

ELECTROPHORETIC PATTERN OF SEED PROTEINS IN *TRIFOLIUM* L. AND ITS TAXONOMIC IMPLICATIONS

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Abstract

The taxonomic delimitations of 61 taxa of the genus *Trifolium* L. belonging to presently accepted five sections, namely *Lotoidea*, *Mistyllus*, *Vesicaria*, *Chronosemium* and *Trifolium* are evaluated, based on numerical analysis of their electrophoretic seed protein profiles. The dendrogram, resulted from the hierarchical cluster analysis of SDS-PAGE profiles of seed proteins conform, with some restrictions, to the present splitting of the genus *Trifolium* into the sections but not into the subsections and series.

Introduction

The genus *Trifolium* L. (Clover) is one of the important genera of Papilionoideae of the Leguminosae with agricultural value. It contains 237 species and represented in all continents (Zohary, 1972b). The Mediterranean region and its adjacent countries are one of the main centres of distribution of *Trifolium* species, and also the centre of domestication and breeding of the cultivated species (Zohary and Heller, 1984).

Several taxonomic treatments were made by botanists to divide the genus into natural groups. Linnaeus (1753) divided the genus into five groups, some of which were later accepted as sections. Seringe (1825) proposed the genus with seven sections. Presl (1832) splitted the genus into nine new genera and all of these genera are retained today as sections. Lojacono (1883) distinguished two subgenera within the genus and divided the first subgenus into 11 sections and the second one into only two sections. Boissier (1873) reduced the number of sections to seven. Hossain (1961) divided the genus into eight subgenera. Another approach was adopted by Zohary and Heller (1984), who recognized eight sections for the genus. The first and largest section is tentatively divided into nine subsections and 13 series. Based on morphological characters alone, it is difficult to distinguish the subordinate taxa of the genus *Trifolium* from one another because they have overlapping variations in terms of the major delimiting morphological and biological characters.

The importance of electrophoretic evidence in plant systematics has been discussed in detail by many workers (Boulter and Derbyshire, 1971; Gottlieb, 1977; Ghareeb *et al.*, 1999; Kamel, 2005). Electrophoretic profiles of seed proteins have been used in different systematic studies (Badr *et al.*, 2000; Zecevic *et al.*, 2000). In Leguminosae many studies have been carried out based on the electrophoresis of seed proteins (Hussein and George, 2002; Hussein *et al.*, 2005). Electrophoretic patterns of total seed proteins as revealed by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS) have been successfully used to resolve the taxonomic and evolutionary problems of some plant species (Ladizinsky and Hymowitz, 1979; Potokina *et al.*, 2000; Ghafoor and Arshad, 2008; Ayten *et al.*, 2009). Badr (1995) and Nikolic *et al.* (2010) studied the electrophoretic seed profiles of some taxa of the genus *Trifolium*. Recently the phylogeny of the genus *Trifolium* was studied based on DNA sequencing (Ellison *et al.*, 2006).

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In the present study, the taxonomic delimitations of 61 taxa of *Trifolium* are re-assessed based on the data resulted from SDS-PAGE profiles of their seed proteins.

Materials and Methods

In the present study, 61 taxa of *Trifolium* have been investigated. Sources of the seeds directly used for protein extraction are given in Table 1. To extract the seed proteins, 0.5 g of mature seeds ground to meal using a mortar and pestle. The meals were homogenized with 0.5 ml of Tris-HCl buffer containing 2% SDS and 10% sucrose at pH 6.8 for overnight at 4°C. The slurry was centrifuged at 9000 rpm for 6 min. The supernatant (protein extract) was taken for loading on 12.5% polyacrylamide gel. Protein samples (20 µl) including loading dye were loaded in the stacking gel. Electrophoresis was carried out under non-reducing conditions in 12.5% polyacrylamide gel. The assay was carried out by an electric supply of 15 mA for 30 min, and then raised to 25 mA for 5-6 h, using a protein marker with low molecular weights. Gels were then stained in Coomassie brilliant blue for 16 h at room temperature, destained and photographed. The bands produced by each sample were counted. The similarity coefficient between the species based on comparisons of their SDS-PAGE profiles was calculated by Jaccard's coefficient using the SPSS program (version 10.1).

The data obtained from the seed protein banding patterns, each species, were subjected to the numerical analysis. The presence or absence of each of the bands (coded as 1 and 0 respectively) was treated as a binary character in a data matrix. The OUTs (Operational Taxonomic Units), produced from the analysis of SDS-PAGE profiles of seed proteins, collected from the investigated taxa of *Trifolium*, resulted in a dendrogram and it was compared with the current taxonomic treatments of the genus *Trifolium*.

Results and Discussion

The banding patterns of *Trifolium* taxa are shown in Figure 1. The seed protein profiles of examined taxa illustrated that bands in between marker weight 116KDs and 55KDs are homogenous in comparison to bands in between 50KDs and 14KDs. The relationships among the taxa of *Trifolium* are presented in Figure 2. The dendrogram resulted from the hierarchical cluster analysis of SDS-PAGE profiles of seed proteins of 61 *Trifolium* taxa conform, with some restrictions, to the splitting of this genus into sections, but not with the sub-sectional arrangement under the section *Lotoidea* and section *Trifolium* considered by Zohary and Heller (1984).

The dendrogram shows that the investigated taxa of *Trifolium* are split into two major clusters. The first major cluster includes 20 taxa belonging to section *Trifolium* and the second major cluster includes 41 taxa belonging to four sections, viz., *Lotoidea*, *Mistyllus*, *Vesicaria* and *Chronosemium*. Within the first major cluster, the taxa are divided into two clusters. The first one included *T. alexandrinum*, *T. caudatum* and *T. canescens* in which *T. alexandrinum* was delimited leaving *T. caudatum* and *T. canescens* as a group. In the second cluster, the taxa are divided into two groups. The first group includes *T. arvense*, *T. bocconeii*, *T. cherleri* and *T. incarnatum*. The second group includes 12 taxa of section *Trifolium*. *Trifolium ligusticum* represents the subsection *Phleoidea*. The similarity between the taxa belonging to section *Trifolium* ranged from 36.4% to 100%. Zohary and Heller (1984) showed that section *Trifolium* ranks second in the number of species, after section *Lotoidea*, which is consistent with the results of this study. It is heterogeneous in appearance but have several distinctive proteins banding pattern after SDS-PAGE. But their agreement in splitting of section *Trifolium* into 17 small and natural clusters by Zohary (1971, 1972a, b), regarded as subsections does not conform to the results of this study (Table 1, Fig. 2). The grouping of *T. caudatum* and *T. canescens*, as well as, the high similarity

Table 1. Sections, subsections and series based on Zohary and Heller (1984) and sources of *Trifolium* samples.

Section	Subsection	Series	<i>Trifolium</i>	Source	Serial no.				
<i>Lotoidea</i>	<i>Loxospermum</i>		8. <i>T. decorum</i> Chiov.	ICLA	9437				
			17. <i>T. multinerve</i> A. Rich.	ICLA	13321				
			19. <i>T. polystachyum</i> Fresen.	ICLA	6298				
	<i>Ochreata</i>			28. <i>T. simense</i> Fresen.	ICLA	324903			
				<i>Lotoidea</i>	Lotoidea	2. <i>T. amabile</i> H.BK	RPIS	262412	
						5. <i>T. burchellianum</i> Ser.	RPIS	369911	
						6. <i>T. burchellianum</i> ssp. <i>johanstonii</i> Gillett (Oliv.)	ICLA IPK	10179 53179	
							7. <i>T. cernuum</i> Brot.		
							10. <i>T. hybridum</i> L.	RPIS	184555
							12. <i>T. masaiense</i> Gillett.	ICLA	896
							14. <i>T. michelianum</i> Savi.	IPK	79181
							15. <i>T. michelianum</i> var. <i>balansae</i> (Boiss.) Azn.	IPK	145176
							18. <i>T. nigrescans</i> ssp. <i>nigrescens</i> Viv.	IPK	117179
							22. <i>T. repens</i> L.	RPIS	282378
							23. <i>T. repens</i> var. <i>giganteum</i> Larg.-Foss.	RPIS	324903
							24. <i>T. occidental</i> Coombe.	IPK	254191
							25. <i>T. semipilosum</i> var. <i>semipilosum</i> Fresen.	ICLA	905
							26. <i>T. semipilosum</i> var. <i>glabrescens</i> Gillett	ICLA	6235
	30. <i>T. thalii</i> Vill.	RPIS	308090						
	<i>Platystylium</i>			1. <i>T. africanum</i> Ser.	RPIS	369885			
				3. <i>T. ambiguum</i> M. Bieb.	RPIS	440689			
				4. <i>T. bilineatum</i> Fresen.	ICLA	8355			
				11. <i>T. isthmocarpum</i> Brot.	IPK	7719			
				16. <i>T. montanum</i> L.	RPIS	234914			
				20. <i>T. ruppellianum</i> var. <i>ruppellianum</i> Fresen.	ICLA	9229			
				21. <i>T. ruppellianum</i> var. <i>lanceolatum</i> Fresen.	ICLA	6260			
				29. <i>T. tembense</i> Fresen.	ICLA	8501			
				Micranthemum			9. <i>T. glomeratum</i> L.	IPK	136180
							28. <i>T. suffocatum</i> L.	IPK	71179
				<i>Calycospatha</i>			13. <i>T. mattirolanum</i> Chivo.	ICLA	8444
31. <i>T. quartinianum</i> A. Rich.							ICLA	9428	
32. <i>T. spumosum</i> L.							IPK	67183	
<i>Mistyllus</i>						33. <i>T. teudneri</i> Schweinf.	ICLA	9720	
						34. <i>T. xerocephalum</i> Fenzl.	IPK		
<i>Vesicaria</i>			35. <i>T. fragiferum</i> L.	RPIS	13322				
			36. <i>T. physodes</i> Stev. ex. M.B.	RPIS	243229				
			37. <i>T. lumens</i> Stev. ex. M.B.	IPK	181189				
			38. <i>T. resupinatum</i> L.	ICLA	9224				
			39. <i>T. tomentosum</i> L.	IPK	138180				
<i>Chronosemium</i>		Agraria	40. <i>T. campestre</i> Schreb.	IPK	98180				
		Filiformia	41. <i>T. dubium</i> Sibth.	IPK	234186				
<i>Trifolium</i>	<i>Intermedia</i>		54. <i>T. heldreichianum</i> (Gib. Belli) Hausskn.	RPIS	419289				
			60. <i>T. Medium</i> var. <i>medium</i> L.	RPIS	259988				
			61. <i>T. medium</i> var. <i>sarosiense</i> (Hajsl.) Savul.	RPIS	179191				

Table 1 Contd.

Section	Subsection	Series	<i>Trifolium</i>	Source	Serial no.
	<i>Alpestris</i>		43. <i>T. alpestre</i> L.	RPIS	210191
	<i>Stellata</i>		56. <i>T. incarnatum</i> L.	ILCA	7018
	<i>Trichoptera</i>		48. <i>T. bocconei</i> Savi.	IPK	81187
	<i>Phleoidea</i>		59. <i>T. ligusticum</i> Balb. ex. Loisel.	IPK	137189
	<i>Lappacea</i>		51. <i>T. cherleri</i> L.	IPK	135182
			55. <i>T. hirtum</i> All.	IPK	213175
			57. <i>T. lappaceum</i> L.	IPK	140182
	<i>Arvensia</i>		47. <i>T. arvense</i> L.	IPK	40186
	<i>Angustifolia</i>		44. <i>T. angustifolium</i> L.	IPK	419304
			47. <i>T. purpureum</i> Loisel. var. <i>desvaxii</i> (Boiss).	IPK	143182
			53. <i>T. dichroanthum</i> Boiss.	IPK	130179
	<i>Alexandrina</i>		42. <i>T. alexandrinum</i> L.	ILCA	6810
			46. <i>T. apertum</i> Bobrov.	IPK	44182
	<i>Urceolata</i>		58. <i>T. leucanthum</i> M. Bieb.	IPK	131177
	<i>Clypeata</i>		52. <i>T. clypeatum</i> L.	IPK	129192

ILCA = International Livestock Center for Africa at Addis Ababa, Ethiopia; RPIS = Regional Plant Introduction Station, Pullman, Washington, USA; IPK = Institut für Pflanzengenetik und Kulturpflanzenforschung, Germany.

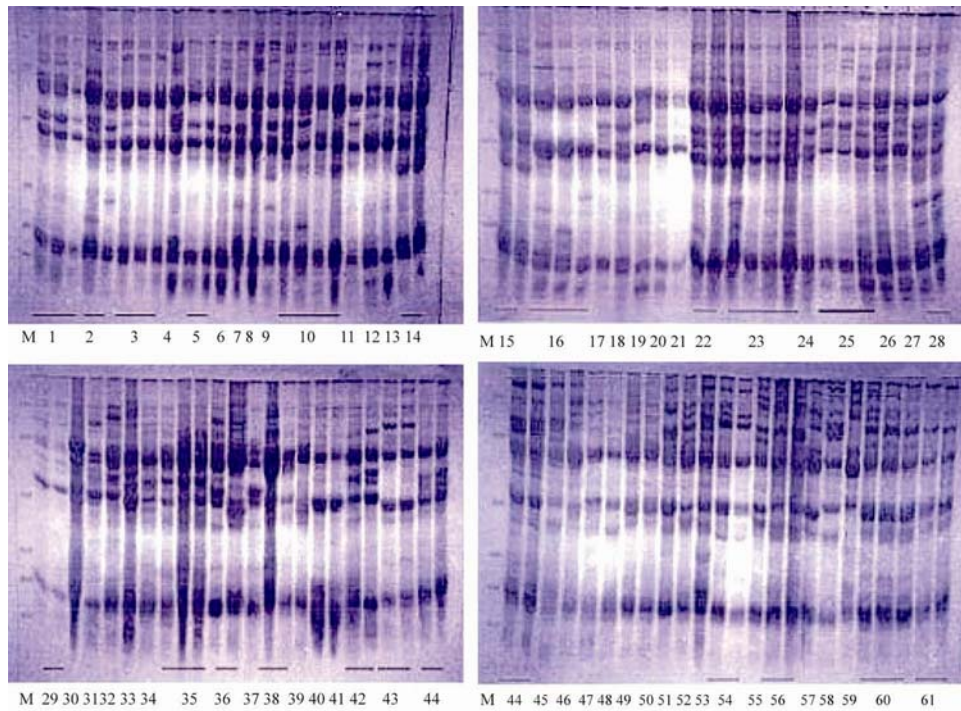


Fig. 1. Electropherograms produced by SDS-PAGE analysis of seed proteins of 61 *Trifolium* taxa, under non-reducing conditions, numbered as in Table 1. M = Marker protein standards.

(95.7%) between them support their position in subsection *Ochroleuca*. On the other hand, *T. alexandrinum* show low similarity (36.4%) with *T. apertum*, although the obtained results, in the

present work, referred that both the two species still delimited under the same section *Trifolium*, it may be claimed that the inclusion of them in the same subsection *Alexandrina* is inconsistent and needs further investigation. Three species *T. cherleri*, *T. hirtum* and *T. lappaceum* representing subsection *Lappacea* are distant from one another. This result implies that it may be better to treat them under separate subsections. Although *T. angustifolium*, *T. purpureum* var. *desvauxii* and *T. dichroanthum* belonging to subsection *Angustifolia*, *T. angustifolium* and *T. purpureum* var. *desvauxii* grouped together but *T. dichroanthum* grouped with *T. clypeatum* showing similarity (87.0%). Among the three taxa *T. heldreichianum*, *T. medium* var. *medium* and *T. medium* var. *sarosiense* comprising the subsection *Intermedia*, the two varieties of *T. medium* shows no difference with each other with a similarity of 100% and *T. heldreichianum* differs from these two varieties with a similarity of 52.05%. The present data show that the taxonomic delimitations in section *Trifolium* requires reconsideration and the number of its subsections as proposed by Zohary (1971, 1972a, b), should be reduced.

The second major cluster comprising of four sections (*Lotoidea*, *Mistyllus*, *Vesicaria* and *Chronosemium*) is divided into two large clusters. One includes 30 taxa belonging to the section *Lotoidea* and other includes 11 taxa belonging to the sections *Mistyllus*, *Vesicaria* and *Chronosemium*. Within the large cluster of section *Lotoidea* the taxa combine variously and form six similarity groups as described below. The pairs of taxa *T. tembense* and *T. thalii*, *T. mattirolianum* and *T. polystachyum*, and *T. isthmocarpum* and *T. masaiense* are consequently segregated as separate groups. The remaining taxa of the section are separated into three groups. The first group is formed by *T. africanum*, *T. amabile*, *T. ambiguum*, *T. bilineatum*, *T. burchellianum*, *T. burchellianum* var. *johanstonii*, *T. cernum*, *T. decorum*, *T. glomeratum* and *T. hybridum*. The second group comprised *T. nigrescans* ssp. *nigrescens* and *T. suffocatum* and the third group is formed by the remaining 12 taxa of the section *Lotoidea*. These groupings of taxa also show that the members included in the subsections *Loxospermum*, *Ochreata*, *Lotoidea*, *Platystylium* and *Calycospatha* or that included in the series *Lotoidea*, *Platystylium* and *Micrantheum* by Zohary and Heller (1984) do not belong to these subsections or series (Table 1, Fig. 2). The similarity between the taxa belonging to this section ranged from 34.3% to 100%. Among these taxa, *T. ruppellianum* var. *lianruppeelum* and *T. repens* shows no difference respectively with *T. ruppellianum* var. *lanceolatum* and *T. repens* var. *giganteum*, rather a similarity of 100%. *Trifolium semipilosum* and *T. semipilosum* var. *glabrescens* presented the same similarity (100%). *Trifolium michelianum* differs from *T. michelianum* var. *balansae* with a similarity of 96.4%. These results show that the nine subsections and 13 series recognized in section *Lotoidea* by Zohary and Heller (1984) based on morphological characters should be reconsidered. Their view to consider this section as the most primitive group of the genus should be justified by its robust phylogeny. George and Hussein (2002) separated tribe *Ononideae* based on chromosome study, as well as the seed proteins analysis of 10 taxa of tribe *Trifolieae*. Badr (1995) illustrated that, on the basis of seed protein electrophoresis, section *Lotoidea* appears as a heterogenous group in which species relationship requires reconsideration.

The large cluster formed by 11 taxa following the section *Lotoidea* is segregated into three groups, one of which including *T. quartinianum*, *T. spumosum*, *T. teudneri* and *T. xerocephalum* is consistent with the section *Mistyllus* recognized by Zohary and Heller (1984). The unique structure of the symmetrically vesicular calyx and the persistent corolla, the manifestly bracteolate flowers and 2-4 seeded pod dehiscing suturally, sharply delimits this section from the others (Zohary and Heller, 1984). The other two groups that include the taxa of sections *Vesicaria* and *Chronosemium* and share the similarities between 53.8% and 76.2% do not completely conform to these sections, as recognized by Zohary and Heller (1984). The two taxa *T. resupinatum* and *T.*

tomentosum belonging to the section *Vesicaria* group with the taxa of section *Chronosemium* (Table 1, Fig. 2) which is inconsistent with Zohary and Heller (1984).

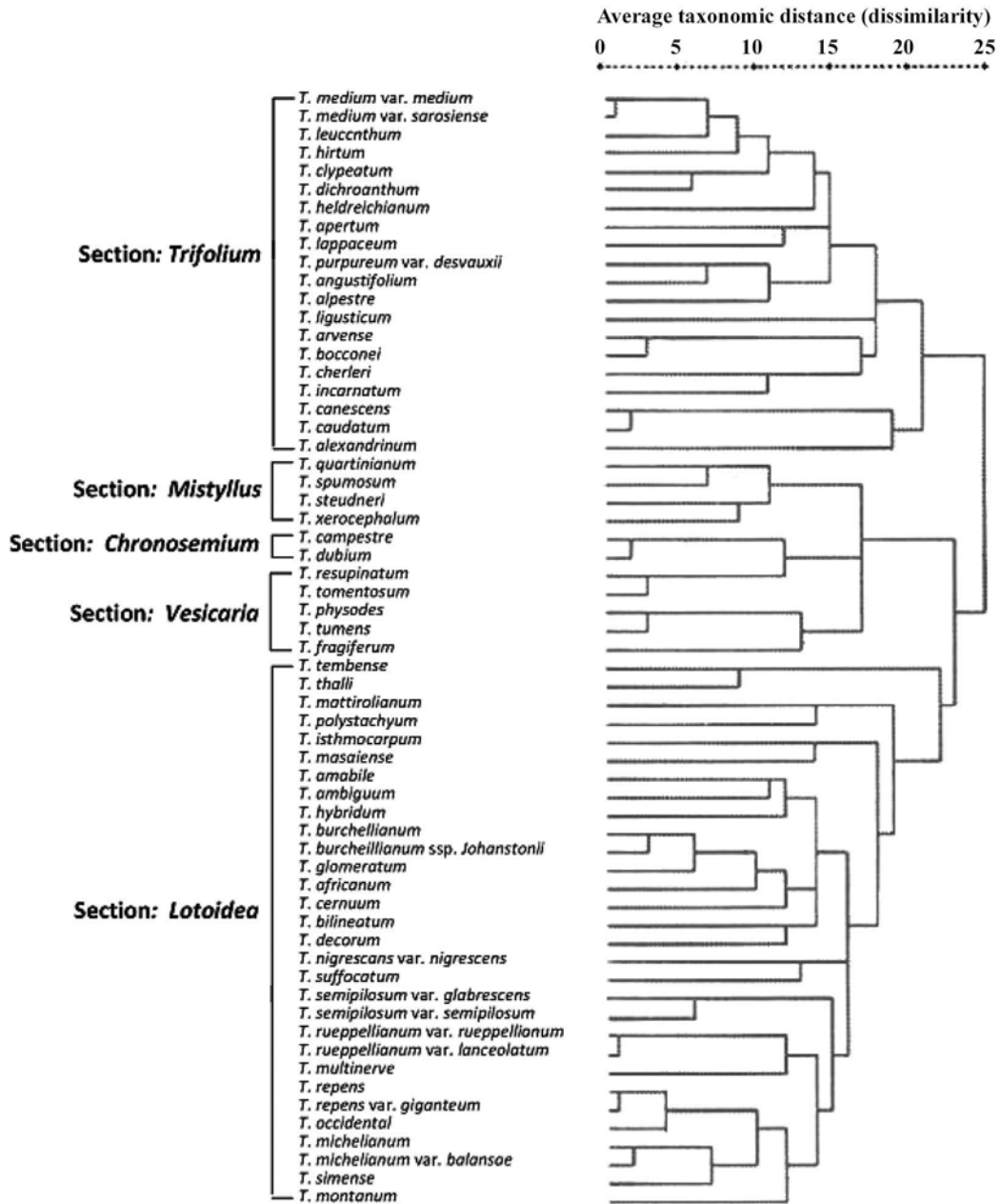


Fig. 2. Dendrogram illustrating the average taxonomic distance (dissimilarity) between the *Trifolium* taxa studied, based on the SDS-PAGE of seed protein characters under non-reducing conditions; numbered as in Table 1.

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