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Protein profile and species relationship in *Trifolium*

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Abstract

The genus *Trifolium* commonly called clovers comprises of 290 annual and perennial species. The important annual types grown in tropical climate are *T. resupinatum*, *T. subterranum* and *T. alexandrinum*. Egyptian clover (*T. alexandrinum*) is the most important winter season legume of India. Genetic improvement programmes on Egyptian clover have not been substantially successful due to little crossability among species in natural condition or through conventional breeding techniques. Hence, present investigation was carried out with a view to work out the genomic relationship based on protein analysis among various species of *Trifolium*. Fifteen genotypes representing 12 species of the genus *Trifolium* were subjected to leaf protein analysis using vertical Polyacrylamide gel electrophoresis system and genetic similarity were estimated. Several species specific bands were also observed such as band numbers 4 and 9 in *T. hybridum*, band number 10 in *T. angustifolium*, band number 15 in *T. vesiculosum*, band number 23 in *T. alexandrinum* and band number 24 and 26 in *T. resupinatum*. The similarity matrix showed the maximum similarity of three *T. alexandrinum* accessions with *T. apertum* (87% similarity). *T. apertum* and *T. purpureum* clustered with four accessions of *T. alexandrinum* in one cluster. *T. alexandrinum* along with *T. apertum* showed 74% similarity with *T. purpureum* which also showed similarity with *T. alexandrinum* in terms of its erect nature, branching pattern and profuse growth in isoclimatic condition. *T. alexandrinum* showed least similarity with *T. pratense* (26 to 33%) followed with *T. hybridum* (30%) and *T. echinatum* (37-46 %). *T. alexandrinum* has 12 bands, out of which 11 are common with *T. apertum* and 9 common with *T. purpureum*. Work carried out in this laboratory indicated that the morphological characteristics of *T. apertum* also closely resembled with *T. alexandrinum* and its crossability with *T. alexandrinum* (using embryo rescue) supports the finding of leaf protein analysis that the two species have close affinity.

Key words: *Trifolium*, Egyptian clover, *T. alexandrinum*, protein profile, species relationship

Introduction

The genus *Trifolium* commonly called clovers, comprises of 290 annual and perennial species, of which 25 are agriculturally important as cultivated forage and pasture crops [1]. The important perennial pasture clover *T. repens* (white clover), *T. hybridum* (alsike clover), *T. pratense* (red clover) and *T. ambiguum* (caucasian clover) are widely distributed in the temperate and subtemperate regions of the world. The annual types *T. resupinatum* (persian clover), *T. subterranum* (subterranean clover) and *T.. alexandrinum* (Egyptian clover or berseem) are commonly cultivated as winter annuals in the tropical and subtropical regions. The genus is believed to have evolved through ancestral heterophyletic origin [2] with hybridity playing little role [3].

Egyptian clover (*T. alexandrinum*) is the most important winter season legume of tropical and subtropical world. In India, it is cultivated in an area of around two million hectares. There has not been any substantial development in its genetic improvement programme in the last two decades, the major bottleneck being the narrow genetic base. The interspecific incompatibility is another problem in transfer of desirable traits from related genera. The strong post-zygotic barrier among the species resulting in the embryo-endosperm incompatibility, makes the embryo rescue technique one of the potential tool for successful interspecific hybridization [4]. For hybridization, it is important to have an understanding of the affinity among different species of the genus and to identify the species showing high degree of similarity with the target species i.e. *T. alexandrinum*. The present investigation was carried out with a view to work out the genomic relationship of *T. alexandrinum* with other species of *Trifolium* based on protein analysis.

Material and methods

Fifteen genotypes representing 12 species of the genus *Trifolium* were grown at experimental farm. The genotypes studied were *T. echinatum* (EC 425078), *T. hybridum* (EC 425032), *T. hirtum* (EC 425045), *T. purpureum* (EC 425069), *T. vesiculosum* (EC 402168), *T. pratense* (EC 401721), *T. resupinatum* (SH 98-15),

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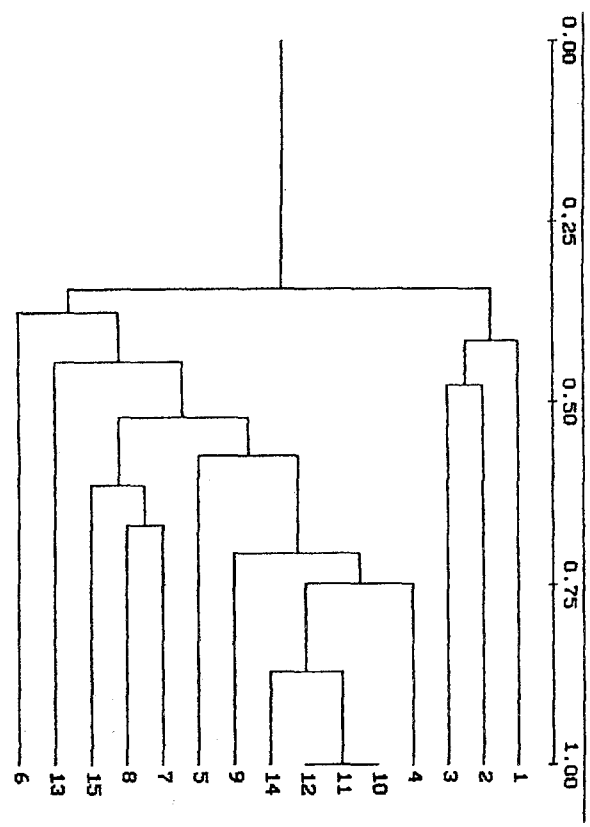
T. repens (EC 400385), *T. alexandrinum* (JHB 99-32-1, JHB 99-32-2, JHB 99-32-3, JHB 99-25), *T. angustifolium* (EC 425062), *T. apertum (EC 401712), T. lappaceum* (EC 402165). These genotypes were subjected to leaf protein analysis using vertical Polyacrylamide gel electrophoresis system.

Forty ml of 12% resolving gel was prepared by adding Acrylamide (30%, 16ml), Tris HCl (10 ml), H_2O (14ml), TEMED (20µl), Ammonium per Sulfate (10%, 200µl). This was overlayed by 10 ml of 4% stacking gel prepared by adding Acrylamide (30% 1.3 ml), Tris HCl (2.5 ml), H_2O (6.2 ml), TEMED (10µl), APS (10%, 50µl). 'Genei' vertical migration chamber was used for electrophoresis with running gel electrode buffer (Tris-Glycine pH 8.3). 200µl of freshly prepared sample mixed with traces of bromophenol blue dye was loaded in wells and a constant current of 20 mA was applied till tracking dye crossed the stacking gel followed with electrophoresis at 40 mA. The gel was fixed in 3.5% Perchloric acid for overnight and stained in staining solution (40 mg of comossie brilliant blue G-250 in 100 ml 3.5% Perchloric acid) for 3 to 4 hours. Only clear and unambiguous bands were recorded based on their relative mobility and bands were numbered from the well i.e. the slowest band as one. Binary data matrix was generated taking '1' as presence and '0' as absence. The genetic similarity were estimated using Dice's similarity co-efficient [5] and further analysed by SAHN clustering and UPGMA analysis. The statistical calculations were done using NTSYS programme.

Results and discussion

The electrophoretic leaf protein banding pattern of 12 species of *Trifolium* carried out on native PAGE revealed a total of 28 electrophoretic bands (Table 1). None of the species showed identical banding pattern. Out of four samples of *T. alexandrinum*, three samples were of self progeny of accession number JHB 99-32 and one of JHB 99-25, The two samples of selfed progeny and that of JHB 99-25 showed identical banding pattern i.e. presence of band 1, 2, 3, 5, 6, 16, 18, 19, 22, 23, 25 and 27 whereas one plant in the self progeny was represented by band numbers 2, 3, 16, 17, 19, 22, 23, 25 and 27. The maximum number of bands present in any species was 13 in *T. purpureum* (1, 2, 3, 5, 6, 7, 8, 11, 16, 17, 18, 19 and 27) and *T. vesiculosum* (1, 2, 3, 11, 12, 14, 15, 16, 17, 20, 22, 25 and 27) and the minimum number of bands were found in *T. echinatum* (16, 19, 22, 28 followed with 6 bands in *T. pratense*. Band number 3 was most commonly represented and was found in 11 out of 12 species studied. Several species specific bands were also observed such as band numbers 4 and 9 in *T. hybridum*, band number 10 in *T. angustifolium*, band number 15 in *T. vesiculosum*, band number 23 in *T. alexandrinum* and band number 24 and 26 in *T. resupinatum*.

The similarity matrix showed the maximum similarity of three *T. alexandrinum* accessions with *T. apertum* (87% similarity) (Table 2). These three *T. alexandrinum* samples together with *T. apertum* showed 70% similarity with the plant of the selfed *T. alexandrinum* (JHB 99-32-1). SAHN clustering analysis revealed that these 12 species could be grouped in six clusters (Fig. 1). The first cluster comprising of *T. echinatum*, *T. hybridum* and *T. hirtum* showed 34% similarity with group of cluster number 2 to 6. *T. apertum* and *T. purpureum* were clustered with four accessions of *T. alexandrinum* in cluster 2. The four samples of *T. alexandrinum* along with *T. apertum* showed 74% similarity with *T. purpureum*. The second cluster was



List of accessions
1. *T. echinatum* (EC 425078), 2. *T. hydridum* (EC 425032), 3. *T. hirtum* (EC 425045), 4. *T. purpureum* (EC 425069), 5. *T. vesiculosum* (EC 402168), 6. *T. pratense* (EC 401721), 7. *T. resupinatum* (SH 98-15), 8. *T. repens* (EC 400385), 9. *T. alexandrinum* (JHB 99-32-1), 10. *T. alexandrinum* (JHB 99-32-2), 11. *T. alexandrinum* (JHB 99-32-3), 12. *T. alexandrinum* (JHB 99-25), 13. *T. angustifolium* (EC 425062), 14. *T. apertum* (EC 401712), 15. *T. lappaceum* (EC 402165)

Fig. 1. Dendrogram based on cluster of leaf protein electrophoresis data showing genetic relatedness among accessions of *Trifolium* species

Table 1. Protein banding pattern in different *Trifolium* species

Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Specie																												
T. repens (EC 400385)	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-
T. pratense (EC 401721)	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+
T. resupinatum (SH 98-15)	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	+	-	+	-	-
T. alexandrinum (JHB 99-32-1)	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	-	+	-	+	-
T. alexandrinum (JHB 99-32-2)	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	+	-	+	-
T. alexandrinum (JHB 99-32-3)	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	+	-	+	-
T. alexandrinum (JHB 99-25)	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	+	-	+	-
T. apertum (EC 401712)	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	+	-
T. hydridum (EC 425032)	-	-	+	+	-	-	-	+	+	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+
T. echinatum (EC 425078)	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+
T. purpureum (EC 425069)	+	+	+	-	+	-	+	+	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-
T. angustifolium (EC 425062)	-	-	+	-	-	+	+	-	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+	-
T. lappaceum (EC 402165)	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+
T. hirtum (EC 425045)	-	+	+	-	+	+	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	-	-	+
T. vesiculosum (EC 402168)	+	+	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	+	-	+	-	-	+	-	+	-

Table 2. Similarity matrix based on protein banding pattern in different *Trifolium* species

Accessions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T. echinatum (EC 425078)	1.000														
T. hydridum (EC 425032)	0.400	1.000													
T. hirtum (EC 425045)	0.429	0.476	1.000												
T. purpureum (EC 425069)	0.235	0.417	0.522	1.000											
T. vesiculosum (EC 402168)	0.235	0.333	0.435	0.538	1.000										
T. pratense (EC 401721)	0.200	0.353	0.435	0.538	0.421	1.000									
T. resupinatum (SH 98-15)	0.000	0.273	0.476	0.583	0.333	0.471	1.000								
T. repens (EC 400385)	0.000	0.222	0.353	0.600	0.500	0.462	0.667	1.000							
T. alexandrinum (JHB 99-32-1)	0.462	0.300	0.421	0.545	0.636	0.267	0.300	0.500	1.000						
T. alexandrinum (JHB 99-32-2)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000					
T. alexandrinum (JHB 99-32-3)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000	1.000				
T. alexandrinum (JHB 99-25)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000	1.000	1.000			
T. angustifolium (EC 425062)	0.167	0.316	0.222	0.476	0.381	0.316	0.400	0.471	0.500	0.500	0.500	0.500	1.000		
T. apertum (EC 401712)	0.267	0.364	0.476	0.833	0.583	0.353	0.545	0.667	0.700	0.870	0.870	0.870	0.526	1.000	
T. lappaceum (EC 402165)	0.308	0.400	0.421	0.727	0.455	0.533	0.600	0.625	0.444	0.571	0.571	0.571	0.353	0.700	1.000

57.3% similar with cluster number 3 of *T. vesiculosum*. Cluster 4 comprised of *T. resupinatum*, *T. repens* and *T. lappaceum*. In this cluster *T. resupinatum* and *T. repens* showed 66.7% similarity and together they were 61.3% similar with *T. lappaceum*. This cluster was 52.2% similar with group of cluster 2 and 3. Cluster number 5 of *T. angustifolium* was 44.2% similar with group of cluster numbers 2 to 4. Similarly the group of cluster numbers 2 to 5 showed 37.3% similarity with cluster number 6 of *T. pratense*. Cluster number 1 and the group of clusters 2 to 6 showed 34.3% similarity.

On the basis of similarity index, it was inferred that the *T. alexandrinum* lines showed maximum affinity with *T. apertum* (70-87%) followed with *T. purpureum* (72%) and *T. vesiculosum* (56-62%). *T. purpureum*, also showed similarity with *T. alexandrinum* in terms of its erect nature, branching pattern and profuse growth in isoclimatic condition. Fifty four per cent similarity of *T. alexandrinum* was also observed with *T. hirtum*. *T. alexandrinum* accessions showed maximum distance from *T. pratense* (26 to 33% similarity) followed with *T. hybridum* (30% similarity) and *T. echinatum* (37-46%). It is also to note that *T. alexandrinum* has 12 bands, out of which 11 are common with *T. apertum* and 9 common with *T. purpureum*. *T. angustifolium*, *T. pratense*, *T echinatum*, *T. hybridum* and *T. hirtum* formed small cluster of 1 to 2 species and were placed quite away from the cluster of *T. alexandrinum*.

Seed protein electrophoretic profile study has placed *T. alexandrinum* (Miskawi) in a separate group [6], whereas *T. alexandrinum* (Fahli) was placed with *T. lappaceum*, *T. pratense*, *T. medium* and *T. hirtum* in spite of the fact that SDS PAGE analysis of seed protein showed that Miskawi and Fahli were similar for

all the bands. The SDS PAGE also exhibited similarity for all the bands except one with *T. pratense* and *T. medium* which were otherwise 100% similar showing close phylogenetic relationships. Such close relationship was also been suggested earlier [7, 8, 9]. However in the present study *T. alexandrinum* showed little similarity with *T. pretense*. Close affinity was reported between *T. repens* and *T. nigrescence* [10] where the third generation fertile backcross progenies showed significant variation for vegetative and reproductive traits. Similarly successful backcross development between *T. repens* and *T. ambiguum* also indicate genomic similarity [11].

Work carried out in the same laboratory (unpublished work) indicated that the morphological characteristics of *T. apertum* also closely resembled with *T. alexandrinum* and its crossability with *T. alexandrinum* (using embryo rescue) supports the finding of leaf protein analysis that with the two species have close affinity. *T. alexandrinum* has been reported to owe its origin to a few wild species such as *T. salmoneum*, *T. berytheum* and *T. apertum* [12,13].

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