Range Mgmt. & Agroforestry 40 (2) : 215-226, 2019
ISSN 0971-2070

Genetic diversity and structural variation among tall fescue (*Festuca arundinacea*) grass genotypes using morphological and molecular markers

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Received: 18th July, 2018 Accepted: 16th August, 2019

Abstract
Considering the potential forage value of tall fescue grass in Indian Himalayan region, the present research was aimed to study the genetic diversity of 36 tall fescue genotypes using morphological and molecular markers. Analysis of variance revealed significant difference among the genotypes. PCA divided the tall fescue genotypes into three distinct groups, explained 76.43% of the total variation. Remarkably both RAPD and SSR revealed high polymorphism, while it was higher for EST-SSR (89.78%) than RAPD (85.13%). Polymorphism information content (PIC) for RAPDs ranged from 0.37 (OPE 20) to 0.50 (OPB 6 and OPD 14); while for EST-SSRs, PIC values ranged from 0.32 (NFA113) to 0.50 (NFA001, NFA036, NFA045). In neighbour-joining tree using RAPD and SSR, 4 and 5 clusters were clearly distinct, indicating the genetic differentiation among the tall fescue genotypes. Use of morphological and molecular markers for the characterization of Himalayan tall fescue grass revealed that these markers may be used for the identification and classification of 36 diverse genotypes and may also be considered for their wider application as it will avoid repetition of genetically similar genotypes in hybridisation breeding programme.

Keywords: Himalayan region, Morphological traits, RAPD, SSR, Tall fescue

Introduction

Tall fescue grass (*Festuca arundinacea*) is a perennial, cool-season, high yielding and drought tolerant bunch grass which is being grown throughout the temperate regions of the world (Sleper, 1985). Majority of commercial tall fescue grass cultivars are allohexaploid (2n = 6x = 42) with the genomic constitution of $PPG_1G_1G_2G_2$ (Sleper and West, 1996). It is predominantly cross-pollinated species with a high level of self-incompatibility and thus highly heterozygous. In India, it is cultivated in Himalayan states like Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh where temperate/alpine pastures are abundant. It is valued for its ability to tolerate hot, dry conditions as well as frost conditions, pasture pest attack and to produce leafy green herbage over the summer. Hence, it is preferred over other winter season grasses for its better-stockpiling properties which involves fertilisation of pastures in late summer or early autumn with nitrogen, thus allowing them to grow, and then grazing them during late fall and winter (Matches, 1979).

For successful crop improvement, characterization of germplasm is necessary to know the value of germplasm, so that it can be effectively utilized in crop improvement. The genetic improvement of tall fescue grass can be potentially achieved through the use of non-domesticated germplasm to diversify breeding populations by the incorporation of the novel and superior allele content. Further, information on the magnitude of genetic diversity helps us to identify superior genotypes for hybridization (Humphreys *et al*., 2005).

Morphological traits have been critical for diversity analysis over environments. However, due to phenotypic plasticity in relation to environment accuracy on diversity analysis may not equivalent to the actual. (Mondini *et al.,* 2009). Thus molecular markers have been successfully used to assess the genetic diversity in different crops (Belaj *et al.,* 2007), which are not influenced by environmental variations. Among molecular marker, random amplified polymorphic (RAPD) marker is a simplest, dominant and rapid marker to elucidate the extent of genetic diversity present in the germplasm (Williams *et al.,* 1990). Whereas simple sequence repeats (SSRs) have become the marker of choice for the study of genetic diversity, genetic mapping and genotype fingerprinting due to their high reproducibility, codominance, polymorphism and transferability among related species and genera (Mahalanobis, 1936; Matches

1979). SSRs derived from Expressed Sequence Tags (EST), have a higher rate of transferability across species than its genomic counterpart and is thus well suited for application in cross-species studies. Further, EST-SSRs analyse polymorphism that is associated with the coding regions of the genome, therefore capable of detecting the expressible traits based genetic diversity available inside (Eujayl *et al.,* 2002; Theil *et al.,* 2003).

Considering the potential forage value of tall fescue grass in Indian Himalayan regions, the present study was undertaken to know the extent of genetic variation available in experimental materials, correlation among the mophological traits and diversity of 36 genotypes of tall fescue grass using morphological and molecular markers (RAPD and SSR). This information will be helpful for selection and utilisation of diverse genotypes to enhance variability and productivity of tall fescue grass through breeding.

Materials and Methods

Plant material and experimental layout: The plant material used in the present study was a collection of 36 tall fescue grass genotypes of diverse origin (Table 1). For the morphological study, the experiment was laid out in a randomized complete block design with three replications. The plot size was kept at 3 m×0.3 m with a row to row and plant to plant spacing of 30 cm. Each genotype was raised in one row of 3m length, and each hill was planted with three root slips of the genotype. Recommended package of practices was followed for raising the crop.

Morphological evaluation: The data were recorded on 11 agro-morphological traits for two cuts (2014-2015). These include green forage yield per plant (g), dry matter yield per plant (g), Plant height (cm), Tillers per plant, leaf stem ratio, leaf length (cm), leaf width (cm), number of leaves per plant, stem thickness (cm), crude protein content (%) and crude protein yield per plant (g). Traits were measured as per standard protocol on five randomly selected plants from each genotype and the mean from each genotype was used for statistical analysis. For the analysis of morphological data, mean values of 2-cuts observations were taken. The data were subjected to descriptive statistics, ANOVA, principal component analysis (PCA) and cluster analysis. The statistical analysis was performed using the software PROC GLM SAS (Prasad *et al*., 2007) and StatistiXL version 1.10. The genetic divergence of tall fescue genotypes was estimated using Mahalanobis D^2-statistics (Mahalanobis, 1936). The morphological data used in this study was based on the mean values recorded for 2 cuts (2014-2015).

Molecular analysis: For molecular analysis, genomic DNA was isolated from young leaf tissue (0.5–1 g) of 36 different genotypes of tall fescue using the CTAB method (Murray and Thompson, 1980). The leaf tissue was rinsed in deionised water, dried on tissue paper discs and ground to a fine powder in liquid nitrogen in autoclaved pre-cooled pestles and mortars. DNA quality was evaluated by electrophoresis on 0.8% (w/v) agarose gel and concentration was determined spectrometrically (NanoDrop Spectrophotometer).

SSR and RAPD genotyping: A set of reported 49 EST-SSRs primers (Saha *et al*., 2004) were selected to test the polymorphism in 36 tall fescue genotypes and 23 informative EST-SSRs were chosen for genotyping. Similarly, 25 RAPD primers were used to test polymorphism, in which 12 informative RAPD primers were chosen for genotyping. Polymerase chain reaction was performed in final volume of 10 µl containing 3.8 µl of sterilized distilled water, 2.0 µl template DNA (25 ng/µl), 1.0 µl of RAPD primer (5 µM) and 0.5 µl (forward) and 0.5 µl (reverse) of EST-SSR primer (5 µM), 1.0 µl $MgCl_2$ (25 mM), 1.0 µl 10X PCR buffer, 1.0 µl dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP) and 0.2 µl *Taq polymerase* (2 U/µl). The amplifications were carried out in Eppendorf thermocycler (Eppendorf, Germany) for

Table 1. Details of the plant material used along with source

Source	Genotypes
NBPGR, New Delhi (India)	EC-1942, EC-178188, EC-178185, EC-178184, EC-178181
Selections derived from composite populations of indigenous and exotic collections available at CSKHPKV, Palampur (India)	Sel-6, Sel-8, Sel-11, Sel-47, Sel-48, Sel-49, Sel-50, Sel-61, Sel-63, Sel-66, Sel-67, Sel-68, Sel-69, Sel-70, Sel-71, Sel-84, Sel-85, Sel-86, Sel-87, Sel-88, Sel-89, Sel-90, Sel-91, Sel-92, Sel-93
Hima-1×Hima-4	Hima-15, Hima-3
CSKHPKV, Palampur (India)	Hima-1(check), Hima-4(check), Hima-14 (check), EC-178182 (Palam Fescue-1) (check)

RAPD and SSR as per Saha *et al*. (2004). The gels were stained using ethidium bromide solution and documented in the Biovis Gel Doc system.

Data analysis: From the amplified DNA of 36 genotypes of tall fescue, RAPD and SSR marker profiles were generated and the presence or absence of each RAPD and SSR band of particular molecular weight was scored manually. A binary data matrix with '1' indicating the presence of particular molecular weight and '0' indicating its absence was generated separately for each primer. The binary data was used to generate a similarity matrix using Jaccard's coefficient as under $J_{ij} = C_{ij}/(n_i + n_j - c_{ij})$; where 'Cij' is the number of positive matches between two genotypes, while ni and nj are the total number of bands in genotype i and j respectively, in SIMQUAL programme of NTSYS–PC package (Setauket, New York) [Rohlf, 1993]. Genetic distances (GD) were calculated as under: $GD = 1 - [C_{ij}/ (n_i+n_j- C_{ij})]$. The binary data generated was used to calculate a genetic dissimilarity matrix using the Jaccard dissimilarity index (d_{ij}) between pairs of accessions (units).

$$d_{ij} = \frac{(b+c)}{a+(b+c)}$$

where d_{ij} represents the dissimilarity between units i and j.

From the dissimilarity matrix, the Principal coordinate analysis was carried out and the Neighbor-Joining tree (UnWeighted neighbour-joining) was computed using the DARwin software version 5.0 (Perrier and Jacquemoud-Collet, 2006). Branch robustness was tested using 1000 bootstraps. The polymorphism information content (PIC) of each primer pair was calculated according to the formula given by Botstein *et al*. (1980) and implemented in Cervus version 3.0. Various genetic diversity estimates such as expected heterozygosity (He), observed heterozygosity (Ho), Shannon information index (I), etc were calculated with the help of POPGENE version 1.32 (Yeh and Boyle, 1997). Software program STRUCTURE version 2.3.4 (Pritchard *et al*., 2000) was used to infer the genetic structure. The maximal value of LnP (D), the posterior probability of data as per Evanno *et al*. (2005), was obtained using STRUCTURE HARVESTER (Earl and von Holdt, 2011). An analysis of molecular variance (AMOVA) was performed using GenAlex 6.4 program (Peakall and Smouse, 2006).

Results and Discussion

Morphological characterization: The gene pools, the reservoir of genetic diversity are often exploited to study variability and identification for traits of importance for its possible utilization in breeding programs. The summary statistics including mean, standard error, range and coefficient of variation (CV) for 11 agro-morphological traits, were recorded (Table 2).

A large variation was observed for all the 11 morphological traits. The frequency distribution of 11 quantitative traits in tall fescue genotypes were also recorded (Fig 1). The mean value for green forage yield/plant (g) ranged from 61.13 (Sel-66) to 105.62 (Hima-3). The dry matter yield (g) ranged from 13.55 for EC 1942 to 19.94 for Hima-3. Value of plant height (cm) was highest (49.22) for Sel-48 and as low as 30 cm for Sel-66. The average tillers/plant was 38.72 and ranged from 33.08 for Sel-66 to 49.16 for Sel-85. Stem thickness was maximum (0.49) for sel-68 and minimum (0.35) for Sel-48 with an average value of 0.42. The average leaves per plant were 92.54 varying from 76.84 to 117.59 for Sel-67 and Sel-85, respectively. Leaf stem ratio was maximum (1.69) for Hima-3 and

Table 2. Descriptive statistics detected on all genotypes for all the agro-morphological traits

Traits	Mean± S.E.	Range	CV	Mean squares (genotypes)
Green forage yield per plant (g)	83.40±5.12	61.13-105.62	10.79	291.05**
Dry matter yield per plant (g)	16.00±0.72	13.55-19.94	7.91	8.77**
Plant height (cm)	40.42±2.37	30.00-49.22	10.31	52.65**
Tillers per plant	38.72±2.28	33.08-49.16	10.34	48.28**
Stem thickness (cm)	0.42±2.28	0.35-0.49	6.61	0.004**
Leaves per plant	92.54±5.57	76.84-117.59	10.58	310.82**
Leaf stem ratio	1.37±0.07	1.09-1.69	9.71	0.06**
Leaf length (cm)	33.60±2.22	25.58-41.28	11.61	33.84**
Leaf width (cm)	0.87±0.02	0.77-0.94	4.42	0.003*
Crude protein content (%)	9.41±0.49	7.59-11.37	9.10	2.74**
Crude protein yield per plant (g)	1.54±0.13	1.06-2.30	14.72	0.27**

minimum (1.09) for EC-178185. Genotype Sel-49 had 41.28 cm long leaf as compared to Sel-66, which had 25.58 cm long leaf. The leaf width ranged from 0.77 for Sel-71 to 0.94 for Sel-11. The crude protein content was highest in Sel-6 (11.37) and lowest in Sel-8 (7.59). The average crude protein yield/plant were 1.54 and ranged from 1.06 for Sel-11 to 2.30 for Hima-3. Presence of variation in the studied material was also evident from the coefficient of variation.

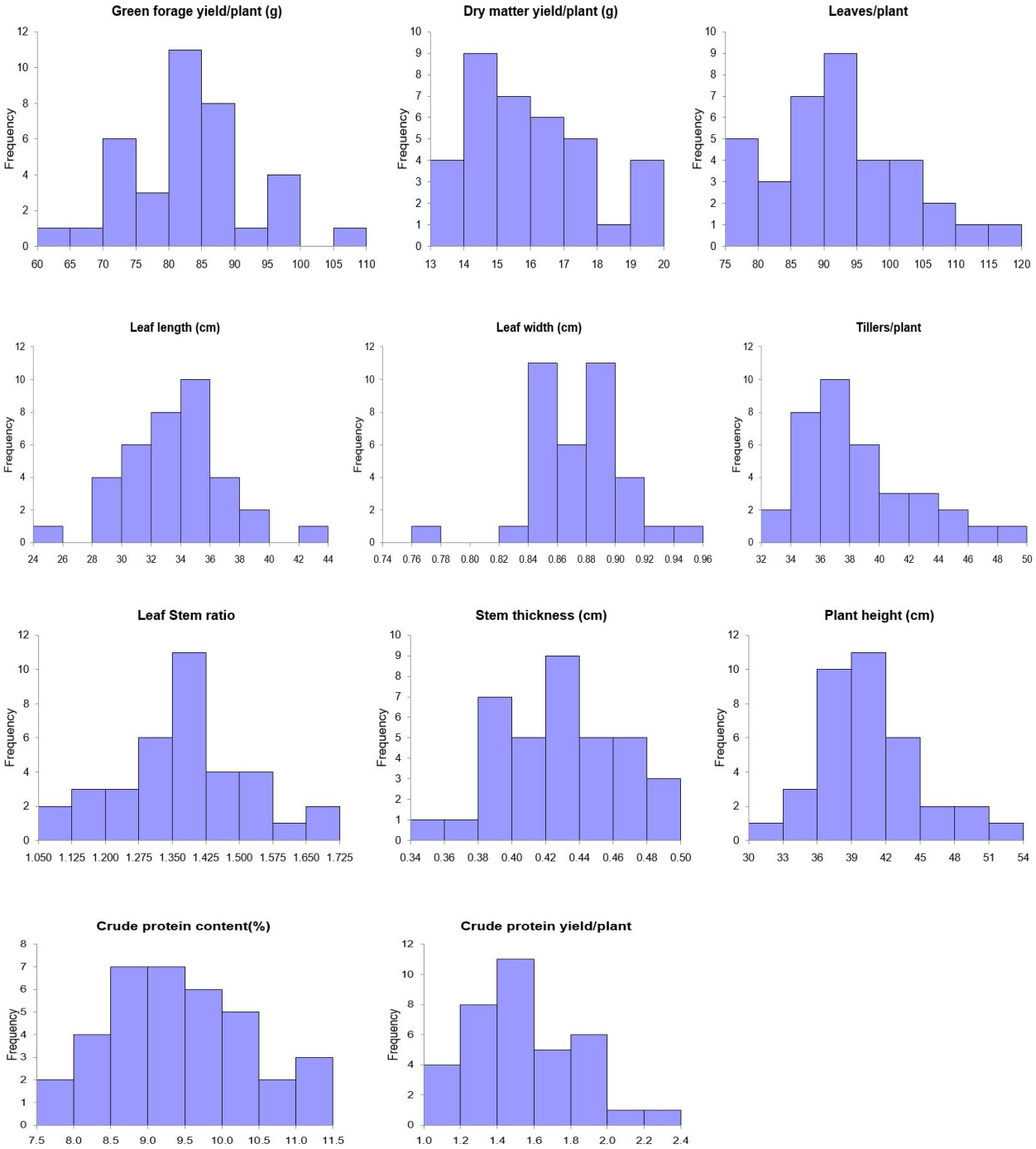

Fig 1. Frequency distribution of 11 aquantitative measured traits in 36 Tall fescue genotypes

Table 3. Correlation coefficients among quantitatively measured traits in 36 tall fescue genotypes

Traits		Dry matter yield per plant	Plant height	Tillers per plant	Stem thickness	Leaves per plant	Leaf stem ratio	Leaf length	Leaf width	Crude protein content	Crude protein yield per plant
Green forage yield per plant	P	0.86**	0.59**	0.50**	-0.16	0.50**	0.19*	0.61**	0.02	0.41**	0.72**
	G	0.99**	0.62**	0.47**	-0.23*	0.47**	0.77**	0.66**	0.14	0.64**	0.98**
Dry matter yield per plant	P		0.53**	0.40**	-0.16	0.49**	0.21*	0.51**	0.03	0.37**	0.80**
	G		0.62**	0.48**	-0.23*	0.50**	0.61**	0.63**	0.13	0.52**	0.90**
Plant height	P			0.03	-0.24*	0.04	0.21*	0.90**	-0.07	0.14	0.37**
	G			-0.22*	-0.38**	-0.30**	0.66**	0.98**	-0.24*	0.16	0.44**
Tillers per plant	P				-0.20*	0.90**	0.06	0.10	-0.01	0.30**	0.43**
	G				-0.38**	0.99**	0.43**	-0.21*	0.22*	0.02**	0.50**
Stem thickness	P					-0.16	-0.11	-0.17	0.32**	-0.01	-0.09
	G					-0.29**	-0.35**	-0.24*	0.74**	0.04	-0.15
Leaves per plant	P						0.09	0.11	-0.02	0.25**	0.42**
	G						0.50**	-0.35**	0.10	0.37**	0.56**
Leaf stem ratio	P							0.13	-0.01	0.23*	0.27**
	G							0.65**	-0.27*	0.42**	0.57**
Leaf length	P								0.05	0.22*	0.39**
	G								-0.01	0.40**	0.58**
Leaf width	P									-0.02	-0.02
	G									0.02	0.08
Crude protein content	P										0.80*
	G										0.83**

*Significant at P<0.05

**Significant at P<0.01

Persual of the analysis of variance (ANOVA) revealed significant differences for all the traits studied (Table 2) which suggests the prevalence of a high genetic variability among the genotypes. Presence of variation in the tall fescue genotypes infers a greater scope for possible exploitation and selection of genotypes in commercial cultivars.

***Association, cluster and PCA studies*:** The inter-character relationship is essential for indirect selection of the characters that are not easily measured and for those that exhibit low heritability. In the present study, most of the characters were statistically significant at both 1% and 5% level (Table 3). Phenotypic and genotypic correlation coefficients estimate revealed that green forage yield per plant found significantly and positively correlated with dry matter yield per plant, plant height, tillers per plant, leaves per plant, leaf stem ratio, leaf length, crude protein content and crude protein yield per plant, whereas negative and significant association with stem thickness was observed at genotypic level. This indicates that selection for these traits would be effective to increase the green forage yield per plant as reported by Sharma *et al.* (2018).

Sleper *et al*. (1977) reported that tiller per plant was significantly correlated with the total forage yield per plant. Ramakrishnan *et al.* (2013) noted that green fodder yield per plant was significantly positively correlated with tillers per plant, leaves per plant and dry matter content, while positively correlated with leaf stem ratio and crude protein in *Panicum maximum.* Dendrogram constructed based on morphological traits using squared Euclidean distance and group average clustering method showed two clear groups along with one outlier. All the checks along with exotic genotypes were evenly distributed among both the clusters indicating huge diversity among themselves. Further, all the selections fall under both the clusters (cluster-I and II) indicating the presence of two different gene pools (Fig 2). However, morphological markers are limited in number so we could not rely on them solely and thus needs further validation of this grouping using molecular markers.

Principal component analysis (PCA) was performed to identify the most significant traits which contributed towards variation. Principal components were considered significant for eigenvalues greater than or equal to 1.0. As a result, a total of 76.43 per cent variation was explained by the first three principal components. The first principal component (PC1) was the most important and revealed 43.03 per cent of the total variance which was mainly contributed by green forage yield per plant, dry matter yield per plant and crude protein yield per plant (Table 4). PC2 accounted for 19.29 per cent variation and was attributed mainly due to leaves per plant and tillers per plant. PC3 contributed 14.11 per cent of the total variance through leaf width and stem thickness. The loading plot was drawn for PC1 and PC2 also indicated the importance of different morphological traits and genotypes for explaining the variance among accessions and understanding species relationship (Fig 3).

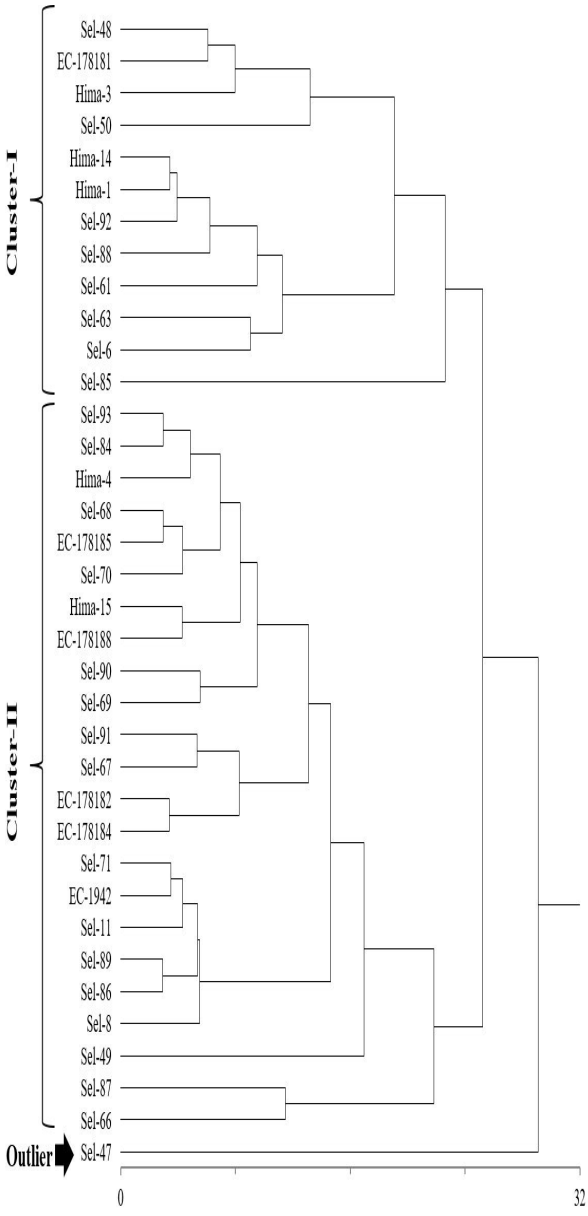

Fig 2. Dendrogram based on morphological traits constructed using squared Euclidean distance and group average clustering method

Table 4. Eigenvectors for the first three components of different traits in 36 genotypes of tall fescue grass

Variable	Eigen vectors		
	PC1	PC2	PC3
Eigen value	4.73	2.12	1.55
Variation (%)	43.03	19.29	14.11
Cumulative (%)	43.03	62.32	76.43
Green forage yield per plant (g)	0.94	-0.02	0.11
Dry matter yield per plant (g)	0.91	-0.03	0.11
Leaves per plant	0.53	0.78	-0.12
Leaf length (cm)	0.67	-0.63	0.06
Leaf width (cm)	0.04	0.08	0.80
Tillers per plant	0.54	0.75	-0.14
Leaf stem ratio	0.58	-0.10	-0.21
Stem thickness (cm)	-0.28	0.03	0.85
Plant height (cm)	0.58	-0.72	-0.13
Crude protein content (%)	0.65	0.10	0.22
Crude protein yield per plant	0.91	0.08	0.17

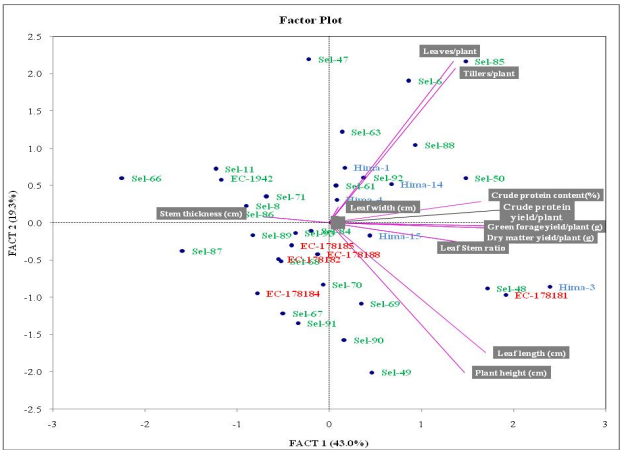

Fig 3. Biplot of different variables and genotypes on PC1 and PC2

The traits that were main contributors to account for a variance may be utilised to broaden the genetic base by bringing an additional source of variability for successful improvement of tall fescue grass. The scattered diagram showed that maximum genotypes were unique as they fall in different corners of biplot. None of the variable fall in the left corner of the lower half of the biplot while many genotypes fall in this region which indicates that some variables were there which may involve in the variance of these genotypes but were not taken into the study.

Molecular characterisation: In the present investigation, two types of molecular marker (RAPD and SSR) were utilized and compared to assess the genetic diversity of 36 genotypes of tall fescue. Details of polymorphic primers, the total number of bands, total number of the polymorphic band, percentage polymorphism and polymorphic information content (PIC), revealed by each of the RAPD and EST-SSR primers are also recorded (Table 5-6).

Table 5. Number of scorable and polymorphic RAPD bands obtained in the PCR amplified DNA of tall fescue grass genotypes generated by 12 primers

Primers	Nf	Np	Polym-orphic bands (%)	Polymorphism information content
OPA 18	5	4	80.00	0.47
OPB 6	4	3	75.00	0.50
OPB 15	5	5	100.00	0.46
OPB 17	6	5	83.33	0.47
OPC 16	6	5	83.33	0.43
OPC 20	6	5	83.33	0.49
OPD 14	6	4	66.66	0.50
OPZ 17	8	8	100.00	0.48
OPA 19	7	7	100.00	0.46
OPG 05	9	9	100.00	0.46
OPG 01	10	10	100.00	0.43
OPE 20	4	2	50.00	0.37
Total	**77.00**	**68.00**	**1021.22**	**5.52**
Mean	**6.42**	**5.67**	**85.13**	**0.46**

N_f: Number of fragments; N_p: Nnumber of polymorphic fragments

A total of 25 RAPD primers were screened for the PCR amplification, 13 primers did not produce amplified polymorphic products, and thus RAPD analysis of 36 genotypes of tall fescue was done using 12 RAPD polymorphic primers. A total of 77 alleles were amplified with 12 selected RAPD polymorphic primers, of which 68 (85.13%) were polymorphic (Fig 4) with an average of 5.67 polymorphic alleles per primer ranging between 2 (OPE-20) to 10 (OPG-01). Five RAPD primers showed 100% polymorphism, whereas OPE-20 (50%) was least polymorphic. The high level of polymorphism might be attributed to the wide geographical distributions of the included accessions.

Initially, 49 microsatellite regions were evaluated in this study. However, only 23 polymorphic EST-SSRs were selected for further analysis. A total of 70 alleles were amplified with 23 selected EST-SSR polymorphic primers, of which 70 (89.74%) were polymorphic (Fig 5) with an average of 3.04 polymorphic alleles per primer. The number of polymorphic alleles per locus ranged from 1 to 5, with an average number of polymorphic alleles 3.04.

Table 6. Number of scorable and polymorphic EST-SSR bands obtained in the PCR amplified DNA of tall fescue grass genotypes generated by 23 primers

Primers	Nf	Np	Polym-orphic bands (%)	Polymorphism information content
NFA001	3	3	100.00	0.50
NFA007	2	2	100.00	0.47
NFA013	4	4	100.00	0.47
NFA036	4	4	100.00	0.50
NFA045	3	2	66.67	0.50
NFA052	3	3	100.00	0.50
NFA058	3	2	66.67	0.49
NFA064	4	4	100.00	0.50
NFA068	2	2	100.00	0.50
NFA072	3	3	100.00	0.50
NFA091	4	4	100.00	0.44
NFA101	2	2	100.00	0.45
NFA102	5	5	100.00	0.49
NFA113	5	4	80.00	0.32
NFA115	3	2	66.67	0.50
NFA123	4	4	100.00	0.44
NFA127	3	3	100.00	0.39
NFA130	2	1	50.00	0.50
NFA133	4	3	75.00	0.49
NFA142	5	4	80.00	0.49
NFA150	2	2	100.00	0.50
NFA154	5	4	80.00	0.48
NFA157	3	3	100.00	0.49
Total	**78.00**	**70.00**	**2065.00**	**10.85**
Mean	**3.39**	**3.04**	**89.78**	**0.47**

N_f: Number of fragments; N_p: Number of polymorphic fragments

The primer NFA-102 generated maximum number of alleles (5) and showed 100 per cent polymorphism, while NFA130 produced a minimum number of alleles (2) and showed 50 per cent of polymorphism. SSR profile of a representative primer was recorded (Fig 5). The usefulness of markers is described mainly through the percentage of polymorphic fragments. The pattern of polymorphism revealed by these two marker systems was different and both the marker systems showed a high level of polymorphism, thus the more significant potential of these markers for analysis and discrimination of tall fescue genotypes. High levels of polymorphism depicted that the different genotypes under study were genetically diverse. Level of polymorphism was higher for EST-SSR (89.78%) than RAPD (85.13%), thus revealing SSR being more efficient marker system because of its hyper-variable nature.

Polymorphism information content (PIC) values, a parameter associated with the discriminating power of markers, for RAPDs ranged from 0.37 (OPE 20) to 0.50 (OPB 6 and OPD 14) with an average of 0.46 (Table 5); while for EST-SSRs, PIC values ranged from 0.32 (NFA113) to 0.50 (NFA001, NFA036, NFA045, NFA052, NFA064, NFA068, NFA072, NFA115, NFA130 and NFA150) with an average of 0.47 (Table 6). Comparison of PIC values for two marker systems indicated that both the marker systems were equally effective in determining polymorphisms though the magnitude of EST-SSR was slightly higher than RAPD. However, PIC of 0.62-0.84 was observed which was higher than those found in our study

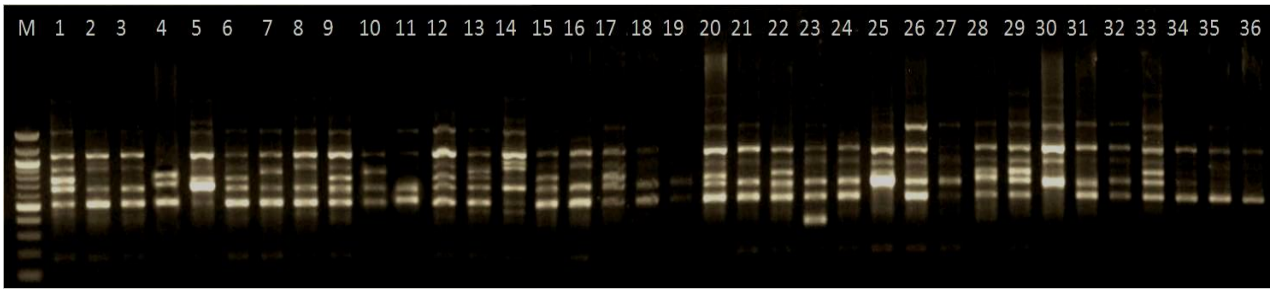

Fig 4. RAPD profile of tall fescue grass genotypes using primer OPZ-17, lane M 100 bp ladder

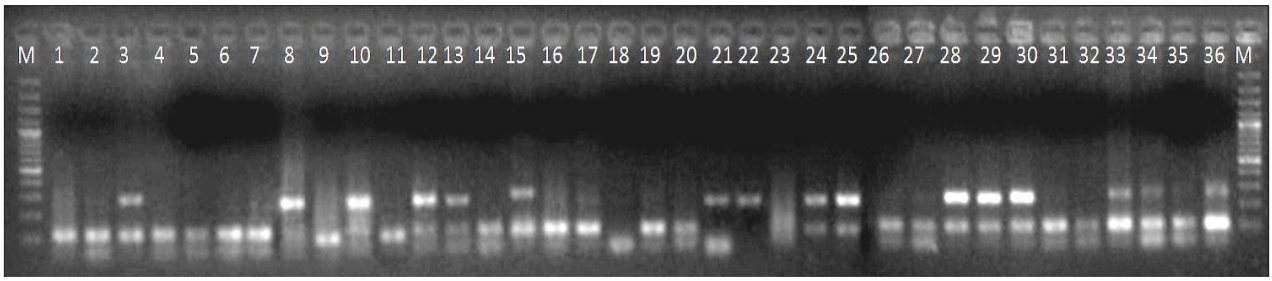

Fig 5. EST-SSR profile of tall fescue grass genotypes using primer EST-SSR 101, lane M 100 bp ladder

carried out in tall fescue grass (Kirigwi *et al*., 2008; Cuyeu *et al.,* 2013).

Cluster analysis: In neighbour-joining tree constructed using RAPD data (Fig 6), four main clusters were distinguished. Cluster-I consisted of 16 genotypes comprised of almost all the exotic lines collected from NBPGR, New Delhi *viz.,* EC-1942, EC-178182 (Palam Fescue-1), EC-178184, EC-178188 and EC-178181 except EC-178185 which fall under Cluster-III and almost all the checks used *viz.*, Hima-4, Hima-14, Hima-1 and Hima-3, whereas cluster II, III and IV comprised of 20 genotypes which consisted of all the selections collected from various regions indicating the presence of enormous diversity among themselves.

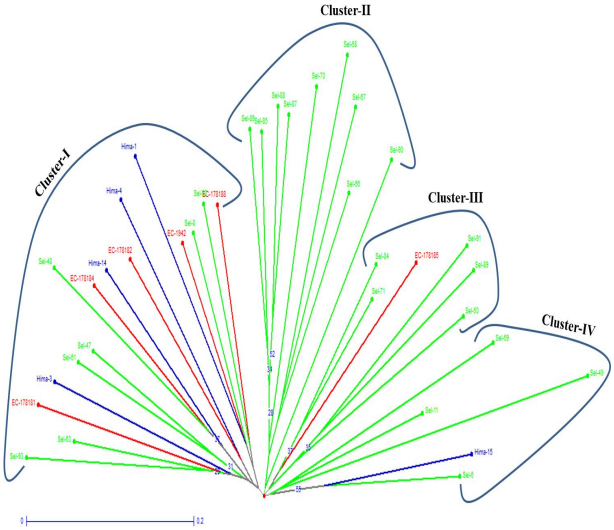

Fig 6. Radial neighbour-joining tree based on 77 alleles from 12 RAPD loci among 36 tall fescue genotypes

Further, the neighbour-joining tree was constructed using SSR data (Fig 7) which illustrated the overall genetic relationships among the 36 genotypes evaluated and clustered the accessions in five distinct clusters. All the exotic lines collected from NBPGR, New Delhi fall in different clusters viz. Cluster-II, IV and V which depicted that there must be some diversity among these exotic genotypes also which was not described by RAPD markers.

Similar was the case with genotypes collected from CSK HPKV, Palampur and selections derived from composite populations of indigenous and exotic collections. Clustering of checks used in almost one cluster indicated that these were closely related to each other, depicting a narrow genetic base between these commercial cultivars. It is thus imperative to exploit the genetic diversity present in wild relatives and landraces for breeding new varieties and broadening the genetic variation in commercial tall fescue cultivars.

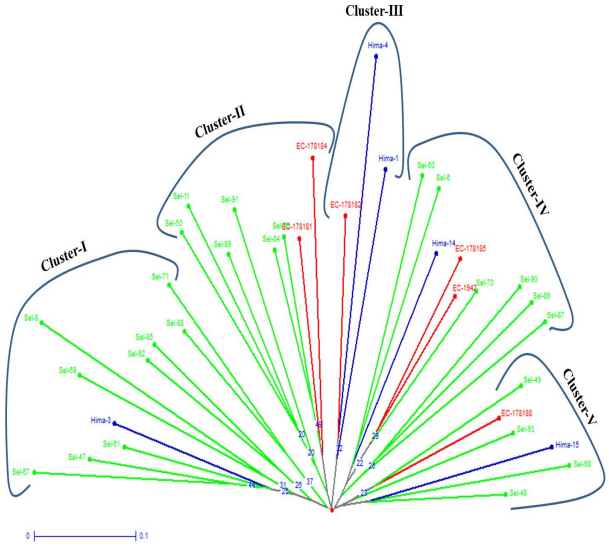

Fig 7. Radial neighbor-joining tree based on 70 alleles from 23 SSR loci among 36 tall fescue genotypes

Based on the above results, clustering differences were evident between morphological and molecular (SSR and RAPD) derived marker systems. Squared Euclidean distance and group average clustering method helped in grouping different genotypes into 2 clusters resulted in an underestimation of diversity, while RAPD and EST-SSR markers grouped them into four and five clusters, respectively. The molecular and morphological differentiation was observed in faba bean (Rebaa *et al*., 2017). Neighbour-joining tree constructed based on RAPD and EST-SSR markers indicated that genetic differentiation exists between indigenous and exotic collections and also depicted the importance and efficiency of both markers in distinguishing the available tall fescue gene pool in Indian Himalayan regions. The discriminating pattern revealed by RAPD data, grouped all exotic genotypes and the genotypes derived from CSKHPKV, Palampur in the same cluster whereas EST-SSR fall them in different clusters which showed that EST-SSR is a more reliable tool than RAPD markers.

Further, exotic cultivars grouped with other selections indicated that they may be closely related to each other but divergent from other species. The higher genetic diversity of landraces or selections which fall in different clusters is favourable for genetic marker development, construction of segregating populations, functional gene

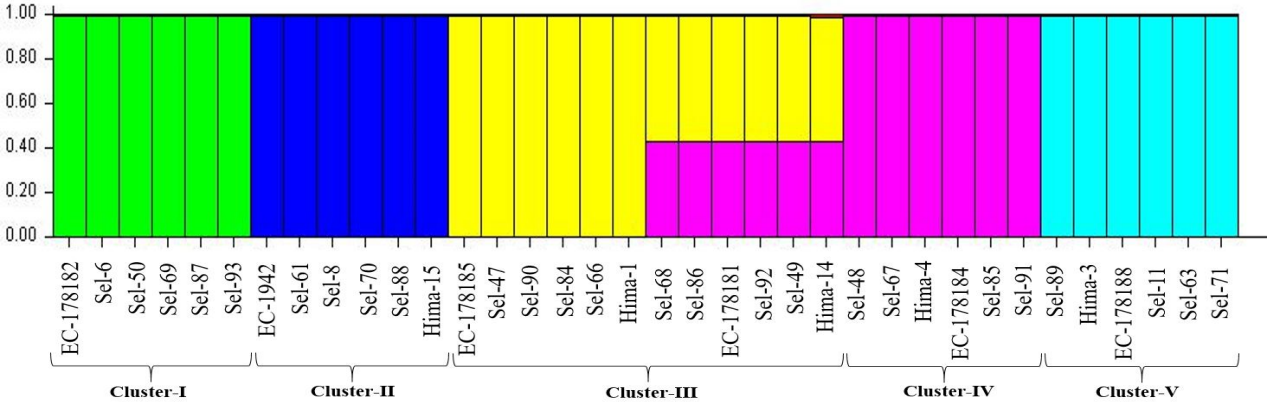

Fig 8. Genetic structure of 36 tall fescue genotypes as inferred by STRUCTURE v2.3.3 with 35 pooled (RAPD and SSR) markers data set

cloning and association mapping. Selections that revealed genetic divergence with commercial cultivars and exotic genotypes might be used in the conventional ongoing polycross breeding programme to enrich the commercial gene pool of tall fescue forage grass and develop novel cultivars with valuable traits. Further, the SSR markers may be used to predict genetic diversity in synthetic progenies, allowing efficient selection of parental genotypes and thus enhancing tall fescue breeding endeavours. The usefulness of SSR markers to predict genetic diversity in synthetic (Syn1) progeny of tall fescue was revealed by Amini *et al*. (2011), and they concluded that genetic diversity detected among parental plants using phenotypic traits or molecular markers is transmitted directly to their synthetic progenies. Similarly, in perennial ryegrass, the selection of genetically diverse parents based on molecular markers was shown to lead to a better agronomic performance in first and second-generation progeny (Kolliker *et al*., 2005).

Bayesian genetic structure: Delta K, which is used to determine the best fit value of K, was computed by STRUCTURE harvester for the given range, *i.e*. 1-10 and the highest value was shown at K=5. Therefore, STRUCTURE analysis was conducted for K=5, which largely clustered accessions based on their type (Fig 8). Exotic lines were distributed in all clusters except Cluster-I indicating the huge level of diversity among them which was also depicted by SSR markers. In addition to assigning individuals to different clusters based on allele frequencies, it also detected the extent of admixture within accessions. These results suggested that the genetic divergence among the genotypes was high, which could be due to the primary differences in the pedigree of different genotypes. These estimates showed that various allelic combinations were present within the population and exchange rates of alleles were very high within-population than among the population.

Conclusion

The use of morphological and molecular markers for the characterisation of Himalayan tall fescue grass revealed molecular markers are more effective and efficient for diversity analysis and may be used for the identification of diverse genotypes that may be the carriers of many new traits. Further, the classification of 36 tall fescue grass genotypes may be of the broader application as it will avoid repetition of genetically similar genotypes in hybridisation breeding programme. Based on D^2 clustering, molecular clustering and mean values maximum diversity was observed for Sel-4, Sel-85, Sel-88, EC-178181, Sel-48 and Hima-3 which indicated that these genotypes can be used as superior parents in polycross breeding programme for the development of synthetics in tall fescue grass.

Acknowledgement

Authors gratefully acknowledge Director Research, CSK HPKV, Palampur for financial aid to carry out this investigation.

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