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Genetic Diversity and Lineage Based on SSR Markers of Two Genomic Resources among *Trifolium* Collections Held within the Australian Pastures Genebank

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Abstract

Trifolium alexandrinum, an important forage legume, suffers from narrow genetic base. The present investigation was envisaged to reveal the inter- and intra-species genetic diversity and lineage among 64 accessions, representing a global collection, of *T. alexandrinum;* it's two probable progenitor species (*T. salmoneum* and *T. subterraneum*) and the three genetically distant species (*T. repens*, *T. vesiculosum*, *T. michelianum*). A set of Simple Sequence Repeats (SSR) primer-pairs developed from *T. alexandrinum* have shown to amplify alleles across the species under study, suggesting utility of the newly developed resource for assessing molecular diversity among *Trifolium* species. These SSRs markers together with previously reported SSRs, derived from *T. repens*, enabled to reveal high intra-species polymorphism in *T. alexandrinum* and successfully discriminate different species investigated in this study. The diverse accessions determined herein provide a superior resource for further breeding of *T. alexandrinum*. High allelic similarity of *T. alexandrinum* with *T. subterraneum* and *T. salmoneum* indicated close relatedness among the species, suggesting polyphyletic evolution of *T. alexandrinum*.

Keywords

Egyptian Clover, Evolution, Genetic Resource, Molecular Diversity

1. Introduction

Genus *Trifolium*, of the family Leguminosae (Fabaceae), comprises more than 237 annual and perennial species [1]; of which 20 are recognized for forage value. *T. alexandrinum* (Egyptian clover or Berseem), one of the most important forage crops, is cultivated across the globe. It is grown in a wide range of environments across Egypt, India, Australia, Pakistan and Middle East countries [2]. It is a popular fodder crop among growers because of its several desirable attributes: high biomass yield (120 t/ha green), protein content (23%), dry matter digestibility (65%) and multi-cut nature (4 to 8 cuts). Besides, it improves soil fertility through symbiotic nitrogen fixation and thus reduces carbon footprint in production and application of nitrogen fertilizers in agricultural landscape. During last 30 years, significant genetic progress has been made in this crop that enabled to the development of improved cultivars. However, in addition to genetic bottleneck during domestication, selective breeding has further narrowed down the genetic base of *T. alexandrinum*, especially in breeding germplasm.

Various methods such as morphological, biochemical and molecular markers have been deployed to assess the extent of genetic diversity in crop plants including Indian collection of the *Trifolium* [3] [4] [5] [6]. These studies have shown a limited genetic and molecular diversity among *T. alexandrinum* accessions, suggesting that novel alleles for useful traits such as resistance to biotic and abiotic stresses are required to make rapid genetic gain in this species.

Germplasm collections held at various genebanks provide diverse source of naturally occurring genetic variation which can be exploited for trait improvement. Australian Pastures and West Australian Gene Banks have a collection of diverse accessions of *T. alexandrinum*, *T. repens* (white clover), *T. subterraneum* (subterranean clover), *T. salmoneum*, *T. vesiculosum* (arrow clover), and *T. michelianum* (balansa clover). So far, these accessions have not been characterized. Therefore, it is important to characterize this set of germplasm so that forage breeders can target accessions of interest and utilize them effectively in their national breeding programs. SSR markers have been extensively used in the genome and genetic analysis of various crops due to their high repeatability, codominant inheritance, abundance and multiallelic nature in plant genomes. However, unlike major food crops, limited genetic and genomic resources exist for improvement of forage crops especially for *T. alexandrinum*. In this study, we 1) isolated and characterized SSR markers in *T. alexandrinum*, and 2) investigated the level of polymorphism and the extent of molecular diversity among 69 accessions of *Trifolium*. In addition, we used existing SSR markers developed from *T. repens* to investigate the molecular diversity in *Trifolium*.

2. Materials and Methods

2.1. Plant Materials

Seeds of 72 accessions of *Trifolium* were procured from the Western Australian and Southern Australian gene banks (part of the Australian Pastures Gene-

bank-APG); however, three of them did not germinate (Table 1). Seed were sown in plastic pots in glass house condition at Wagga Wagga Agricultural Institute, Wagga Wagga, NSW Australia maintained at 18˚C ± 4˚C (Figure 1). In order to validate the genetic identity of accessions, passport information on growth habit, shape of cotyledonary and the first true leaf; and number of leaflets at the seedling stage, was recorded at seedling stage. The plants were cut; leaving 5 cm growth from the base (soil surface) after 45 and 65 days of sowing to observe the regeneration potential.

2.2. DNA Isolation

Fifteen days after germination, 2 to 3 g of young leaf tissue was collected from at least 10 plants from each accession and frozen in liquid nitrogen. DNA was extracted following the phenol-chloroform extraction method [7].

2.3. Isolation of SSR

This work was accomplished at the Indian Grassland and Fodder Research Institute, Jhansi, India. A library enrichment protocol [8] based on the PCR amplification of genomic DNA with 5' anchored degenerate microsatellite primers KKVRVRV(GA)10, KKVRVRV(GGT)5, KKVRVRV(CA)10, KKVRVRV(AAT)6, KKVRVRV(GTG)6, KKVRVRV(GACA)5, and KKVRVRV(CAA)6 (where K = G/T, V = G/C/A, R = G/A), was used to amplify *T. alexandrinum* (cv. Wardan) genomic DNA following [9]. Positive recombinant clones were used for plasmid DNA isolation following [7]. DNA sequencing (ABI 3700) of the clones was done using the Big Dye Terminator reaction kit (Applied Biosystems, USA).

2.4. Primer Design and PCR Amplifications

Primers flanking SSR motifs were designed using the PRIMER 3 software [10] having 18 - 24 nucleotides, Tm = 50˚C - 62˚C with an optimal of 56˚C, 100 - 400 bp PCR product, and an optimal 40% G + C content. Primers were designated as IGFRI-SSR 1 to 15 and details are provided in Table 2. These primer-pairs, in addition to those developed previously from *T. repens* [11] were used for genetic diversity study. SSR primer sequences were synthesized from Sigma-Aldrich (Australia); the forward primers were tailed with M13 sequence (19 bp long) and labeled with fluorescent dyes following [12]. PCR amplifications were performed under the conditions described previously ([11]. SSR polymorphism was analyzed using Beckman Coulter CEQ™ 8000 Genetic Analysis System according to [12]. Fragment analysis was done using three fluorescent labeled dyes *i.e.* D2, D3 and D4 with size standard 400 and SSR alleles scored on CEQ capillary electrophoresis.

2.5. Data Analysis

Fragments amplified with SSR primer-pairs were scored into binary format ("1" for presence and "0" for absence). Genetic similarity, based on allelic data, was

Table 1. *Trifolium* accessions used in study and cluster groups as per dendogram (**Figure 2**).

Pot No.	Species	Primary Name	APG Accession	Status	Country of Origin	Cluster Group
SA GRC Lines						
1			593[c]	Gp		
2			594	Gp	Afghanistan	A
3			595	Gp	Afghanistan	B1
4			596 [c]	Gp		
5			598	Gp	Turkey	B1
6			667	Gp	Portugal	B1
7			668	Gp	Portugal	B1
8			669	Gp	Portugal	B1
9			670	Gp	Portugal	B1
10			671	Gp	Portugal	B1
11			673	Gp	Portugal	B1
12			674	Gp	Portugal	B1
13			675	Gp	Portugal	B2
14			676	Gp	Portugal	B1
15			677	Gp	Portugal	B1
16			678	Gp	Portugal	B2
17			679	Gp	Portugal	B2
18	*T. alexandrinum*		700	Gp	Israel	B2
19			6168	Gp	Portugal	B1
20			8579	cv	Israel	B1
21			8582	Gp	Israel	D
22			14,247[c]	cv		
23			15,890	Gp	Syria	B2
24[a]			15,892	Gp	Iraq	G
25			19,675	Gp	Afghanistan	B2
26			19,678	Gp	Afghanistan	B1
27			24,502	cv	Morocco	B2
28			24,503	cv	Morocco	B1
29			24,545	Gp	Tunisia	B1
30[a]			32,668	Gp	Turkey	F
31			33,621	Breeder line	Australia	B1
32			33,622	Breeder line	Australia	B2
33[b]			33,747	cv		J
34			33,875	cv		B1
35			35,688	cv		E

Continued

36[b]			36,369	Gp	Israel	I
37			37,099	cv	Saudi Arabia	D
38			41,596	Gp	Morocco	B2
39			42,936	cv	Italy	C
40			45,313	Gp	Pakistan	B2
41			45,314	cv		B2
42			45,315	cv		B1
43			45,316	cv		B2
44			45,317	cv		B1
45			45,318	cv		B1
46			45320	cv	Italy	B2
WA GRC Lines						
47		138978	80,647	Gp	Morocco	B2
48		139496	75,354	Gp	USA	B2
49		144658	77,737	Gp	Israel	C
50		018742	76,777	Gp		B1
51		034544	73,590	Gp	Israel	E
52[b]		086555	77,740	Gp	Israel	B1
53		086558	77,741	Gp	Israel	B2
54		086566	77,742	Gp	Portugal	B1
55[b]		086756	77,743	Gp	Israel	B1
56[b]	*T. alexandrinum*	087277	75,011	Gp		B2
57[b]		087361	68,947	Gp		H
58		93MAR264ALE	73,299	Gp	Morocco	B1
59		93MAR60ALE	73,308	Gp	Morocco	E
60		CQ1166	63,256	Gp		B1
61		CS/1/82	73,410	Gp		B1
62		Italy.ALE	77,746	Gp		C
63		L59-72	77,747	Gp		B1
64[b]		LA YAPA INTA	75,014	Gp		H
65		Sacromonte	62,041	Gp		B1
66		Warden	76,306	cv	India	B1
67	*T. salmoneum*	087360	73,734	Gp		B1
68	*T. subterraneum*	Dalkeith subclover	17,496	cv		B1
69	*T. alexandrinum*	Elite II	35,688	cv		J
70	*T. vesiculosum*	Arrow leaf clover	78,434	cv		K
71	*T. michelianum*	Boltabalansa	32,860	cv		L
72	*T. repens*	Haifa white	63,892	cv		K

[a]Prostrate and slow growing; [b]Morphologically close to *T. salmoneum*; [c]No germination; Gp: Germplasm; cv: Cultivar.

Figure 1. *Trifolium* germplasm growing in glasshouse at NSW DPI, Wagga Wagga: A general view (A), *T. repens* (B), *T. michelianum* (C), *T. alexandrinum* (no 36369) (D), *T. alexandrinum* (No 15892) (E), *T. salmoneum* (087360) (F), *T. alexandrinum* (087361) (G), *T. alexandrinum* (No 32668) (H), Variation in leaf shape and marker (I), *T. alexandrinum* (J), *T. vesiculosum* (K), *T. subterraneum* (L).

estimated following [13]. Dendogram was prepared following SAHN clustering based on unweighted pair-group method with arithmetic average (UPGMA) method using the NTSYS PC software (http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html). The clusters in the dendogram were identified by drawing phenetic line at 0.5 similarity coefficient and considering the dedogram topologies, *i.e.* groups joining the other major group coinciding with the cutting points at root level. Polymorphic information content (PIC) was calculated as per [14].

3. Results and Discussion

3.1. Development of Genomic SSR Markers in Egyptian Clover

Amplicons generated through the five degenerate primers having (GA)10, (CT)10, (GTG)6, (GACA)5 and (CAA)6 repeats, were cloned and 89 colonies obtained. Of the 59 positive clones, 46 had inserts containing one or more SSR repeat motifs, suggesting that our approach for library construction was effective in isolation of SSR. Redundant clones visualized after BLAST analysis were removed. Although sequence analysis revealed all recombinants to possess terminal microsatellite repeats, to avoid any base pair degeneracy in degenerate primers, synthesis of forward and reverse primers was done; which helped in get-

ting better cross-species reaction. The SSR motifs comprised of mononucleotide (T), dinucleotide (CA), tetranucleotides and compound repeats (Table 2). In all, 15 SSR primer pairs (IGFRI-SSR1 to 15), including 5 SSRs having compound repeats, were designed (Table 2). Degenerate primers are largely designed based on the common repeat sequences observed in plant genome and have been reported

Table 2. Sequences of *T. alexandrinum* primer pairs of SSR developed.

Locus/Primer name	SSR motif	Expected fragment size (bp)	Primer sequence (5' to 3')
IGFRI-SSR1	(AACC)3	139	GATGCTGGAATTGGAAGAGAAT(F)
			CTTGAACCAACCAACCAGTACA(R)
IGFRI-SSR2	(AACC)3N*.(GGTT)3		GCTGTGTGATTACTGCTTGGAG(F)
			GCTGATCTTATCTCTAATGGGAAGAG(R)
IGFRI-SSR3	(ACCA)3	189	AACTTCTTCCCCATCAGTTTCA(F)
			ACCAACCAACCAAGATGACC(R)
IGFRI-SR4	(AACC)3N*.(GGTT)3	317	GTTAAGAAATCCTGTGGGCAAG(F)
			GAAGAAAGGAGCGAAAACAGAC(R)
IGFRI-SSR5	(ATGT)12N*.(AACC)3	337	CATCGGTTGGTTGGTTGG(F)
			TCGTACATTAACATGCGTGACC(R)
IGFRI-SSR6	(GGTT)3	296	ATTAAAACCGAACCAACCAACC(F)
			AAGATGTGACCAACCAACCAAC(R)
IGFRI-SSR7	(AACC)3N*.(GGTT)3	320	GGTTAATTGGTCACGCATGTT(F)
			TTGAAGCAATCTAGTCAGGCAG(R)
IGFRI-SSR8	(AACC)3	225	GAAAGGAGGCCACACAGAACT(F)
			TCATACAACCAACCAACCAAGA(R)
IGFRI-SSR9	(TTGG)3	259	ACTTAAACCAACCAACCGGAA(F)
			GCCCCATATTCCCTCACTAAAC(R)
IGFRI-SSR10	(T)10	300	GAAATCTTGGTTGGTTGGTTGT(F)
			CACTAAAGGGTTCCATTCCATT(R)
IGFRI-SSR11	(T)10	151	AATGGAATGGAACCCTTTAGTG(F)
			TGCATGTGGAAAATACCTTCAG(R)
IGFRI-SSR12	(CCAA)3N*.(GGTT)3	274	AACTCCCCTCTCCTCTGCTAGT(F)
			CATGATATACGGACCACCTGC(R)
IGFRI-SSR13	(AACC)3	240	GGTCACGCATGTTAATGTACGA(F)
			CATAACCAACCAACCGGAACT(R)
IGFRI-SSR14	(GGTT)3	167	TGAACCAACCAACCTGGAGT(F)
			GGCAGCATTAGCCTTTCTTTTA(R)
IGFRI-SSR15	(CA)20	229	GGGGACTCTCTCTCTCTCTCTC(F)
			GCGTGATTCCTTTCCACA(R)

*N - A/C/G/T, bp = base pair.

working with different legume and grass species [9]. Thus, the results demonstrate the utility of degenerate 5' anchored primers as simple, fast and cost effective as reported earlier also by [9].

3.2. SSR Polymorphism and Cross Transferability in *Trifolium*

Of the 15 IGFRI-SSR primer pairs (Table 2), derived from *T. alexandrinum* in this study, seven amplified the fragments of expected sizes in Egyptian clover. We further analyzed 7 polymorphic SSR markers to reveal allelic diversity in 69 accessions of the different *Trifolium* species. A total of 20 alleles were detected by the seven IGFRI-SSR markers (Table 3). The size of SSR alleles varied from

Table 3. SSR alleles scored on CEQ capillary electrophoresis.

Primer name	*T. alexandrinum* (bp)	*T. salmoneum* (bp)	*T. subterraneum* (bp)	*T. vesiculosum* (bp)	*T. michelianum* (bp)	*T. repens* (bp)	Accessions with doubtful identity (bp)	PIC	No. of fragments
***T. repens* primers**									
A02H09				211 - 213	213	211 - 213		0.99	2
A01C10	287 - 289	287 - 289	287 - 289	238 - 289		238 - 289	287 - 318	0.82	8
A04F01	182 - 202	182 - 192	192	192 - 234	192	192 - 234	192	0.89	7
A01H11	138 - 175	138 - 144	138 - 144	175 - 245	138 - 169	169 - 242	138 - 144	0.95	8
A02D12	237 - 247			146 - 148	148	146 - 247	237 - 247	0.97	6
A05A09	168 - 197			193 - 197	168 - 205	176 - 197	179 - 205	0.98	4
B01B05	121 - 269	121 - 265	123	235 - 269	263 - 267	235 - 269	121 - 269	0.89	10
A06E06	131 - 161	131 - 161	131 - 161	137 - 175	131 - 144	137 - 175	131 - 263	0.91	13
A06B04	165 - 194	173 - 175		173 - 175	185 - 194	173 - 199	165 - 199	0.84	8
A02D07	165 - 182	165	165	165 - 182		165 - 182	165 - 182	0.97	7
B02E01	133 - 177	139 - 143		135 - 137	135 - 177	127 - 137	177	0.97	7
B01E07	137 - 189		181	233 - 235	235	233 - 235	137 - 235	0.89	6
A04B12	254 - 258	254 - 258	254 - 258	258 - 278	278	266 - 278	250 - 258	0.84	8
A03B05	122 - 166	126 - 130	126 - 136	126 - 166	122 - 134	122 - 166	128 - 166	0.96	10
A02H03				232 - 234		232 - 240	236	0.99	5
***T. alexandrinum* primers**									
IGFRI-SSR3	133 - 189					173 - 189	165 - 189	0.97	7
IGFRI-SSR7	251		251	259		251		0.99	2
IGFRI-SSR8	250 - 262							0.99	2
IGFRI-SSR11	168 - 172	168 - 172	168 - 172	168 - 172	168 - 172	168 - 172	168 - 172	0.74	3
IGFRI-SSR13	168 - 176	168 - 176	168 - 176	168 - 176		168 - 176	168 - 176	0.78	2
IGFRI-SSR14	158 - 170						158	0.99	2
IGFRI-SSR15	228 - 232	228 - 232	228 - 232	228 - 232			228 - 232	0.68	2

bp: base pair.

133 to 262 bp among *T. alexandrinum* accessions as well as across the different species. The number of alleles/SSR ranged from two to seven, with an average of 2.85 allele/SSR locus. The number of alleles/SSR among *T. alexandrinum* accessions ranged from one to seven with 2.71 alleles per SSR locus. PIC values observed with these primers ranged from 0.99 to 0.68. Primer IGFRI-SSR 8 was species specific and polymorphic with *T. alexandrinum.* IGFRI-SSR 14 amplified fragments with *T. alexandrinum* and the two off type (prostrate and slow growing) *T. alexandrinum* accessions 15,892 and 33,747 (**Figure 1(E)** & **Figure 1(H)**). The remaining five IGFRI-SSRs amplified fragments with other species also. Alleles of IGFRI-SSR 11, 13 and 15 were represented among most of the accessions under study, thus, indicating common ancestral contribution of the alleles. *T. michelianum* was found to possess the most common fragments of IGFRI-SSR 11 only (**Table 3**), hence, showed high degree of dissimilarity with other accessions. These results suggest that SSR markers, derived from *T. alexandrinum*, are suitable to effectively discriminate different *Trifolium* species.

To validate the usefulness of *T. repens* primers and to assess molecular diversity among *Trifolium* species, we analyzed second set of 15 SSR markers developed from *T. repens* [11]. Thirteen out of these 15 primers amplified fragments with *T. alexandrinum*; all 15 amplified with arrow clover; 10 with *T. salmoneum*, 9 with *T. subterraneum* and 13 with *T. michelianum*. A total of 109 alleles were scored (**Table 3**) which was attributed to the diverse genetic background of the accessions under the present study. More than 0.9 PIC values were noticed for 8 out of 15 primer pairs and the minimum PIC value of 0.82 was noticed with primer A01C10. Most of these primers amplified fragments with other species also. A02H09 and A02H03 primers did not amplify the DNA from *T. alexandrinum*, *T. salmoneum*, *T. subterraneum* and the morphologically *T. salmoneum* look-alike accessions (**Table 1**), thus suggesting a genetic relatedness among them. A few primers produced 6 to 10 alleles among *T. alexandrinum* accessions (**Table 3**) which was attributed to diverse genetic base of the *T. alexandrinum* accessions and that the accessions from different place of collection might be having allelic contribution from different *Trifolium* species. It is also possible that the primers could bind at many locations due to pleiotropism or non-specificity of the primers. Molecular studies on two ecotypes of Berseem as done by [15] also found that QTLs for different traits were mapped in the same region on map, thus, indicating common genetic control and possibility of pleiotropism and/or tight linkage of different polygenes or QTLs. The extent of genetic diversity in the present set of germplasm was larger than that assessed by morphological and isozyme studies among Indian collection of *T. alexandrinum* germplasm [7] [16]. This can be attributed to factors such as the diverse source of the germplasm, the efficacy of SSR primers and possible different pathway of origin of these germplasm lines.

In all, the two sets of primers proved to be effective in revealing the intra and the interspecies diversity. Additionally, high polymorphism exhibited among *T. alexandrinum* accessions indicated suitability of these SSRs for further allelic

study of the species.

3.3. Genetic Relationship among Accessions

In order to investigate the genetic relationship among accessions of *T. alexandrinum* and related species, we generated dendogram using clustering method. Twelve distinct clusters were formed containing a varying number of accessions (Figure 2). *T. alexandrinum* accession No 594 formed independent cluster "A" (Figure 2), although this accession was morphologically similar to other Egyptian clover accession. Cluster "B", the largest cluster was divided in two sub-clusters "B1" and "B2" containing 33 and 17 accessions respectively. The majority of *T. alexandrinum* accessions together with *T. salmoneum*, *T. subterraneum* and the three *T. salmoneum* type accessions (75,011, 77,743, 77,740) grouped in this cluster. Cluster "C", "D" and "E" contained three, two and three *T. alexandrinum* accessions respectively. Although, *T. alexandrinum* accessions represented different geographical places, no trend of clustering based on place

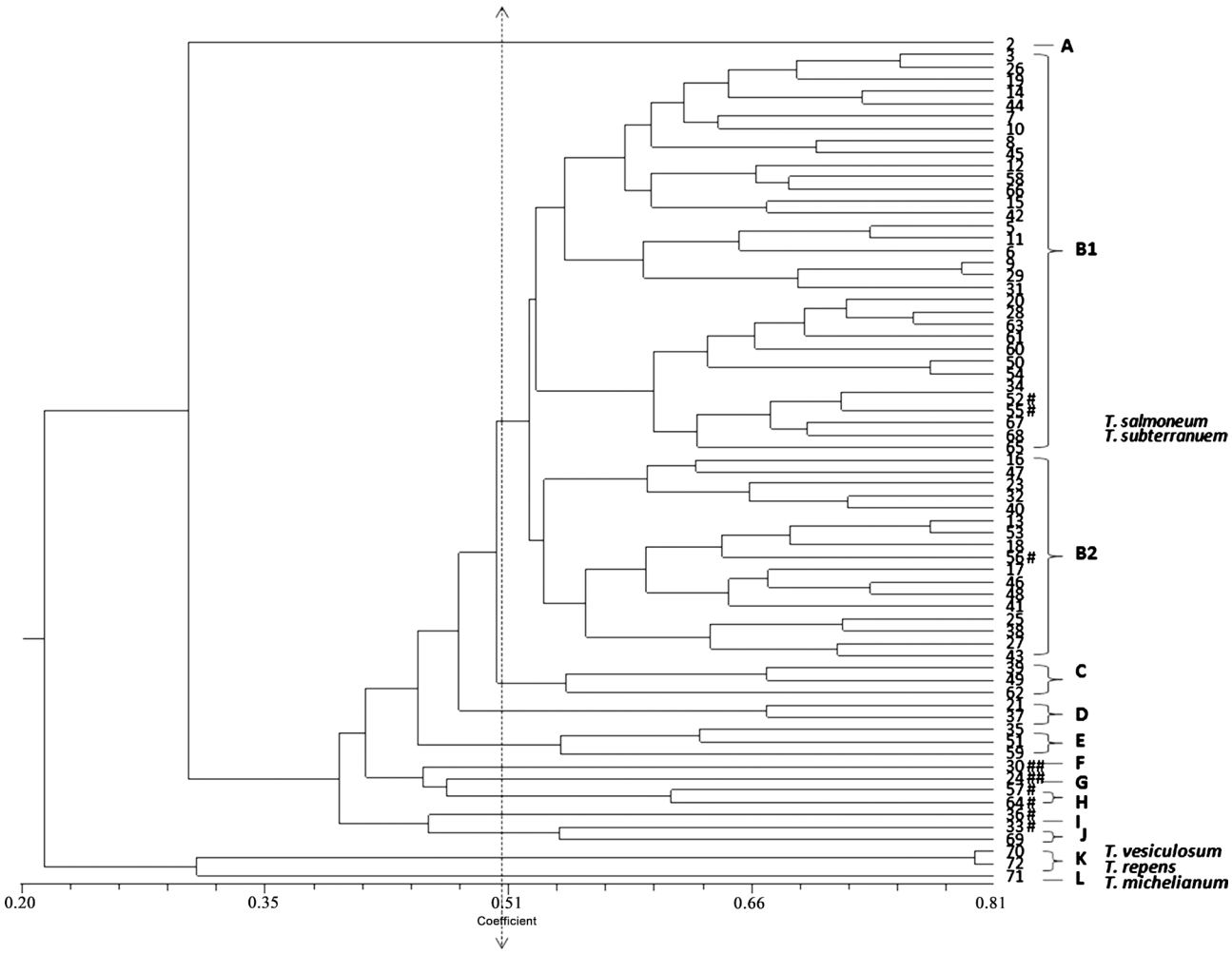

Figure 2. Dice similarity based dendogram showing clustering of *Trifolium* accession: Similarity coefficient is on "x" axis and accession numbers as per serial numbers in Table 1 on axis "y"; Dotted phenetic line drawn at 0.5 similarity coefficient; #: *T. alexandrinum* accession close to *T. salmoneum* plant type; ##: prostrate and slow growing *T. alexandrinum* accessions.

of collection was established. This might be because of movement of germplasm and its intermixing with native germplasm. Two prostrate and slow growing *T. alexandrinum* accessions 32,668 and 15,892 formed independent clusters "F" and "G" respectively. These two accessions were having no morphological similarity with *T. alexandrinum. T. alexandrinum* accessions, possessing morphological similarity with *T. salmoneum*, 68,947 and 75,014 formed cluster "H"; accession 36,369 formed independent cluster "I" whereas accession 33,747 formed cluster "J" with an *T. alexandrinum* cv Elite II. Cluster "F", "G" and "H" grouped together before joining the major cluster. Similarly, cluster "J" and "I" also grouped together before joining the major cluster. These *T. alexandrinum* accessions which differed in morphology were placed closely in the dendogram. Cluster "K" possessed the two species *T. vesiculosum* and *T. repens* whereas cluster "L" was represented with *T. michelianum* only.

The genus *Trifolium* has been divided in eight sections [1]. In the present study, clustering of *T. subterraneum* belonging to section Trichocephalum of the genus, with *T. alexandrinum* and *T. salmoneum* of section *Trifolium*, indicated that the three species possessed relatedness which was congruent with earlier reports [3] [16] [17] [18] [19]. In fact, Lotoidea is the largest section and has served as source taxa for the evolution of many other sections [1]. This is also supported with development of fertile intersection interspecific crosses [10] [20] [21]. Thus, this study reaffirms the high relatedness of the species as well as possibility of polyphyletic origin of *T. alexandrinum*.

Origin and ancestry of *T. alexandrinum* has remained controversial. *T. berytheum* [22] [23], *T. echinatum* [24], *T. apertum* [18] and *T. salmoneum* [23] were considered as its progenitor. In the present study, clustering of *T. salmoneum* and the *T. salmoneum* look-a-like accessions with *T. alexandrinum* accessions also indicated close affinity between the two species and that *T. salmoneum* might have contributed in evolution of *T. alexandrinum*. Clustering of *T. alexandrinum* Elite II, an Australian cultivar, with two *T. salmoneum* type accessions also suggested its ancestry with the former. In fact, the accessions mentioned as "*T. salmoneum* type" were phenotypically closer to *T. salmoneum* and possessed round cotyledonary leaves similar to *T. salmoneum*. Thus, the molecular data based similarity was congruent with phenotype of the plants.

Boundaries among different *Trifolium* species are extremely difficult to define because of the range of diversity caused by primary polymorphism [25]. Hence, identification of species is an important aspect in *Trifolium* genetic resource study. Occurrence of a few plants, either with serrated margin first leaf or bifoliate/trifoliate first leaf among accessions 668, 45,313, 669, 35,688, 73,308 and 77,740 of *T. alexandrinum*, were considered as admixtures which occurs as samples are collected from the field. However, the diversity as result of admixture can also be conserved because the basic objective of any gene bank is to conserve genetic diversity. Occasional occurrence of plants with a few multifoliate leaves in accessions 673, 6,3256 and 33,875 was in line with such reports in many *Tri-*

folium species. The genetics of the trait is not known, although pentafoliate trait was recently fixed in *T. alexandrinum* [26].

Thus, this study developed a set of primer-pairs which have shown to amplify alleles from *T. alexandrinum*, *T. salmoneum*, *T. vesiculosum*, *T. michelianum*, *T. repens* and *T. subterraneum*, suggesting that this newly developed resource is useful for assessing molecular diversity among accessions of at least six *Trifolium* species. The seven SSR markers from *T. alexandrinum* genomic resource and 15 from *T. repens* genomic resource were able to distinguish different accessions of *T. alexandrinum* and the species under study. Allelic dissimilarity among morphologically similar accessions (accession 594, a typical *T. alexandrinum*, forming independent cluster) and clustering together of morphologically distinct accession (Elite II and accession Nos. 33,747 and 36,369) shows the efficiency of SSR primers in the study. High PIC value of SSR proves that these markers were suitable to differentiate among accessions. Cluster analysis indicated suitability of SSR markers for genome analysis. The study could successfully establish the larger diversity of the *T. alexandrinum* gene pool as superior resource for further breeding and enriching the SSR markers repertoire for further genetic study. The study also established the relatedness of *T. subterraneum* and *T. salmoneum* accession with *T. alexandrinum*, indicating their role in the evolution of *T. alexandrinum*.

4. Limitations of Study

Five species of *Trifolium*, other than *T. alexandrinum*, were represented by single accession because of primary interest to characterize the *T. alexandrinum* germplasm. The interspecific lineage discussed in the study may be more emphatically established with representation of more number of accessions of related species. Although the limited number of SSR primers used in the study could effectively differentiate among species, studies with more SSRs will help exposing intra-species variation and contribution of different species in development of cultivars/ germplasm.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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