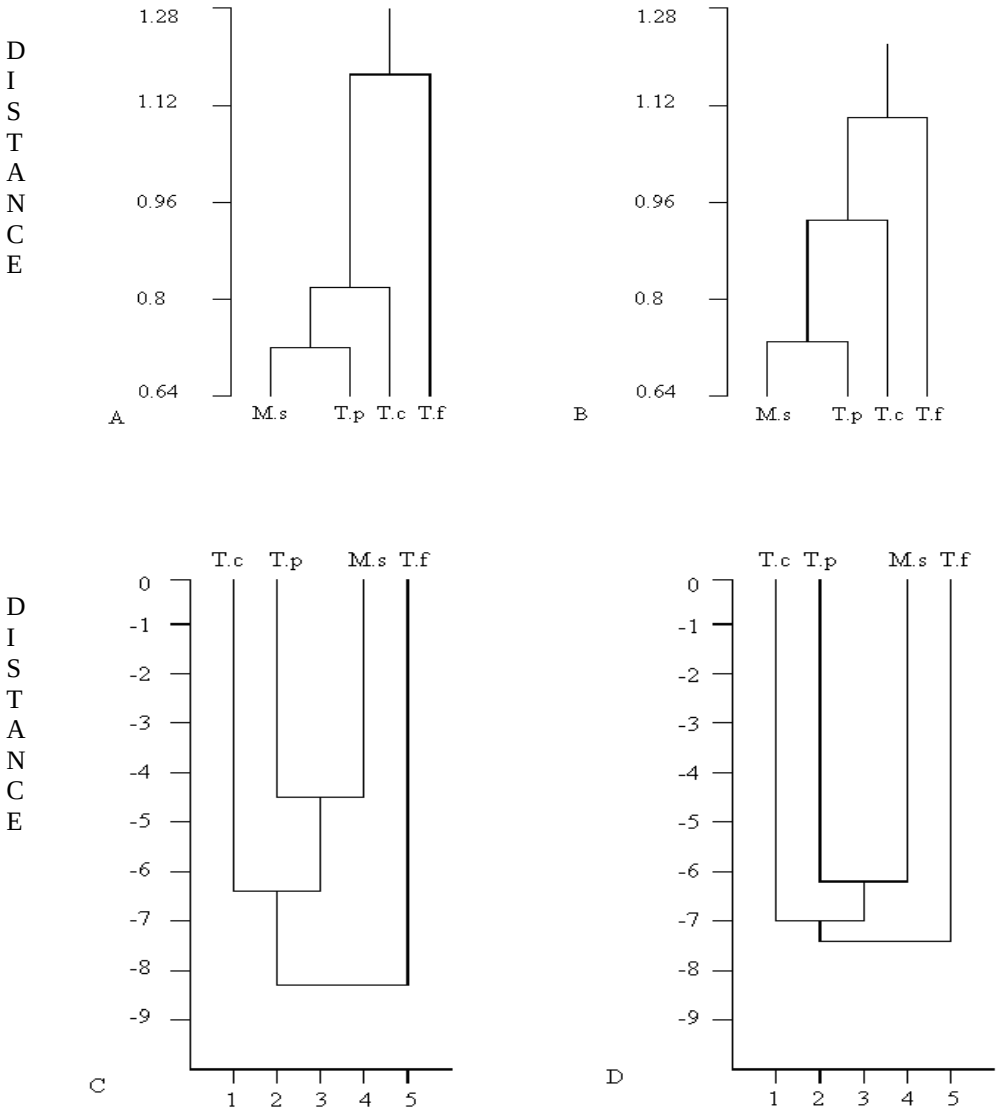

Fig. 1. Dengrogram from 1D-SDS-PAGE (A) and (C) using both NTSYS-pc program and PAST program, respectively. Dendrogram either from 2D-peptide mapping or from both 1D-SDS-PAGE and 2D-peptide mapping (B) and (D) using NTSYS-pc program and by PAST program, respectively; T.f, *T. foenum-graceum*; T.c, *T. cylindracea*; T.p, *T. polyceratia*; M.s, *M. sativa.*

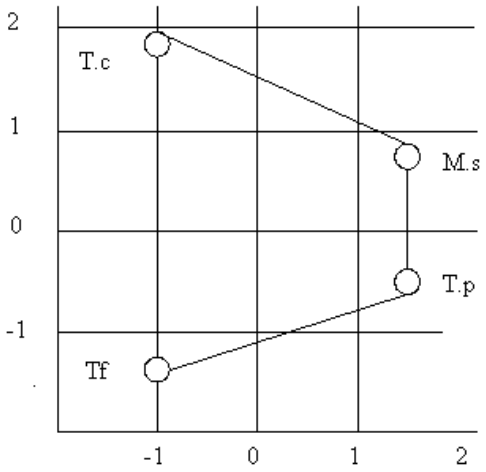

Fig. 2a. Two dimensional plot of the first two components of the four studied species using 1D-SDS-PAGE; T.f., *T. foenum-graceum*; T.c., *T. cylindracea*; T.p. *T. polyceratia* and M.s., *M. sativa*.

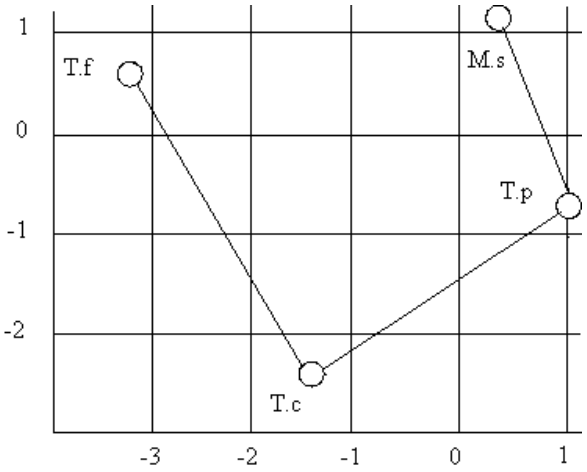

Fig. 2b. Two dimensional plot of the first two components of the four studied species using either 2D-peptide mapping or both 1D-SDS-PAGE and 2D-peptide mapping; T.f., *T. foenum-graceum*; T.c., *T. cylindracea*; T.p. *T. polyceratia* and M.s., *M. sativa*.

In the current study, cluster analysis and PCA using protein data obtained from both 1D-SDS-PAGE and 2D-peptide mapping or from the data of both two types demonstrated that the similarity between both *T.*

cylindracea and *T. polyceratia* on one hand and *M. sativa* on the other is more than that to *T. foenum-graecum*. This notable demarcation in the case of 1D-SDS-PAGE more than that of 2D-peptide mapping may be due to the more specificity of the later technique and the generalized form of proteins in the former one.

The results of 1D-SDS-PAGE signify the following points:

1. Both *Trigonella* and *Medicago* share three common bands at 67, 31 and 14.4 KDa, which is congruent with their grouping in subtribe Trigonellinae (Small, 1987).
2. *Trigonella cylindracea*, *T. polyceratia* and *Medicago sativa* share one band at 27.5 KDa, while *T. foenum-graecum* is distinguished by three specific bands at 43, 22.5 and 18 KDa.
3. There are two bands common to *T. polyceratia* and *M. sativa* at 37 and 23 KDa, while, a single band is shared between *T. cylindracea* and *M. sativa* at 20KDa.

From the acquired results, it can be deduced that peptide mapping for each of the studied species is unique and can be used for the discrimination of each species. Wilson *et al.* (1991) concluded that peptide mapping as a technique for systematic research appears most useful at the intergeneric level. It can be also noticed that proteins of *T. foenum-graecum* and *M. sativa* are less affected by trypsin (20µl for 2hours) than the two other species (5µl for 1hour), which reflect the presence of highly resistant proteins in the two former species contrary to the highly susceptible ones in the two later species.

The 2D-peptide mapping of the four examined species, signify the following points:

1. There are no common peptide bands among the four species. On the other hand a peptide of 25 KDa is common to *T. cylindracea*, *T. polyceratia* and *M. sativa* together.
2. The presence of one 27.3 KDa band in *T. polyceratia* and *M. sativa* and one 45 KDa band in *T. cylindracea* and *M. sativa*.
3. *Trigonella foenum-graecum* is characterized by the widest range of peptide bands from 63.9 KDa to 12.6 KDa, while the narrowest range, from 45 KDa to 17.4 KDa distinguishes *T. polyceratia.*
4. *Trigonella foenum-graecum* and *T. cylindracea* share five peptides of molecular weights: 36, 34.7, 21, 19.3 and 18.6 KDa and. In spite

of these similarities, *T. cylindracea* segregated from *T. foenum-graecum* in the cluster analysis and PCA, that may be due to: (a) the relatively high number of peptide bands in these two species (12 and 15 bands respectively) or (b) the presence of 3 shared peptides between *T. foenum-graecum* and *M. sativa* at 30, 22 and 16 KDa.

The present paper suggested the transposition of *T. polyceratia* to *Medicago*, and for *T. cylindracea* recommended more studies for clear-cut decision. Given the good congruence between morphological characteristics and both 1D-SDS-PAGE and peptide mapping (fingerprint) which maximize the usage of these characters in solving taxonomic problems especially those closely related.

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