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Genetic diversity based on multivariate analyses for breeding strategies in *Trifolium*

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Abstract
Data on eighteen agro-morphological traits were recorded in twenty-five genotypes of *Trifolium* belonging to seventeen species and were analyzed using Mahalanobis D^2 statistics and Principal Component Analysis (PCA) for precise grouping. Wide range of genetic diversity was observed within and between species of *Trifolium* for agro-morphological traits studied. Internode length, leaves per plant and green fodder yield per plant contributed maximum towards the genetic divergence. Cluster analysis based on Tocher's method, categorized the cultivars into five groups. Based on PCA, the first five components explained over 88% of total variation. Scatter plot using first two components also confirm the grouping done by cluster analysis. *T. pratense*, *T. ambiguum*, *T. lappaceum* can be used as donor for genetic up gradation of *T. alexendrinum*. *T. compestre* and *T. resupinatum* (shaftal) were found more diverse and hence chances of making successful interspecific hybrids are less.

Keywords: Cluster analysis, Genetic diversity, PCA, Powdery mildew, *Trifolium*,

Abbreviations: BP: Branches/plant; **CP**: Crude protein; **CPYP**: Crude protein yield/plant; **DF**: Days to 50 per cent flowering; **DMYP**: Dry matter yield/plant; **GFYP**: Green fodder yield/plant; **IL**: Internode length; **LP**: Leaves/plant; **LSR**: Leaf stem ratio; **NP**: Nodes/plant; **PH**: Plant height

Introduction

Trifolium commonly known as clover includes economically valuable species cultivated extensively throughout the world. Characterization and evaluation of *Trifolium* species is utmost important to identify donors for different traits and utilizing species showing affinity towards berseem in interspecific hybridization programme. It is desirable to study the genetic diversity among the species of *Trifolium* before going to interspecific hybridization. Some appropriate methods, cluster analysis, PCA and factor analysis, for genetic diversity identification, parental selection, tracing the pathway to evolution of crops, centre of origin and diversity, and study interaction between the environment are currently available (Mohammadi and Prasanna, 2003; Eivazi *et al*., 2007). Results of using PCA showed that this method is limited when the pattern of variation is not based on a 0 and 1 scores. Therefore, PCA along with other techniques can appropriately be used for grouping (Mohammadi and Prasanna, 2003). The main objective of this study was to capture potential genetic diversity between *Trifolium* genotypes grown in India and grouping them by using cluster analysis and PCA to identify potential donor for genetic upgradation of *Trifolium alexandrinum* (berseem). Berseem is an important forage crop with multicut nature (four to eight cuts), long duration of green fodder availability, high yield, good quality, good digestibility and palatability (Kaushal *et al*., 2003; Zayed *et al*., 2011). The feed and fodder constitute 50-60% of the cost of milk production and cultivated fodders like berseem play important role in the economic milk production (Hazra, 2014).

Materials and Methods

***Experimental material and field evaluation*:** The 25 genotypes of *Trifolium* belonging to 17 *species*, maintained at Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur, Indian Grassland and Fodder Research Institute (IGFRI), Jhansi and Punjab Agriculture University (PAU), Ludhiana were used in the present study (Table 1). The material was planted at the experimental farm, CSK HPKV, Palampur during *rabi* 2009-10. The experiment was laid out in randomized block design with three replications. The plot size was kept 0.8×1.25 m^2, with row to row and plant to plant spacing of 40 and 10 cm, respectively. In annual and perennial spp., the sowing was done during October-November and seed rate was 4-12 kg/ha depending up on the size of the seed. Fertilizer was

applied at the rate of 20 kg N and 60 kg P per hectare at the time of sowing and 3-4 irrigations were done during summers. In case of perennial spp. similar practices were followed except sowing was done only once. Every year recommended doses of fertilizers were applied. Morphological characterization was done on the basis of visual observation for 7 morphological characters *viz*., growth habit, petiole colour, petiole hairiness, leaflet hairiness, leaflet colour, leaf apex shape, flower colour, and 11 agronomic and quality traits *viz*., days to 50 per cent flowering (DF), plant height (PH) (cm), branches/ plant (BP), leaves/plant (LP), nodes/plant (NP), internode length (IL), leaf/stem ratio (LSR), green fodder yield/plant (GFYP), dry matter yield/plant (DMYP), crude protein (CP) and crude protein yield/plant (CPYP).

***Statistical analysis*:** The recorded data on different agronomic and quality traits were statistically analysed as per the procedure given by Panse and Sukhatme (1984) to find out significance of variation resulting from genotypes. Genetic diversity analysis was done using Mahalanobis D^2 statistic and grouping of clusters was done by following Tocher's method given by Rao (1952) and PCA was done using SAS software (SAS Enterprise Guide 9.2). Principal component (PC) axes showing value greater than unity were considered significant (Dagnelie, 1975).

Results and Discussion

Genetic variability and cluster analysis: Analysis of variance revealed sufficient genetic variability for all the traits under study (Table 2). Genetic diversity studies using D^2 statistics grouped 25 genotypes of *Trifolium* into five clusters. Cluster 1, 2, and 4 were multi genotypic, whereas cluster 3 and 5 were mono genotypic. Maximum genotypes were grouped under cluster 2 followed by cluster 1 (Fig.1). Seven genotypes were grouped into cluster 1 include three cultivars of *T. pratense* (PRCS-1, PRC-3, and Kashmir collection), *T. ambiguum cv*. Monal, *T. lappaceum cv.* EC-528542 and two cultivars of *T. alexandrinum cv*. BL-42 and BL-10. Cluster 1 included genotypes with erect, semi erect and prostrate growth habit, all types of petiole color *i.e.* light green, green, green with pink pigmentation, hairy and non-hairy petiole and leaf lets and green, dark green and variegated leaf colors and having tapering and rounded leaf apex, pink and white flowers. This cluster had genotypes with less no. of BP (6.14) and NP (7.72). Twelve genotypes were grouped in cluster 2 *viz*., *T. grandiflorum cv*. EC-528540, *T. spumosum cv*. EC-528549, two cultivars of *T. echinatum* (EC-425048, EC-425075), *T. hirtum cv*. EC-425039, *T. hybridum cv*. EC-425029, *T. vesiculosum cv.* Palampur, *T. apertum cv*. EC-401712, three cultivars of *T. repens* (PWC-22, PWC-3, PWC-20) and *T. angustifolium cv*. EC-425062. Cluster 2 had genotypes with all types of growth habits, petiole color, petiole hairiness, leaflet hairiness, leaf color, leaf apex shape, pink, light pink and white flowers and had highest mean for LSR (0.74%). One genotype *T. compestre cv.* EC-402155 was grouped in cluster 3 which had prostrate type growth habit, green colored hairy petiole and non-hairy leaflets, green leaflet color, rounded leaf apex and yellow flowers and had highest mean for NP. Four genotypes were grouped in cluster 4 *viz*., two cultivars of *T. alexandrinum* (BL305, Wardan), *T. constantinopolinatum cv*. EC-401713 and *T. arvense cv.* EC-528533. Cluster 4 had erect type growth habit, light green and green colored hairy petiole, non-hairy green leaflets with tapering and rounded leaf apex and white flowers. This cluster included genotypes with highest values for yield and related traits. One genotype *T. resupinatum cv.* SH 48 was grouped in cluster 5 and had semi erect growth habit, green colored non-hairy petiole and non-hairy green leaflets with rounded leaf apex and pink flowers. Cluster 5 had highest means for BP (9.73), IL (9.95) and CP percent (25.08) and lowest mean value for DF (151.67 days). Estimates of mean performances of genotypes showed that out of 11 traits studied, cluster 4 had genotypes with maximum desirable traits *viz*., late flowering, highest PH, LP, GFYP (g), DMYP (g) and CPYP (g). Genotypes grouped in cluster 3 had lowest mean values for most of the traits. The clustering pattern showed the presence of sufficient genetic diversity between and within different species of *Trifolium*. The genotypes grouped in the same cluster were less divergent than ones placed in different clusters. Highest intra-cluster distance was observed for cluster one followed by cluster two which means the genotypes were more divergent in these clusters. Highest inter-cluster distance was observed between cluster three and cluster five, indicating that *T. compestre* and *T. resupinatum* (shaftal) were more diverse and chances of making successful interspecific hybrids were less. Similar studies were conducted on *Trifolium* spp. and reported grouping of genotypes into different clusters using D^2 statistics (Kouame and Quesenberry, 1993; Lee *et al*., 1994; Rasso *et al*., 1997; Bulinska, 2000; Drobna and Zakova, 2001; Kaila *et al*., 2009). According to Rahim *et al.* (2010) the hybrids of genotypes with maximum distance resulted in high yield, the cross between these genotypes could be used in breeding programs to achieve maximum

Table 1. Details of the material used in the study

S. No.	Species/ genotypes	Accession no.	Chromosome no.	Source
1	*T. pratense* (Red clover)	Kashmir Collection	14	CSKHPKV, Palampur
2	*T. pratense* (Red clover)	PRCS-1	14	CSKHPKV, Palampur
3	*T. pratense* (Red clover)	PRC-3	14	CSKHPKV, Palampur
4	*T. repens* (White clover)	PWC-22	32	CSKHPKV, Palampur
5	*T. repens* (White clover)	PWC-25	32	CSKHPKV, Palampur
6	*T. repens* (White clover)	PWC-3	32	CSKHPKV, Palampur
7	*T. ambiguum* (Caucasian clover)	Monal	16	CSKHPKV, Palampur
8	*T. apertum* (Open clover)	EC-401712	16	IGFRI, Jhansi
9	*T. compestre* (Hop clover)	EC-402155	14	IGFRI, Jhansi
10	*T. hirtum* (Rose clover)	EC-425039	16	IGFRI, Jhansi
11	*T. grandiflorum*(Large flower hop clover)	EC-528540	16	IGFRI, Jhansi
12	*T. spumosum* (Bladder clover)	EC-528549	16	IGFRI, Jhansi
13	*T. constantinopolinatum* (Constantinopole clover)	EC-401713	16	IGFRI, Jhansi
14	*T. lappaceum* (Bur clover)	EC-528542	16	IGFRI, Jhansi
15	*T. echinatum* (Prickly clover)	EC-425048	16	IGFRI, Jhansi
16	*T. echinatum* (Prickly clover)	EC-425075	16	IGFRI, Jhansi
17	*T. vesiculosum* (Arrow leaf clover)	Palampur	16	CSKHPKV, Palampur
18	*T. hybridum* (Alsike clover)	EC-425029	16	IGFRI, Jhansi
19	*T. arvense* (Haresfoot trefoil)	EC-528533	14	IGFRI, Jhansi
20	*T. angustifolium* (Narrow leafed clover)	EC-425062	16	IGFRI, Jhansi
21	*T. resupinatum* (Shaftal)	SH 48	16	CSKHPKV, Palampur
22	*T. alexandrinum* (Berseem)	BL-42	16	PAU, Ludhiana
23	*T. alexandrinum* (Berseem)	BL-10	16	PAU, Ludhiana
24	*T. alexandrinum* (Berseem)	BL-305	16	PAU, Ludhiana
25	*T. alexandrinum* (Berseem)	Wardan	16	CSKHPKV, Palampur

heterosis. The lowest inter-cluster distance was observed between cluster one and four indicating that genotypes of these clusters were genetically close. *T. pratense*, *T. ambiguum* and *T. lappaceum* could be used as donor species for important forage traits like green and dry matter yield, quality and powdery mildew resistance. Similar findings with respect to different traits were reported by different workers earlier (Singh, 2003; Malaviya *et al*., 2004; Khare *et al*., 2007; Kaila *et al*., 2009).

***Fodder yield and quality*:** On the basis of mean values for fodder yield and quality traits, *T. alexandrinum cv*. Wardan, *T. arvense cv.* EC-528533 and *T. hirtum cv*. EC-425039 were found to be superior. The genotypes of three species in cluster 1 were highly resistant to powdery mildew, whereas *T. pretense* was highly susceptible to powdery mildew. The genotypes grouped in cluster 3, 4 and 5 showed resistant reaction to powdery mildew. In cluster 2, genotypes of *T. repens* showed differential reaction to powdery mildew. *T. repens* cv. PWC 22 showed moderately resistant reaction, *T. repens* cvs. PWC3 and PWC 25 showed susceptibility to powdery mildew.

Correlation studies: Results of Pearson correlation analysis support the use of multivariate analysis. Correlation studies showed significant association between yield and component traits. Significant positive associations were observed between PH with LP (0.721**), NP (0.587**), IL (0.593**), GFYP (0.587**), DMYP (0.503**) and CPYP (0.530**). Branches per plant were positively associated with NP (0.510**). Leaves per plant were positively correlated with GFYP (0.678**), DMYP (0.615**) and CPYP (0.656**). Green fodder yield/plant was positively associated with DMYP (0.882**) and CPYP (0.873**). Significant positive associations were observed among DMYP and CPYP (0.953**). Correlation studies showed that PH and LP were positively associated with GFYP and DMYP. Singh *et al*. (1999) reported positive correlation between plant height and yield, similar to the findings of the present study.

Table 2. Analysis of variance for various traits in different *spp*. of *Trifolium*

Source of variation	df	Traits								
		DF	PH(cm)	BP	LP	NP	IL(cm)	LSR	GFYP(g)	DMYP(g)	CP(%)	CPYP (g)
Replication	2	42.65	45.99	2.52	391.59	0.16	0.24	0.01	39.83	10.91*	7.39	0.66*
Genotype	24	223.94**	779.14**	8.36**	17040.49**	15.79**	16..11**	0.10*	542.50**	59.09**	21.01**	2.94**
Error	48	26.26	35.79	1.04	285.34	1.53	0.10	0.01	13.34	3.10	6.46	0.20

*Significant at 5% level ; **Significant at 1% level

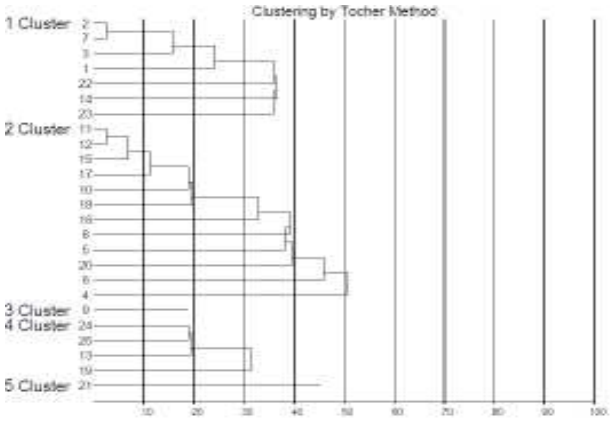

Fig 1. Dendrogram showing grouping of 25 *Trifolium* genotypes generated using D^2 cluster analysis (Tocher's method)

***Principle component analysis*:** In the PCA, eigenvalues greater than unity were shown by first five principal components (Table 3). PC 1 accounted for 38.17% of total variations and was positive indicator of GFYP, DMYP, CPYP and to lesser extent PH. The component 2 accounted for 18.37% variations and was mainly associated with NP and BP and component 3 summarized 13.12% of variation and was positive indicator of LSR, CP% and BP. The component 4 resumed 9.69% of total variations and was associated with DF and PC 5 contributed 9.24% variations and was positive indicator of CP% and IL. Five components cumulatively account for 88.59% of the total variations. Comparing these results with table 3 indicated that traits with largest impact on the components showed highest rate of variation hence can be used in grouping genotypes effectively. The graphic presentation of relationship among different genotypes of different species, on the basis of evaluated traits, were shown for PC1 and PC2 which account for 56% of total variation (Fig. 2). Different genotypes were broadly

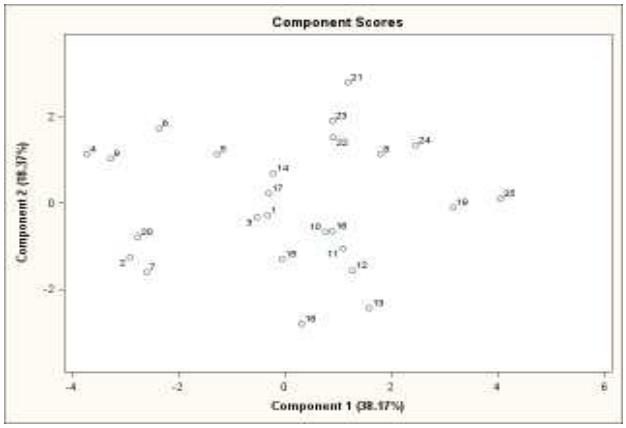

Fig 2. Scatter plot grouping of *Trifolium* genotypes based on PC1 and PC2 of the principal component analysis

Table 3. Eigenvalues, proportion of variability and traits contributing to first five PCs in studied *Trifolium* genotypes

	PC1	PC2	PC3	PC4	PC5
Eigen values	4.20	2.02	1.44	1.06	1.02
Percentage of variance	38.17	18.37	13.12	9.69	9.24
Cumulative percentage	38.17	56.54	69.66	79.35	88.59
		Coefficient of variates			
DF	0.147	-0.377	0.156	0.628	-0.038
PH(cm)	0.395	0.241	-0.089	0.238	0.272
BP	0.140	0.380	0.407	0.046	-0.459
LP	0.424	0.045	0.213	0.162	0.086
NP	0.146	0.567	0.113	0.269	-0.097
IL (cm)	0.210	0.170	-0.496	0.158	0.482
LSR	0.055	-0.418	0.486	0.135	0.310
GFYP (g)	0.439	-0.163	0.030	-0.167	-0.133
DMYP (g)	0.421	-0.198	-0.175	-0.240	-0.231
CP (%)	0.020	0.214	0.477	-0.430	0.540
CPYP (g)	0.430	-0.132	-0.055	-0.367	-0.077

categorized into five groups. Two genotypes belonging to *T. arvense* (EC-528533) and *T. alexendrinum* (Wardan) clearly stand out for PC1 values, corresponding mainly to high LP (272.67), GFYP (57.42 q/ha), DMYP (19.43 q/ha) and CPYP (4.62). Genotypes belonging to *T. apertum* (EC-401712), *T. alexendrinum* (BL-42, BL-10 and BL-305) and *T. resupinatum* formed another group which was similar in morphological traits except petiole and leaflet color with positive loading for PC1 and PC2. This group had maximum PH (63.70cm), BP (8.4) and NP (11.2). *T. constantinopolinatum* (EC-401713), *T. hybridum* (EC-425029), *T. hirtum* (EC-425039), *T. grandiflorum* (EC-528540), *T. spumosum* (EC-528549) *T. vesiculosum* (Palampur) and *T. echinatum* (EC-425048, EC-425075) grouped in to third group with positive loadings for PC1 and negative loadings for PC 2. This group comprised of late flowering genotypes. Among these *T. constantinopolinatum* (EC-401713) took maximum time to flower *i.e.* 169 days. Genotypes with longer vegetative stage can be introduced in cultivation as forage crop. *T. repens* (PWC-3, PWC-22 and PWC-25), *T. compestre* (EC-402155), *T. lappaceum* and *T. vesiculosum* formed fourth group with positive loadings for PC 2 and negative loadings for PC 1. This group further divided into two subgroups. First group included genotypes of *T. repens* (PWC-3, PWC-22 and PWC-25), *T. compestre* (EC-402155) which were early flowering, short in height and with less internode length and these genotypes could be used for over seeding in pasture. Second group included *T. lappaceum* (EC-528542) and *T. vesiculosum* (Palampur) which had average PH, high LP and high CP. *T. pratense* (Kashmir collection, PRC-1 and PRC-3), *T. ambigum* (Monal) and *T. angustifolium* (EC-425062) had negative values for both PC 1 and PC 2 formed fifth group which had two subgroups. *T. pratense* (Kashmir PRC-1), *T. ambigum* (Monal) and *T. angustifolium* (EC-425062) formed one group with shortest PH and *T. pratense* (Kashmir collection and PRC-3) formed another group with high LSR. These results were confirmed with cluster analysis. Earlier different workers used both Principal Component and cluster analyses to group *Trifolium* accessions (Caradus *et al*., 1990; Jahufer *et al*., 1997; Khare *et al*., 2007; Singh *et al*., 2007).

Conclusion

T. alexendrinum (BL-42, BL-10 and BL-105) collected from PAU, Ludhiana were in one group with positive loadings for PC1 and PC2. Different collections of *T. pratense* were in one group and of *T. repens* in another group from CSK HPKV, Palampur. Different species collected from IGFRI, Jhansi were put into different groups. *T. alexandrinum cv*. Wardan, *T. arvense cv.* EC-528533 and *T. hirtum cv*. EC-425039 were found superior for yield and quality traits along with resistance to powdery mildew.

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