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Morphological and RAPD markers - mediated assessment of genetic diversity amongst various *Trifolium* species and identification of potential ideotypes for genetic upgradation of berseem under changed climate in mid-hills of north-west Himalayas

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Abstract

Genetic diversity among 25 genotypes belonging to nine species of genus *Trifolium* was evaluated on the basis of agro-morphological traits using Mahalanobis D^2 statistic and RAPD markers with the objective of identifying the species showing affinity with berseem in order to enhance its genetic base for its genetic upgradation. Mean values revealed superiority of SH 48 (shaftal), Wardan, Bundel berseem 2, Bundel berseem 3, Saidi and Fahli (berseem), PRC 3 (red clover), EC 401713 (constantinopole clover) and Palampur (arrowleaf clover) for various fodder traits. D^2 statistic grouped the 25 genotypes into four clusters. In contrast, RAPD analysis grouped the genotypes into three clusters and further sub-clusters corresponding to different species. Both D^2 statistic and RAPD analysis revealed low genetic diversity among white clover and berseem genotypes whereas, red clover genotypes were found more divergent. *T. apertum* and *T. constantinopolitanum* exhibited more affinity towards berseem than any other species.

Key words: *Trifolium*, genetic diversity, morphological characterization, RAPD markers

Introduction

In India, berseem (*Trifolium alexandrinum* L.) is the most important winter season fodder legume, grown in about two million hectares due to its multicut nature, long duration of green fodder availability, high fodder yield, nutritional quality, high digestibility and palatability. Despite its popularity, not much success has been achieved in its upgradation in the past two decades due to its narrow genetic base [1]. Hence, there is an urgent need to enhance the genetic diversity of *T. alexandrinum* cultivars through interspecific hybridization. But a major bottleneck in interspecific hybridization is the post-zygotic cross compatibility barriers among different *Trifolium* species. Therefore it is important to characterise and evaluate different species of the genera showing affinity towards *T. alexandrinum,* to be utilized in interspecific hybridization programmes [2].

In this investigation, genetic diversity studies based on morphological characters supplemented with RAPD markers [3] were carried out to identify suitable species for genetic amelioration of berseem.

Materials and methods

Plant material

The material consisted of 25 genotypes belonging to nine species of *Trifolium viz*., *T. alexandrinum, T. repens* L., *T. pratense* L., *T. resupinatum* L., *T. apertum* Bobrov., *T. vesiculosum* Savi., *T. lappaceum* L., *T. constantinopolitanum* Ser. and *T. ambiguum* M.Beib. representing a few cultivated genotypes, indigenous and exotic accessions (Table 1).

Morphological analysis

All the *Trifolium* species were evaluated in respect of seventeen agronomic and quality traits during *rabi*, 2007–08 at the Experimental Farm of the Department of Crop Improvement, CSK HPKV, Palampur. The genotypes were raised in randomised block design with three replications in two rows (3 m length) plot with a row spacing of 50 cm and plant spacing of 10 cm. Observations were recorded on the basis of ten randomly competitive plants in each replication at 50% flowering stage for agronomic traits *viz.,* days to 50% flowering, number of branches/ plant, number of nodes/

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Table 1. *Trifolium* genotypes used for the present study

S.No.	Species/ Genotypes	Chromosome no.
A.	*T. alexandrinum* (Berseem)	16
1	Wardan	
2	Bundel Berseem 2 (JHB 146)	
3	Bundel Berseem 3 (JHTB 96 4)	
4	Saidi	
5	Fahli	
B.	*T. resupinatum* (Shaftal)	16
6	SH 48	
C.	*T. repens* (White clover)	32
7	Palampur Composite	
8	PWC 22	
9	PWC 20	
10	EC 476924	
11	PWC 27	
12	PWC 12	
D.	*T. pratense* (Red clover)	14
13	PRC 3	
14	Britta	
15	Milvus	
16	Aberruby	
17	EC 596080	
18	EC 596029	
19	EC 596087	
20	EC 596041	
E.	*T. ambiguum* (Caucasian clover)	16
21	Monal	
F.	*T. apertum* (Open clover)	16
22	EC 401712	
G.	*T. constantinopolitanum* (Constantinopole clover)	16
23	EC 401713	
H.	*T. lappaceum* (Bur clover)	16
24	EC 528542	
I.	*T. vesiculosum* (Arrowleaf clover)	16
25	Palampur	

plant, internode length (cm), plant height (cm), petiole length (cm), leaflet length (cm), leaflet width (cm), leaf: shape ratio, leaf : stem ratio, green fodder yield/ plant (g) and on the basis of composite sample from 10 plants for dry matter %, dry matter yield/ plant (g), crude protein (CP)%, CP yield/plant (g), neutral detergent fibre (NDF)% and acid detergent fibre (ADF)%. Genetic diversity analysis was done using Mahalanobis D^2 statistic [4] and dendrogram was constructed using Tocher's method.

RAPD analysis

DNA extraction from leaf samples of *Trifolium* was done using CTAB DNA extraction protocol [5]. Polymerase chain reactions were performed in final reaction volume of 25 µl containing 2.0 µl dNTP (0.2 mM each of dATP, dGTP, dCTP and dTTP), 0.2 µl Taq DNA polymerase 5 U/ µl (Fermentas Inc.), 2.5 µl 10X PCR Buffer, 1.5 µl $MgCl_2$, 1.0 µl of primer (10µM), 2.0 µl of DNA sample and 15.8 µl of sterilized distilled water. DNA amplification was carried out on Eppendorf thermocycler with the temperature conditions as initial denaturation at 94°C for 5 minutes followed by 39 cycles of denaturation at 94°C, annealing at 34°C for 1 minute and extension at 72°C for 2 minutes followed by final extension of 10 minutes at 72°C. Amplification products were rapidly cooled at 4°C and separated by agarose gel electrophoresis. Agarose (1.2% w/v) gels were prepared in 1X TAE Buffer, 40 mM Tris-aceate, 1 mM EDTA (pH 8.5). Electrophoresis was carried out at 5 V/cm. 19 µl of PCR product was loaded along with loading dye. The ethidium bromide stained gels were observed under UV transilluminator and photograph of gel was taken using gel documentation system (Biovis).

Sixty randomly chosen RAPD primers marker profiles were generated by manually scoring presence or absence of RAPD bands. A binary data matrix with '1' indicating the presence of band and '0' indicating absence of band was generated separately for each primer. The binary data was used to generate a similarity matrix using Jaccard's coefficient, Jij = Cij / (ni + nj - Cij), where, Cij is the number of positive matches between two genotypes, while ni and nj is the total number of bands in the genotype i and j, respectively, using SIMQUAL programme of NTSYS-PC package [6]. The data was subsequently used to construct a dendrogram using the UPGMA alogorithm in SHAN programme of NTSYS-PC package.

Results and discussion

Analysis of variance revealed the presence of significant variability among different genotypes for all the agronomic and quality traits studied. The genotypes, *T. resupinatum cv.* SH 48 (shaftal), *T. alexandrinum cv.* Wardan, Bundel berseem 2, Bundel berseem 3, saidi and fahli, *T. pratense cv*. PRC 3 (red clover), *T. constantinopolitanum cv.* EC 401713 (constantinopole

clover) and *T. vesiculosum cv*. Palampur (arrowleaf clover) were identified as superior genotypes for fodder traits. Whereas, PWC 22, PWC 20, EC 476924, PWC 27 and PWC 12 of *T. repens* and Britta, EC 596080, EC 596087 and EC 596029 of *T. pratense* were found superior for quality traits (Table 2). Variation for agronomic and quality traits has also been reported by earlier workers [7, 8].

Genetic diversity

Morphological analysis: On the basis of D^2 values for all possible pairs, cluster analysis of 25 genotypes of genus *Trifolium* was done and dendrogram was made using Tocher method. The cluster analysis revealed that the genotypes were grouped into four clusters (Fig. 1). Cluster I consisted of 16 genotypes, which include five cultivars of *T. alexandrinum viz.,* Wardan, Bundel berseem 2, Bundel berseem 3, Saidi and Fahli; six genotypes of *T. pratense viz.,* PRC 3, Milvus, EC 596080, EC 596029, EC 596087 and EC 596041; *T. ambiguum cv.* Monal; *T. apertum cv*. EC 401712; *T. constantinopolitanum cv*. EC 401713; *T. lappaceum cv.* EC 528542 and *T. vesiculosum cv*. Palampur. Cluster II had six genotypes belonging to *T. repens viz.*, Palampur Composite, PWC 22, PWC 20, PWC 27, EC 476924 and PWC 12. Cluster III comprised of only two exotic cultivars of *T. pratense viz*., Aberruby and Britta. Cluster IV had only one genotype of *T. resupinatum* i.e. SH 48. Maximum intra-cluster divergence was found between cluster 1 (15.53) (Table 3) and the genotypes belonging to this cluster can be suitably used in interspecific hybridization programmes . Although most of the diversity studies have been done among cultivars of same species, but different species were grouped into different clusters on the basis of genetic diversity among them. [9-11]

Molecular analysis

Thirty six primers generated a total of 547 bands, of which, 543 (99.26 %) were polymorphic (Table 4, Fig. 3, 4). On an average 15 bands were generated per primer. The genetic similarity coefficient among genotypes ranged from 0.28 to 0.81. The average similarity coefficient was 0.54. RAPD profile grouped the genotypes into three main clusters A, B and C (Fig. 2). The clusters were further divided into sub-clusters.

Table 2. Promising species and accessions identified on the basis of mean values for fodder yield and quality traits

Species		Characters
Berseem	Wardan	GFY/plant, DMY/plant, CPY/plant, NDF, ADF, DM %, CP %
	Bundel berseem 2	GFY/plant, DMY /plant, CPY/plant, DM %, CP %
	Bundel berseem 3	GFY/plant, DMY /plant, CPY/plant, DM %, CP %
	Saidi	GFY/plant, DMY /plant, CPY/plant, ADF, DM %, CP %
	Fahli	GFY/plant, DMY /plant, CPY/plant, ADF, DM %, CP %
Shaftal	SH 48	GFY/plant, DMY/plant, CPY/plant, ADF, DM %, CP %
White clover	Palampur composite	NDF, ADF, CP %, DM %
	PWC 22	NDF, ADF, CP%, DM %
	PWC 20	DM %, CP %
	PWC 27	NDF , CP %
Red clover	PRC3	Leaf: stem ratio, GFY/plant, CPY/plant, NDF, ADF, CP %
	Britta	ADF, CP %
	EC 596080	DM %, CP %
	EC 596029	NDF, ADF, CP %
	EC 596087	CP %
Caucasian clover	Monal	Leaf: stem ratio, NDF, CP %
Open clover	EC 401712	GFY/plant, DMY/plant,
Constantinopole clover	EC 401713	GFY/plant, DMY/plant, CPY/plant, DM %
Arrowleaf clover	Palampur	Leaf: stem ratio, GFY/plant, DMY/plant, CPY/plant

*GFY - green fodder yield, DMY – dry matter yield, CPY – crude protein yield, NDF – neutral detergent fibre , ADF - acid detergent fibre, DM – dry matter, CP – crude protein

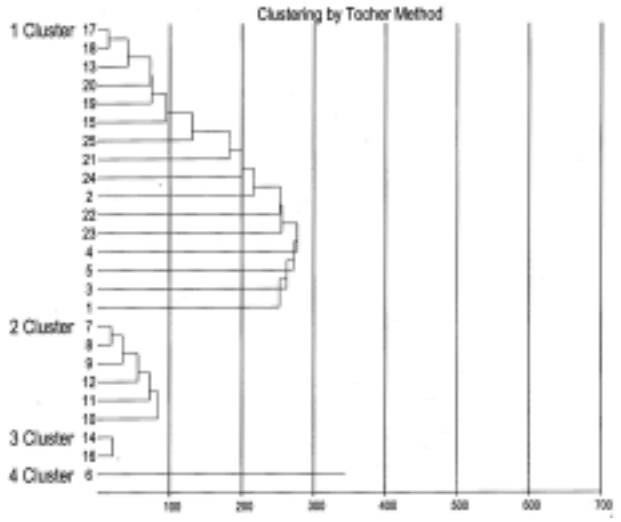

Fig. 1. Dendrogram showing grouping of 25 *Trifolium* species generated using D^2 cluster analysis as per Tocher method

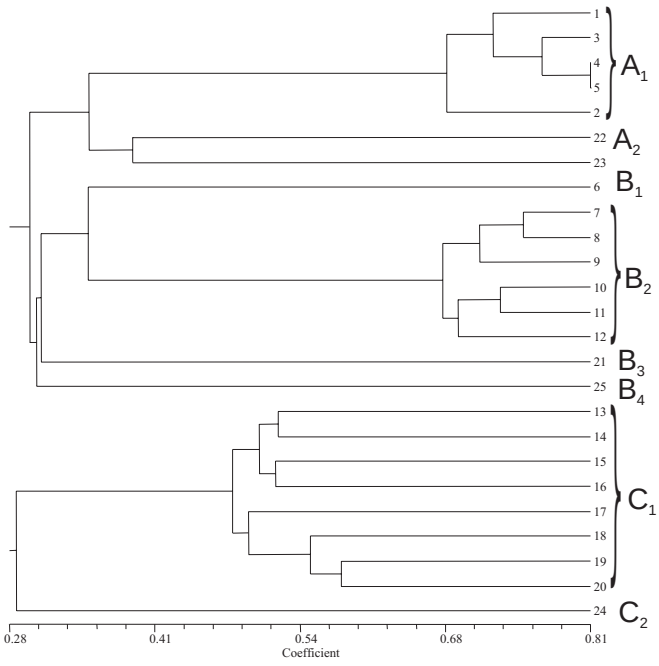

Fig. 2. Dendrogram of 25 genotypes belonging to genus *Trifolium* generated by RAPD data using the UPGMA method

The five cultivars of *T. alexandrinum viz.,* Wardan, Bundel berseem 2, Bundel berseem 3, Saidi and Fahli were grouped into A_1 whereas, *T. apertum cv.* EC 401712 and *T. constantinopolitanum cv.* EC 401713 in A_2 showing genetic similarity of 35 percent. Cluster B grouped 9 genotypes belonging to four species into four sub clusters, having similarity coefficient 30.6 per cent. *T. resupinatum cv.* SH 48, *T. ambiguum cv.* Monal and *T.*

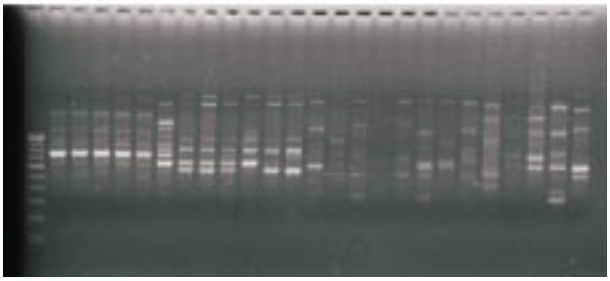

Fig. 3. RAPD profile of *Trifolium genotypes* using primer OPL 12, lane M 500 bp DNA ladder

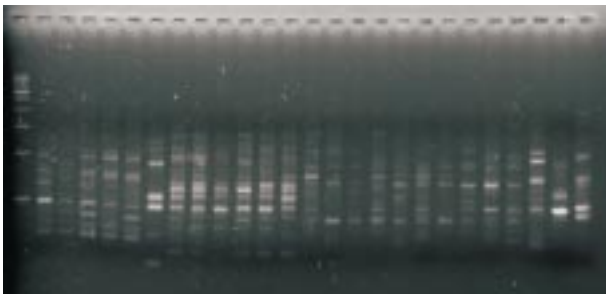

Fig. 4. RAPD profile of *Trifolium genotypes* using primer OPZ 20, lane M 500 bp DNA ladder

Table 3. Average intra- and inter-cluster distance on the basis of D^2 cluster analysis

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	**15.53**			
Cluster 2	21.76	**8.99**		
Cluster 3	22.51	33.70	**4.62**	
Cluster 4	23.48	34.28	28.52	**0.00**

Values in bold are intra-cluster distances

vesiculosum cv. Palampur were placed in sub-clusters B_1, B_3 and B_4 respectively. Whereas, sub-cluster B_2 comprised of all six genotypes of white clover *viz., T. repens cv.* Palampur Composite, PWC 22, PWC 20, PWC 12, PWC 27 and EC 476924. The third cluster included *T. pratense* genotypes *viz.* PRC 3, Britta, Milvus, Aberruby, EC 596080, EC 596029, EC 596087 and EC 596041 in sub cluster C_1 and *T. lappaceum cv.* EC 528542 in sub-cluster C_2 with similarity of 28.5 per cent. Similarly, RAPD markers were used for taxonomic studies in different species of *Trifolium* by other workers [12, 13].

Comparison of both RAPD and D^2 analysis helped in removing the obscure effect of environment playing role at phenotypic level as, the species placed in one cluster on the basis of D^2-statistic *viz., T. pratense*, *T. ambiguum*, *T. vesiculosum* and *T. lappaceum* were

Table 4. RAPD amplicons and polymorphism detected by 36 decamer primers among 25 genotypes of *Trifolium*

S. No.	Primer	Sequence	*N_b	*N_p	*N_m	% Polymorphic bands
1	OPA 04	5'-GGA AGT CGC C-3'	17	17	0	100.00
2	OPA 13	5'-CAG CAC CCA C-3'	11	11	0	100.00
3	OPAK 15	5'-ACC TGC CGT T-3'	14	14	0	100.00
4	OPAL 12	5'-CCC AGG CTA C-3'	18	18	0	100.00
5	OPAP 13	5'-TGA GAA GCG G-3'	12	12	0	100.00
6	OPAQ 04	5'-GAC GGC TAT C-3'	12	12	0	100.00
7	OPAR 19	5'-AAC CCT TCC C-3'	11	11	0	100.00
8	OPAS 16	5'-GTG CCG CAC T-3'	10	10	0	100.00
9	OPC 16	5'-CAC ACT CCA G-3'	12	12	0	100.00
10	OPE 20	5'-AAC GGT GAC C-3'	18	18	0	100.00
11	OPG 03	5'-GAG CCC TCC A-3'	15	15	0	100.00
12	OPG 06	5'-GTG CCT AAC C-3'	17	17	0	100.00
13	OPG 13	5'-CTC TCC GCC A-3'	18	18	0	100.00
14	OPG 17	5'-ACG ACC GAC A-3'	15	15	0	100.00
15	OPG 18	5'-GGC TCA TGT G-3'	16	16	0	100.00
16	OPH 12	5'-ACG CGC ATG T-3'	18	18	0	100.00
17	OPH 13	5'-GAC GCC ACA C-3'	17	16	1	94.11
18	OPH 15	5'-AAT GGC GCA G-3'	14	14	0	100.00
19	OPH 18	5'-GAA TCG GCC A-3'	14	14	0	100.00
20	OPI 20	5'-AAA GTG CGG G-3'	18	16	2	88.88
21	OPK 19	5'-CAC AGG CGG A-3'	17	17	0	100.00
22	OPL 12	5'-GGG CGG TAC T-3'	17	17	0	100.00
23	OPL 19	5'-GAG TGG TGA C-3'	11	11	0	100.00
24	OPL 20	5'-TGG TGG ACC A-3'	18	18	0	100.00
25	OPM 03	5'-GGG GGA TGA G-3'	20	20	0	100.00
26	OPM 07	5'-CCG TGA CTC A-3'	16	15	1	93.75
27	OPM 09	5'-GTC TTG CGG A-3'	14	14	0	100.00
28	OPN 07	5'-CAG CCC AGA G-3'	17	17	0	100.00
29	OPN 14	5'-TCG TGC GGG T-3'	11	11	0	100.00
30	OPN 18	5'-GGT GAG GTC A-3'	11	11	0	100.00
31	OPP 14	5'-CCA GCC GAA C-3'	17	17	0	100.00
32	OPR 13	5'-GGA CGA CAA G-3'	14	14	0	100.00
33	OPW 02	5'-ACC CCG CCA A-3'	16	16	0	100.00
34	OPZ 04	5'-AGG CTG TGC T-3	15	15	0	100.00
35	OPZ 12	5'-TCA ACG GGA C-3'	18	18	0	100.00
36	OPZ 20	5'-ACT TTG GCG G-3'	18	18	0	100.00
Total			547	543	4	99.26
Mean			15.19	15.08	0.11	

*Nb: No. of bands, Np: No. of polymorphic bands; Nm: No. of monomorphic bands.

grouped into different clusters and sub-clusters revealing sufficient diversity among them. Genetic diversity revealed by phenotypic data placed red clover genotypes in different clusters, but at molecular level were grouped together, however genetic differences were revealed by phenotypic and molecular analysis. On the basis of phenotypic results, *T. resupinatum* and *T. repens* exhibited maximum divergence but RAPD analysis placed them in single cluster. Conclusively, *T. alexandrinum* and *T. repens* genotypes had high genetic similarity i.e. 68 per cent and 67.5 per cent respectively. *T. pratense* genotypes were not as divergent as shown by D^2 analysis. *T. apertum* cv. EC 401712 and T. constantinopolitanum cv. EC 401713 exhibited affinity towards *T. alexandrinum* thereby can be used for genetic upgradation of the later in respect of enhanced green fodder yield.

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