The first molecular characterization of the Tunisian-Algerian endemic *Trifolium juliani*Batt. and *Trifolium squarrosum* L. from Dyr El Kef, North West Tunisia

Karim Guenni

karim.guenni@fst.utm.tn

Laboratory of Molecular Genetics, Immunology and Biotechnology (LR99ES12), Faculty of Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia https://orcid.org/0000-0002-6760-9123

Nidhal Chtourou-Ghorbel

Laboratory of Molecular Genetics, Immunology and Biotechnology (LR99ES12), Faculty of Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia https://orcid.org/0000-0002-8289-1956

Salma Sai Kachout

Laboratory of Animal and Forage Production (LR16INRAT01), National Institute of Agronomic Research of Tunis, University of Carthage, Tunis, Tunisia. https://orcid.org/0000-0002-8519-3432

Salah Ben Youssef

Laboratory of Animal and Forage Production (LR16INRAT01), National Institute of Agronomic Research of Tunis, University of Carthage, Tunis, Tunisia. https://orcid.org/0000-0003-4213-048X

Edouard Jean

CAPTE (Collectif d'Acteurs pour la Plantation et la Transition Environnementale), Marseille, France.

Hazem Cherni

Association les amis de CAPTE, Bizerte, Tunisia

Aziza Zoghlami-Khelil

Laboratory of Animal and Forage Production (LR16INRAT01), National Institute of Agronomic Research of Tunis, University of Carthage, Tunis, Tunisia. https://orcid.org/0000-0002-3194-7817

Research Article

Keywords: Trifolium juliani, Trifolium squarrosum, Molecular polymorphism, Genetic differentiation, ISSR and SRAP markers

Posted Date: December 2nd, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8229370/v1

Additional Declarations: The authors declare no competing interests.

Abstract

In Tunisia, clovers represent a valuable genetic resource due to their economic and ecological importance in forage and pastoral systems, particularly within meslin cropping systems. However, certain species, such as *Trifolium squarrosum*, are increasingly threatened with extinction owing to the lack of conservation and management strategies. This study proposes a clarification of the distinction between *Trifolium squarrosum* and *Trifolium juliani*. To substantiate this differentiation, molecular characterization was performed using two types of molecular markers: ISSR (Inter Simple Sequence Repeats) and SRAP (Sequence-Related Amplified Polymorphism). A total of 10 ISSR primers generated 70 polymorphic bands across the two *Trifolium* species, demonstrating a high polymorphism percentage (PPB = 80.46%), with notable variation in band patterns among primers. Conversely, 10 SRAP primer combinations yielded 46 polymorphic bands (PBP = 73.02%). While both marker systems effectively revealed molecular polymorphism, ISSR markers exhibited slightly higher efficiency. Analysis of molecular variance (AMOVA) indicated that genetic diversity primarily partitioned between the two species, reflecting low gene flow and high genetic differentiation. Principal component analysis (PCA) and hierarchical clustering which further supported that distinction, revealed clear genetic structure among the clover species. This structure was also corroborated by robust clustering and high assignment probabilities in the Bayesian inference analysis. This study underscores the efficacy of ISSR and SRAP markers in assessing genetic diversity and differentiating clover species, providing valuable insights into their genetic relationships.

Introduction

Grasslands cover approximately 20 to 40% of the world's agricultural land; they are crucial not only for food production, but also for climate change, mitigation and the provision of key ecosystem services. These services include water regulation, biodiversity preservation, and carbon sequestration within their root systems, which represent about 90% of total grass biomass (Gibson 2008). Forage grasses and legumes are key components of grasslands worldwide. In temperate regions, a relatively small number of species dominate, including *Lolium perenne*, *Festuca* spp., and *Dactylis glomerata* among grasses, and *Trifolium* spp., *Medicago* spp., and *Lotus* spp. among legumes (Ravagnani et al. 2012).

The genus *Trifolium* L. (Clover) belongs to the Fabaceae (Leguminosae) family, within the subfamily Papilionaceae, and is one of the largest genera in the tribe Trifolieae (Ellison et al. 2006). *Trifolium* is grouped with closely related genera, including *Trigonella*, *Medicago*, and *Melilotus*. However, numerous revisions of the genus have been published, and several comprehensive reviews of its taxonomic history are available, such as those by Hossain (1961), Gillet (1970), and Zohary and Heller (1984) (Lamont et al. 2001).

Clover is native to all humid, temperate regions worldwide, except for the Himalayas and Australasia. The three primary centers of diversity for the genus *Trifolium* are Turkey (Mediterranean center), Ethiopia (African center), and northern California, USA (American center) (Zohary and Heller 1984). Recent studies supported a Mediterranean origin for the genus during the Early Miocene, around 16–23 million years ago (Ellison et al. 2006), with the eastern Mediterranean showing the greatest species diversity, including many commercially significant forage species (Russi et al. 1992).

Among the 299 Trifolium species recognized worldwide (POWO 2025), 217 of which are accepted by the ILDIS (International Legume Database and Information System (https://www.ildis.org)), approximately 150 occur in the Mediterranean region. These represent seven of the eight sections of the genus, with most sections having the majority of species concentrated in this area (Zohary and Heller 1984). Several studies have shown the commercial values of about 30 cultivated Trifolium species (Herman 1953; Russell and Web 1976; Evans 1976; Speer and Allinson 1985; vanKeuren and Hoveland 1985), from which the most important cultivated species are: *T. repens* (White Clover), *T. subterraneum*, *T. pratense* (Red Clover), *T. fragiferum*, *T. hybridum*, *T. resupinatum*, *T. incarnatum*, *T. alexandrinum*, *T. dubium*, *T. ambiguum*. The majority of them are native to the Mediterranean, especially in Turkey (Zohary 1970; Zohary and Heller 1984). In North Africa, 33 species have been recorded in Tunisia and 41 species in Algeria (ILDIS 1999), 40 of which are native to Algeria. However, determining the geographical distribution of several clover species remains challenging due to confusion with their cultivated range.

The genus *Trifolium* is economically important within the Fabaceae family, with at least 16 species widely cultivated as forage and green manure crops (Gillett and Taylor 2001). Their symbiotic relationship with *Rhizobium leguminosarum* enables nitrogen fixation through root nodulation, (Sprent 2001), enhancing soil fertility and improving pasture quality in both natural and managed grasslands.

In Tunisia, plant genetic resources are of major economic and ecological importance. Research on spontaneous forage species, particularly Trifolium species, is driven by forage scarcity resources and the low nutritional quality of conventional animal feed, which limits the development of the livestock sector (Ben Salem and Smith 2008). Legumes are especially valued for their high protein content and environmental benefits.

Studies on spontaneous *Trifolium* species in Tunisia, primarily focus on the variability observed between and within species at various levels. Since the 1990s, numerous exploration and collection missions have been conducted, particularly in northern regions, to enhance the value of spontaneous legume species with forage and pastoral potential. Several *Trifolium* species have been identified and are well-represented in Tunisia, especially in the northern areas (Zoghlami et al. 1995). These species constitute a highly diverse and rich genetic resource, encompassing numerous species whose prevalence varied by region (Zoghlami et al. 1995).

Conservation, selection, and improvement efforts for *Trifolium* genetic resources continued into the 2000s. These initiatives enabled the agronomic enhancement and commercialization of species, such as the diploid species *Trifolium subterraneum* L. (Jaritz 1982), officially registered in the national catalog under the name of variety Faïja. Cultivars of *T. subterraneum* are also well integrated into ley-farming systems due to their adaptability to diverse Mediterranean climates and soils (Katznelson 1974; McGuire 1985; Ewing 1996). Although 33 clover species have been recorded in Tunisia (Le Floc'h et al. 2010), only a few have been widely exploited; nevertheless, the annual species *T. alexandrinum* (berseem) has shown promising performance in forage associations combining systems, particularly with triticale (Beji and Khemir 2012; Zoghlami-Khélil et al. 2015).

Recent surveys led to the inclusion of *Trifolium tunetanum* on the IUCN Red List as Critically Endangered (CR). According to Pottier-Alapetite (1979), a Tunisian endemic clover, *Trifolium squarrosum* subsp. *tunetanum*, is reported in the southeastern Kef region and *Trifolium juliani* is a rare and endemic clover species native to Tunisia and Algeria (Pottier-Alapetite 1979). Conservation programs at National Institute for Agronomic Research in Tunisia (INRAT) and the National Gene Bank (BNG) have preserved seeds of both *T. juliani* and *T. squarrosum ssp parnomitanum*, enabling their use in forage trials that confirmed their potential. Due to morphological similarities and overlapping ecological niches, distinguishing *T. juliani* and *T. squarrosum ssp tunetanum* becoming *T. tunetanum*, a specie a part, is challenging. Indeed, several recent surveys have highlighted the close resemblance between these two sympatric taxa, and detailed botanical observations ultimately allowed the clear differentiation of the two taxa *Trifolium juliani* and *Trifolium squarrosum subsp. panormitanum* (Belaifa E and Ghrabi-Gammar Z, personal communication, 2022). The present work applies molecular characterization to examine the genetic diversity within and between the two taxa.

Since to support the phylogeny of the two species, we focused on molecular markers. Unlike morphological traits, molecular markers are stable, environmentally independent, and reflect genome-wide variation (Clegg and Zurawski 1991; Gielly and Taberlet 1996). Our study emphasizes molecular polymorphism and interspecific differentiation using two types of markers: (a) arbitrarily amplified DNA markers (AADs), specifically ISSR markers (Inter Simple Sequence Repeats) (Zietkiewicz et al. 1994), and (b) targeted fingerprinting markers (TFMs), specifically SRAP markers (Sequence-Related Amplified Polymorphism) (Li and Quiros 2001).

ISSR markers were used for the first time to study Trifolium genetic diversity in South American and Eurasian species (Rizza et al. 2007), with subsequent studies confirming their utility across the genus (Paplauskienė and Dabkevičienė 2008; Dabkevičienė et al. 2011; Dabkevičienė and Paplauskienė 2012; Aryanegad et al. 2013; Abate and Tesfaye 2017; Abate 2017; Mostapha Mohamed et al. 2024).

SRAP markers have more recently gained traction for their reproducibility and ability to target coding regions (Yousefi et al. 2018; Youssef et al. 2019; Mostapha Mohamed et al. 2024).

Understanding *Trifolium*'s essential for germplasm management and conservation strategies. Both ISSR and SRAP markers have proven effective for detecting polymorphisms and identifying genotypes of high conservation value. These marker systems are increasingly valuable for exploring key genetic aspects in *Trifolium* species, including their evolutionary origins, centers of diversity, domestication processes, population structure, germplasm characterization, and the dentification of markers linked to agronomic traits. However, their application in assessing genetic diversity within *Trifolium* remains limited, mainly due to the genus's high species richness.

More recently, SRAP markers have demonstrated their effectiveness and versatility in analyzing genetic diversity within clover species (Yousefi et al. 2018; Youssef et al. 2019; Mostapha Mohamed et al. 2024). Understanding the genetic diversity of *Trifolium* is critical for germplasm management and the development of conservation strategies. Studies indicate that both ISSR and SRAP marker systems are well-suited for assessing DNA polymorphism in clover, revealing the existence of diverse genotypes with unique identities

warranting conservation efforts. However, despite their potential, studies employing ISSR and SRAP markers to measure genetic diversity in Trifolium species remain limited, largely due to the extensive number of species within this genus.

Materials and methods

Plant material

The molecular polymorphism analysis was performed on seven young seedlings from each of the two clover species, *Trifolium juliani* and *Trifolium squarrosum ssp parnomitanum*. Seeds were collected in 2021 from Dyr El Kef (36°12'53.323" N, 8°45'38.293" E) (North West of El Kef) (Fig. 1). *Trifolium juliani* and *Trifolium squarrosum ssp parnomitanum*, distinguished in particular by their calyxes during recent surveys (Fig. 2), are currently being propagated in concrete trays at INRAT.

Genomic DNA isolation

Fresh young leaves were sampled, frozen in liquid nitrogen, and ground to a fine powder. Genomic DNA was extracted and purified using the DNAeasy Plant Mini Kit (Qiagen Inc, USA). DNA concentration were measured with a NanoDrop™ 2000/2000c (Thermo Fisher Scientific, USA), and DNA quality was evaluated by electrophoresis on 0.8% agarose gel according to Sambrook et al. (1989).

PCR-ISSR assays

The detection of inter and intraspecific polymorphisms has been performed using 10 ISSR primers. These were designed based on di- or multi-nucleotide repeats complementary to microsatellites (Table 1). The di-nucleotide repeats were anchored at the 3' end with a single degenerating nucleotide. DNA amplification was performed in 25 µL of final volume reaction mixture containing 10X PCR buffer, 1.5 mM of $MgCl_2$ (50mM), 200 µM of dNTPs, 10 pmol of primer, 1.5 U of Taq DNA polymerase (Biomatik, USA), 10 ng of DNA, and double-distilled water. PCR amplifications were performed under the following conditions: one cycle of initial DNA denaturation at 94°C for 5 min, followed by 35 cycles each one including a denaturation step of 30 s at 94°C, a second step of 1 min 30 s for annealing at the primer-specific Tm and an extension step of 1 min at 72°C, followed by a final extension for 5 min at 72°C.

Table 1
Characteristics of ISSR and SRAP primers used for genetic diversity in *Trifolium* species

ISSR primer codes		**Sequence (5'-3')**	**Tm***
Unanchored primers	ISSR1	$(AG)_{10}$	48
	ISSR2	$(AGG)_6$	48
	ISSR3	$(ACTG)_4$	47
Anchored primers at the 3' end by a single degenerate nucleotide	ISSR4	$(AG)_{10}C$	48
	ISSR5	$(AG)_{10}G$	48
	ISSR6	$(AG)_{10}T$	48
	ISSR7	$(CT)_{10}A$	48
	ISSR8	$(CT)_{10}T$	50
	ISSR9	$(CT)_{10}G$	47
	ISSR10	$(TC)_{10}A$	48
SRAP primer codes		Sequence (5'-3')	
Forward primers	me1	TGAGTCCAAA**CCGG**ATA	
	me2	TGAGTCCAAA**CCGG**AGC	
	me4	TGAGTCCAAA**CCGG**ACC	
	me8	TGAGTCCAAA**CCGG**TGC	
	me10	TGAGTCCAAA**CCGG**TTG	
Reverse primers	em2	GACTGCGTACG**AATT**TGC	
	em3	GACTGCGTACG**AATT**GAC	
	em14	GACTGCGTACG**AATT**ACG	

*Tm: Melting temperature for primer, In bold: The core motifs 'CCGG' in the forward primer and 'AATT' in the reverse primer. Underlined: The selective 3' end sequences.

PCR-SRAP assays

Ten SRAP primer-pair combinations were randomly selected from a set of five forward and three reverse primers (Table 1) (Li and Quiros 2001; Zeng et al. 2012). PCR amplifications were performed in a 25 µL reaction volume containing 30 ng genomic DNA, 10X PCR buffer, 0.25 mM each primer (reverse and forward), 3 mM $MgCl_2$, 1 mM dNTPs, 1.5 U of Taq DNA Polymerase (Biomatik, USA), and double-distilled

water. The thermal cycling program consisted of an initial denaturation at 95°C for 5 min; 5 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, followed by 35 cycles of 94°C for 1 min, 47°C for 1 min 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min.

All amplifications for both techniques were carried out in an *Applied Biosystems-Veriti* thermocycler (Thermo Fisher Scientific, USA)

Both PCR products were separated on a 1.5% agarose gel stained with 0.5 pg mL^{-1} ethidium bromide and electrophoresed in 0.5X TBE buffer (pH 8.0) at 90 V for 1 h. The bands were visualized under UV light using a Gel-Doc 2000 image analysis system (Bio-Rad, USA).

Data analysis

Amplified products were compiled into a binary data matrix based on the presence (1) or absence (0) of each selected band to generate the 0/1-matrix. The discriminatory power of the markers in estimating genetic variability was evaluated using several informative indices: number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), expected heterozygosity (H), polymorphism information content (PIC), multiplex ratio (EMR), marker index (MI), discriminating power (D) and resolving power (Rp). These indices were calculated using the iMEC online tool (Amiryousefi et al. 2018). Additionally, genetic diversity was assessed with different parameters, such as the effective number of alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), total genetic diversity (Ht), genetic diversity within population (Hs), genetic differentiation (Gst), and gene flow (Nm) using POPGENE version 1.31 (Yeh et al. 1999). Furthermore, an analysis of molecular variation (AMOVA) was performed in GenAlEx 6.5 software (Peakall and Smouse 2012) to partition variance components within and among *Trifolium* species based on SRAP and ISSR markers.

A dendrogram was constructed using Ward's method (Ward 1963), and a principal component analysis (PCA) was applied to visualize genetic relationships among *Trifolium* genotypes based on Euclidian distances, both implemented in PAST software (Hammer et al. 2001).

Furthermore, the genetic structure of the population was analysed using bayesian clustering approach implemented in *STRUCTURE* version 2.3.4 (Pritchard et al. 2000; Evanno et al. 2005). The optimal of genetic clusters (K) was determined following the ΔK method proposed by Evanno et al. (2005), which identifies the K value with the highest ΔK as the most probable number of clusters. STRUCTURE was run under an admixture model with prior information on sampling locations. For each potential K value (ranging from 1 to 7), 10 independent runs were performed, each consisting of 100.000 burn-in steps followed by 100.000 iterations. The online tool Structure Selector (Li and Liu 2018) was used to summarize and evaluate clustering solutions. To align and average the results from replicate runs, the Clumpak program (Kopelman et al. 2015) was employed using the "Greedy" algorithm with 10.000 random input sequences and an additional 10.000 repetitions to compute pairwise similarity scores (H'). Finally, the cluster membership proportions were visualized using Distruct version 1.1 (Rosenberg 2004).

Results

ISSR polymorphism

The use of 10 ISSR primers produced 87 bands in total, of which 70 were polymorphic (PPB = 80.46%) across the 14 individuals studied (Table 2), highlighting the efficiency of these primers in detecting molecular polymorphism and differentiating between the two clover species. The number of bands generated varied considerably, ranging from 18 bands with the ISSR3 $(ACTG)_4$ primer to only 4 bands with both ISSR5 $(AG)_{10}G$ and ISSR8 $(CT)_{10}T$, indicating differences in the ability of primers to reveal polymorphism within the species.

Although three anchored primers (ISSR5 $(AG)_{10}G$, ISSR8 $(CT)_{10}T$, and ISSR9 $(CT)_{10}G$) exhibited the highest percentage of polymorphism (100%), they produced very few bands, and therefore contributed to limited information. In contrast, the two unanchored primers, ISSR3 $(ACTG)_4$ and ISSR1 $(AG)_{10}$, generated the greatest number of bands and showed high polymorphism levels (83.33% and 80%, respectively), as well as strong resolving power (Rp = 11.571 for ISSR3 $(ACTG)_4$ and 6.714 for ISSR1 $(AG)_{10}$). These two primers were therefore the most informative for distinguishing the genotypes of the two clover species.

Table 2
Summary of polymorphism parameters of ten ISSR primers applied to *Trifolium juliani* and *Trifolium squarrosum*

ISSR primers	Band size (pb)	TNB*	NPB	PPB (%)	H	PIC	EMR	MI	D	Rp
ISSR1	814–2010	10	8	80	0.497	0.371	5.357	1.987	0.715	6.714
ISSR2	750–1515	6	3	50	0.337	0.438	4.714	2.065	0.385	2.571
ISSR3	450–2837	18	15	83.33	0.500	0.370	8.929	3.301	0.755	11.571
ISSR4	519–2174	14	11	78.57	0.487	0.376	8.143	3.064	0.663	9.429
ISSR5	478–1065	4	4	100	0.490	0.375	2.286	0.856	0.678	2.857
ISSR6	418–1380	5	4	80	0.467	0.386	3.143	1.212	0.608	3.429
ISSR7	608–2554	9	7	77.78	0.494	0.373	4	1.491	0.804	4
ISSR8	574–2300	4	4	100	0.459	0.389	2.571	1.001	0.591	2.571
ISSR9	794–2552	6	6	100	0.500	0.370	2.929	1.083	0.765	3.286
ISSR10	266–1527	11	8	72.73	0.497	0.371	5.929	2.200	0.711	5.857
Total		87	70				48	18.259	6.675	52.286
Mean		8.70	7	80.46	0.473	0.382	4.800	1.826	0.667	5.229

*TNB: Total number of bands; NBP: Number of polymorphic bands; PPB (%): Percentage of polymorphism; H: expected heterozygosity; PIC: Polymorphism Information content; EMR: effective multiplex ratio; MI: marker index; D: discriminatory power; Rp: Resolving power

The polymorphism information content (PIC) values ranged from 0.370 for ISSR3 $(ACTG)_4$ to 0.438 for ISSR2 $(AGG)_6$, with an average of 0.382, indicating moderate to high informativeness of the ISSR primers in detecting genetic polymorphism. Figure 3 shows representative ISSR amplification profiles illustrating polymorphic bands that differentiate the two clover species.

SRAP polymorphism

Ten SRAP primer combinations generated a total of 63 bands, 46 of which were polymorphic, corresponding to a polymorphism percentage (PBP = 73.02%) (Table 3). In spite of the effectiveness in detecting molecular

polymorphism and distinguishing the two taxa SRAP markers showed a lower PPB compared to ISSR markers. Among the tested combinations, four primer pairs (me1-em2, me2-em3, me8-em2, and me2-em14) were the most informative (Fig. 4), each producing more than 8 bands, with polymorphism percentages exceeding 70% and resolving power (Rp) values above 6. Overall, SRAP markers showed moderate informativeness, with a mean PIC value and close to 0.5 (0.363) and an average polymorphism percentage (PPB) of 73.02%. Both SRAP and ISSR markers were effective in revealing molecular polymorphism between the two clover species although ISSR markers proved slightly more informative based on their higher performance indices.

Table 3
Summary of polymorphism parameters of ten SRAP primer combinations applied to *Trifolium juliani* and *Trifolium squarrosum*

SRAP primer combination	Band size (bp)	TNB*	NPB	PPB (%)	H	PIC	EMR	MI	D	Rp
me1-em2	282–1549	11	8	72.73	0.493	0.340	6.143	2.089	0.690	6.286
me1-em3	242–1433	4	3	75	0.469	0.352	2.500	0.880	0.614	3
me1-em14	422–850	3	2	66.67	0.408	0.378	2.143	0.811	0.495	1.714
me2-em2	141–1011	5	3	60	0.420	0.374	3.500	1.307	0.513	3
me2-em3	269–1545	10	7	70	0.431	0.369	6.857	2.529	0.531	6
me2-em14	338–1372	8	7	87.50	0.492	0.341	4.500	1.533	0.686	6.714
me4-em2	199–1573	6	5	83.33	0.308	0.414	4.857	2.012	0.347	2
me4-em3	717–1983	3	2	66.67	0.472	0.351	1.857	0.651	0.623	1.714
me8-em2	263–2110	10	7	70	0.485	0.344	5.857	2.015	0.659	5.143
me10-em2	774–2097	3	2	66.67	0.444	0.363	2	0.726	0.561	2
Total		63	46				40.214	14.553	5.717	37.571
Mean		6.3	4.6	73.02	0.442	0.363	4.021	1.455	0.572	3.757

*TNB: Total number of bands; NBP: Number of polymorphic bands; PPB (%): Percentage of polymorphism; H: expected heterozygosity; PIC: Polymorphism Information content; EMR: effective multiplex ratio; MI: marker index; D: discriminatory power; Rp: Resolving power

Analysis of molecular variance and diversity indices of species

The analysis of molecular variance (AMOVA) revealed a pronounced hierarchical structure in the distribution of genetic variation. The majority of the total variance was attributed to differences between the two species, accounting for 82.89% for ISSR and 84.85% for SRAP. In contrast, variation within species represented a considerably smaller proportion of the total genetic diversity (17.11% for ISSR and 15.15% for SRAP) (Table 4). This pattern of partitioning suggests strong genetic divergence between species and limited variability within each species. The pronounced interspecific differentiation is further corroborated by low estimates of gene flow (Nm = 0.198 for ISSR and Nm = 0.116 for SRAP) and high genetic differentiation coefficients (Gst = 0.716 for ISSR and Gst = 0.812 for SRAP) (Table 5).

Table 4
Analysis of molecular variance (AMOVA) for *Trifolium juliani* and *Trifolium squarrosum* based on ISSR and SRAP markers

ISSR markers					
Source of variation	d.f.*	Sum of squares	Mean squares	Estimates variance	Percentage of variation
Among species	1	154.571	154.571	21.449	82.89%
Within species	12	53.143	4.429	4.429	17.11%
Total	13	207.714		25.878	100%
PhiPT	0.829**				
p-Value	< 0.001				
SRAP markers					
Source	d.f.	Sum of squares	Mean squares	Estimates variance	Percentage of variation
Among species	1	120.571	120.571	16.796	84.85%
Within species	12	36	3	3	15.15%
Total	13	156.571		19.796	100%
PhiPT	0.848**				
p-Value	< 0.001				

*d.f.: degrees of freedom; **_p-Value_: significance after 1000 random permutations

Table 5
Genetic indices, genetic differentiation, and gene flow between *T. juliani* and *T. squarrosum* based on ISSR and SRAP data

ISSR markers									
Species	PPB (%)*	Na	Ne	H	I	Ht	Hs	Gst	Nm
T. juliani	9.20	1.092	1.068	0.037	0.054				
T. squarrosum	39.08	1.391	1.255	0.146	0.215				
Average	24.14	1.241	1.161	0.091	0.134				
Total	80.46	1.805	1.594	0.321	0.465	0.321	0.091	0.716	0.198
SRAP markers									
Species	PPB (%)	Na	Ne	H	I	Ht	Hs	Gst	Nm
T. juliani	7.94	1.079	1.059	0.032	0.046				
T. squarrosum	25.40	1.254	1.157	0.089	0.133				
Average	16.67	1.166	1.108	0.060	0.089				
Total	73.02	1.730	1.622	0.323	0.457	0.323	0.061	0.812	0.116

*PPB (%): percentage of polymorphic bands (%); Na: Number of observed alleles per locus; Ne: Effective number of alleles per locus; H: Nei's genetic diversity; I: Shannon diversity index; Ht: total diversity; Hs: mean diversity within populations; Gst: inter-population differentiation index; Nm: Number of migrants (gene flow).

Genetic structuration

The results obtained from principal component analysis (PCA), hierarchical clustering using Ward's method based on Euclidean distances, and Bayesian clustering consistently revealed a clear separation of the two clover species into two major clusters ($K = 2$, $\Delta K > 200$, $H'=0.9$ for ISSR, SRAP and combined datasets), corresponding to groups I and II across the three data matrices (Fig. 5). In the dendrogram, these two main clusters exhibited the highest robustness, with bootstrap values of 100%. Similarly, Bayesian analysis assigned more than 90% of genotypes to their respective clusters, conforming strong genetic differentiation between the species. However, two genotypes (TJ-7 and TS-9) displayed minor admixture signals (> 10%) with the second cluster, suggesting the existence of a weak but detectable shared genetic background, possibly reflecting ancestral polymorphism or limited historical gene flow not fully captured by the dominant markers used.

Discussion

Molecular markers such as SSRs and SNPs are widely used in Fabaceae to assess genetic diversity and population structure, providing critical insights for breeding and conservation strategies (Burstin et al. 2015;

Hong et al. 2021). Their efficiency has been demonstrated in several legume crops, including chickpea, pea, and groundnut, through applications in marker-assisted selection, QTL mapping, and genome-wide association studies (Varshney et al. 2019; Jurado et al. 2024; Istanbuli et al. 2024). Meta-QTL and RNA-seq integration further enhance the resolution of candidate gene discovery (Izquierdo et al. 2023), and a functional markers derived from these studies enable precise introgression of traits into elite cultivars. Genomic prediction models, such as those developed in *Medicago truncatula*, demonstrate the predictive power of genome-wide marker data (Gentzbittel et al. 2015). Moreover, recent advances integrate genome editing with marker-based trait targeting, offering a path toward accelerated trait fixation (Kumar et al. 2024). All together, molecular markers bridge genetic variation analysis and modern breeding tools, facilitating efficient improvement of Fabaceae crops across environments. Despite these advances, dominant markers remain highly relevant, particularly for species with limited genomic resources. Arbitrarily Amplified DNA (AAD) markers - such as ISSR, offer high discriminating power without requiring prior sequence information, whereas Targeted Fingerprinting Markers (TFMs), including SRAP preferentially amplify functional regions, improving both resolution and reproducibility (Li and Quiros 2001; Poczai et al. 2013). These complementary approaches continue to be widely applied in Fabaceae for diversity analysis, genetic structure assessment, and breeding purposes (Uzun et al. 2009; Alghamdi et al. 2012).

Despite being an old technique since 1994 (Zietkiewicz et al. 1994), numerous studies continue to highlight the effectiveness of ISSR markers. These markers are highly efficient in detecting molecular polymorphism without requiring prior sequence information, making them robust and cost-effective tools for assessing genetic diversity in various Fabaceae species (Poczai et al. 2013; Alzate-Marin et al. 2018; Qiang et al. 2018; Nurhasanah et al. 2023; Diallo et al. 2024). In addition, ISSR markers have proven useful in identifying mutant lines in evaluating responses to salt stress, and in supporting their relevance in marker-assisted breeding programs. Overall, ISSR markers offer high discriminating power and valuable applications in breeding and the management of genetic resources within the Fabaceae family.

While SRAP markers have proven to be powerful tools for detecting molecular polymorphism due to their ability to amplify open reading frames, they have been made highly suitable for targeting coding regions in plant genomes (Li and Quiros 2001; Uzun et al. 2009). In Fabaceae, SRAP markers revealed high levels of polymorphism and discriminated effectively among closely related accessions, as demonstrated in *Vicia faba* (Alghamdi et al. 2012). Their high reproducibility and informativeness make them ideal for assessing genetic structure, diversity, and evolutionary relationships (Li and Quiros 2001). Overall, SRAP markers offer a robust and gene-targeted molecular tool for genetic improvement and breeding programs in Fabaceae (Li and Quiros 2001; Alghamdi et al. 2012).

In the local clover species, particularly *Trifolium squarrosum* and *Trifolium juliani* (an endemic species of Tunisia and Algeria), accurate genetic characterization is essential due to their sympatric distribution and significant potential for forage improvement. Differentiating these taxa is a prerequisite for effective conservation strategies and for exploiting inter- and intra-specific genetic variability in breeding programs. The present study assessed genetic diversity and population structure in these species using ISSR and SRAP markers to establish a robust framework for their sustainable use and genetic enhancement.

Both marker systems were chosen for their proven efficiency in diversity studies and demonstrated high levels of polymorphism (> 73%) and substantial polymorphic information content (PIC = 0.382 for ISSR and 0.363 for SRAP), confirming their suitability for detecting molecular variation.. These results are consistent with previous reports on clover species (Abate and Tesfaye 2017; Abate 2017; Yousefi et al. 2018; Youssef et al. 2019; Mostafa Mohamed et al. 2024). Additionally, ISSR markers exhibited slightly higher values for genetic differentiation (H, PIC, EMR, MI, D, and Rp) than SRAP markers, corroborating the observations by Mostafa Mohamed et al. (2024).

The variations in genetic similarity observed across different marker types may reflect differences in their mechanisms which are provided to detect polymorphisms. It may also show the extent of the sequencing genome coverage. Consequently, genetic similarity derived from combined datasets offers a more comprehensive representation of genetic relationships. Unlike ISSR markers, which are randomly distributed across the genome, SRAP markers specifically target open reading frame (ORF) sequences, corresponding to relatively conserved coding regions, thereby focusing on the functional genomic regions. In contrast, random markers such as ISSR capture a broader range of molecular polymorphism can be considerably informative when differentiating closely related species. This difference may explain why ISSR markers provided slightly higher genetic differentiation values than SRAP in the present study.

The molecular analysis of variance (AMOVA) revealed a highly significant partitioning of genetic variation between the two species, confirming their clear differentiation. Intraspecific variance was comparatively low. Yet it highlights the presence of substantial genetic variability within each species. This structuring pattern was further supported by low estimates of gene flow and high genetic differentiation indices, reflecting the self-incompatibility system typical of clovers, favors outcrossing and limits interspecific hybridization (Casey et al. 2010).

Multivariate and clustering analyses consistently corroborated these findings. PCA and Ward’s hierarchical clustering separated all accessions into two major groups corresponding to T. juliani and T. squarrosum, while Bayesian inference identified K = 2 as the most probable number of clusters ($\Delta K > 200$, $H' = 0.9$). Most genotypes were assigned to their respective clusters with high probability (> 90%) although two genotypes (TJ-7 and TS-9) exhibited minor admixture signals, suggesting possible shared ancestry or historical gene flow. These complementary analyses provide strong evidence of species-level differentiation, while also revealing subtle genetic connections among certain genotypes.

Combining ISSR and SRAP data improved structural resolution by integrating signals from random and functional genomic regions, and by providing a holistic view of diversity. The concordance among these approaches further validates the robustness of our results and emphasizes the necessity of combining complementary marker systems in analyses of outcrossing species.

Overall, the clear genetic separation, despite some rare signs of admixture, shows the importance of using combined approaches that consider both species distinctness and occasional interspecific gene flow, to ensure effective conservation and targeted breeding strategies.

Conclusion

This study provides the first molecular characterization of *T. squarrosum ssp parnomitanum* and *T. juliani*, revealing high genetic diversity and a clear species-level distinction using ISSR and SRAP markers. The results highlight the value of complementary markers for studying outcrossing species. These findings establish a foundation for effective conservation and targeted breeding strategies to enhance the genetic potential of clover crops.

Future studies should integrate next-generation sequencing (NGS) and genome-wide association studies (GWAS) with traditional marker-based approaches to achieve high-resolution mapping of adaptive traits in clovers. Combining these genomic tools with interspecific hybridization and genomic selection will enhance the use of the genetic diversity identified in this study, enabling the development of resilient, high-performing forage cultivars tailored for sustainable agriculture.

Declarations

Acknowledgements

The authors are grateful for the valuable contribution of les Amis de CAPTE (Collectif d'Acteurs Pour la Transition Environnementale) for its support to the Project Trefles at Dyr El Kef entitled *"Ensemble pour la restauration des écosystèmes avec des agriculteurs locaux engagés à Dyr el Kef, Tunisie"* with was funded by the CEPF (Critical Ecosystem Partnership Fund) between 2021 and 2024.

The authors would like to express their sincere gratitude to Professor Zeineb Ghrabi-Gammarand her PhD student Emna Belaifa for their consistant contribution to the recognition of collected clover species based on their taxonomic skills.

The authors would like to express their sincere gratitude to Miss Dorra Ben Nasr for helping improve the clarity and flow of this research paper. She is an experienced English teacher who collaborates with several international institutions, including Bourguiba School, Tunisian Private University, and Eight Hospitality Business School. Her support and expertise were greatly appreciated throughout the writing process.

Author contributions

KG supervised and conducted the molecular experiments, performed data analysis, prepared the figures, interpreted the results, and drafted the entire manuscript. AZ-K conceived the study design, was involved in the overall supervision of the project, and contributed to manuscript revision and editing. NC-G supervised the molecular experiments and revised the manuscript. SSK and SBY contributed in all prospections to collect and identify Trifolium species. EJ and HC participated in the sampling of clover specimens for the study as part of the project and also contributed to manuscript revision. All authors have read and approved the final version of the manuscript.

Funding

This research was partially supportet by the Trefles Project hosted at INRAT (at the Laboratory of Animal and Forage Production) (LR16INRAT01) and managed by CAPTE and also benefited from logistical support

provided by the Laboratory of Molecular Genetics, Immunology and Biotechnology (LR99ES12) at the Faculty of Sciences of Tunis.

Data availability

All data supporting the findings are available within the manuscript.

Conflict of interest/competing interests

The authors declare that they have no known competing financial or non-financial interests related to the content of this article.

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Figures

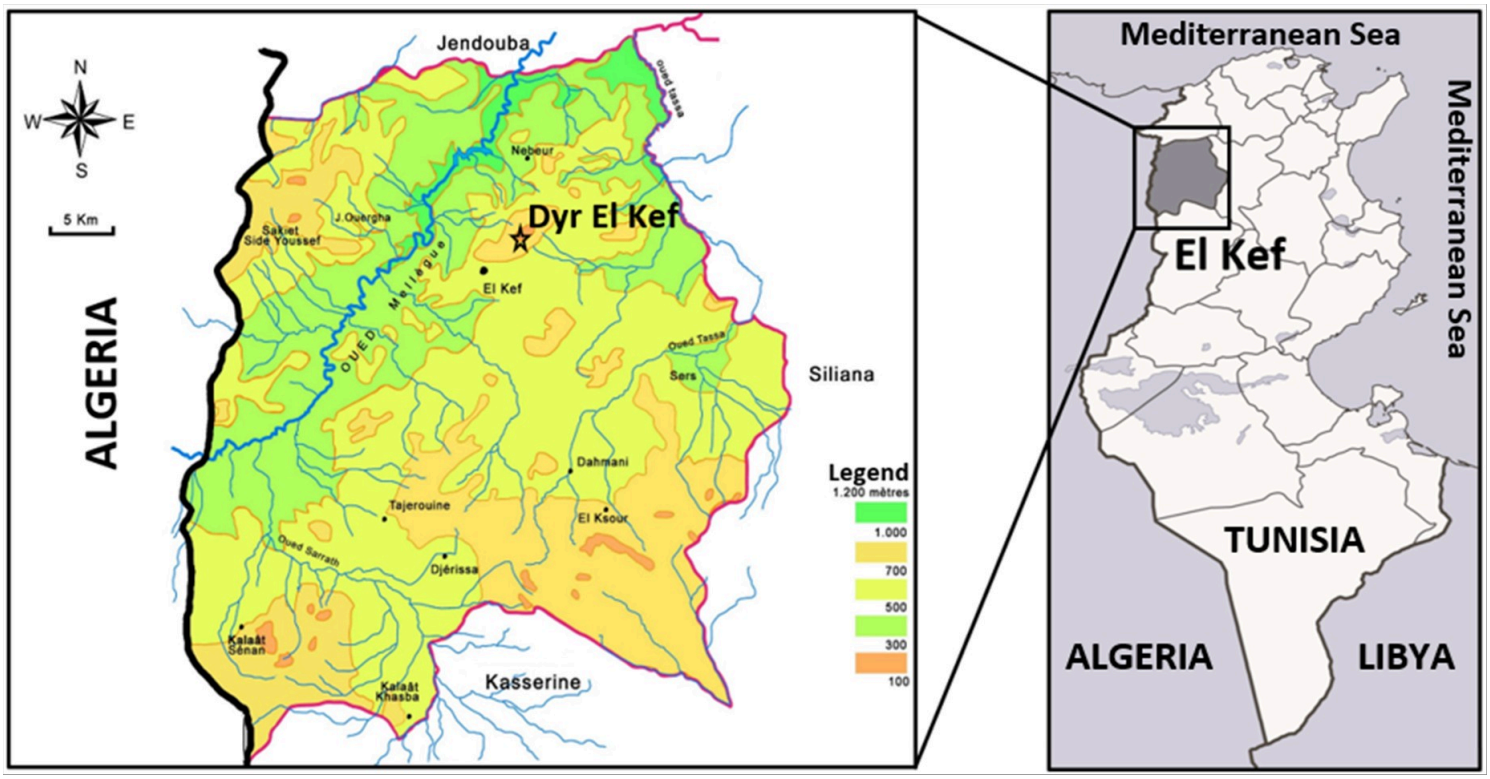

Figure 1

Localisation of Dyr El Kef (Tunisia)

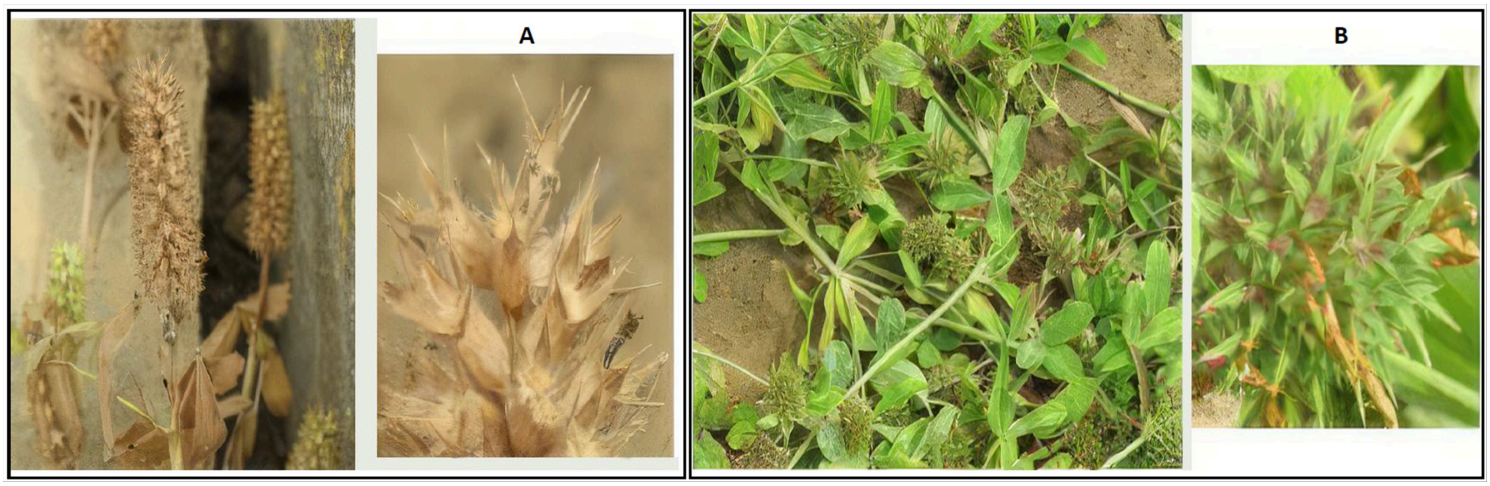

Figure 2

Trifolium juliani (A) and *Trifolium squarrosum ssp. parnomitanum* (B), distinguished in particular by their calyxes during recent surveyscollected in Dyr El Kef

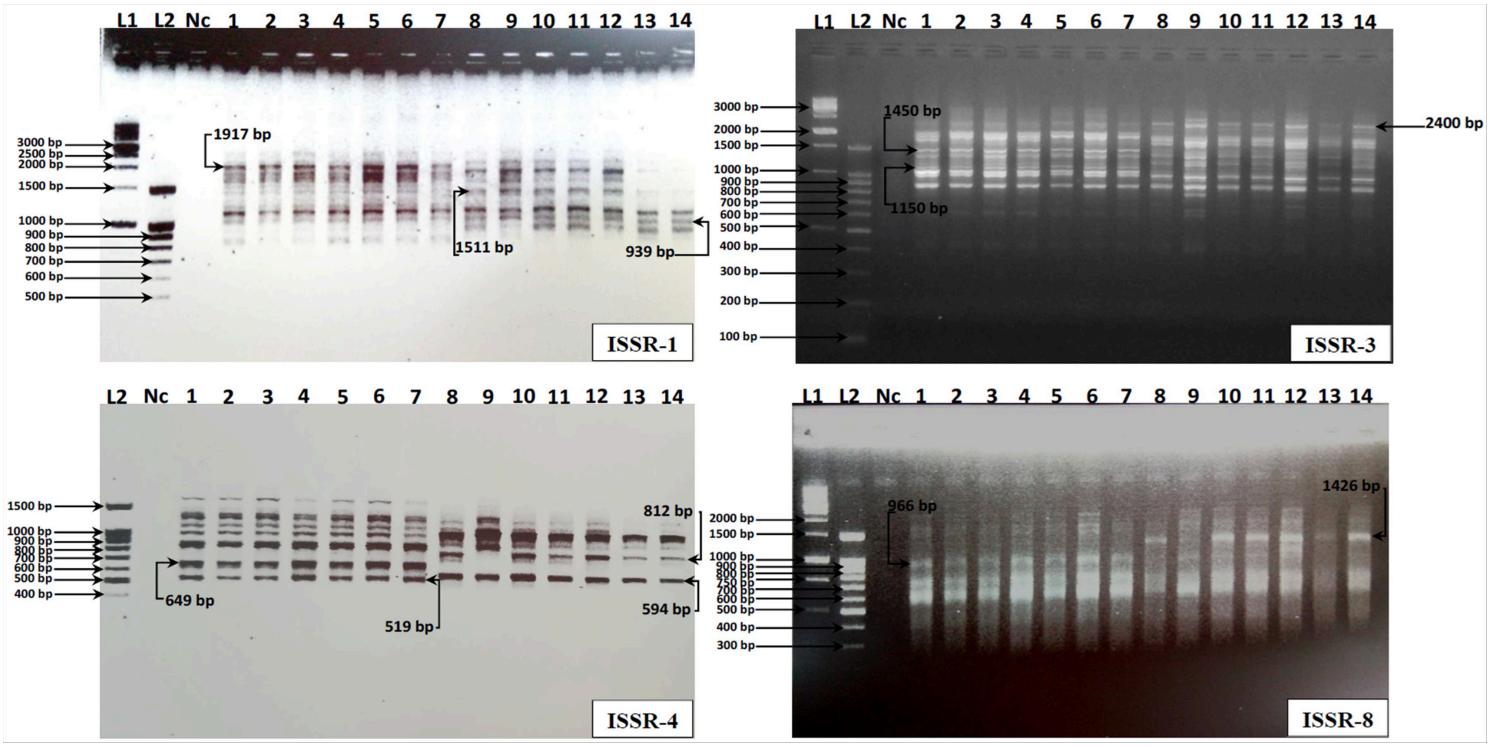

Figure 3

Amplification profiles of representative ISSR markers

Fig. 3 footnotes

L1: 1kb DNA Ladder (BLUE, GeneON, UK, for ISSR-1, ISSR-4 and ISSR-8 / Biomatik, USA, for ISSR-3)

L2: 100 pbDNA Ladder (Plus Blue, GeneON, UK, for ISSR-1, ISSR-4 and ISSR-8 / Biomatik, USA, for ISSR-3)

Nc: Negative control

Numbers 1 to 7 represent *Trifolium juliani* samples, and numbers 8 to 14 represent to *Trifolium squarrosum* samples. Polymorphic bands differentiating the two species are indicated on the gel images. Primer codes are listed in Table 1

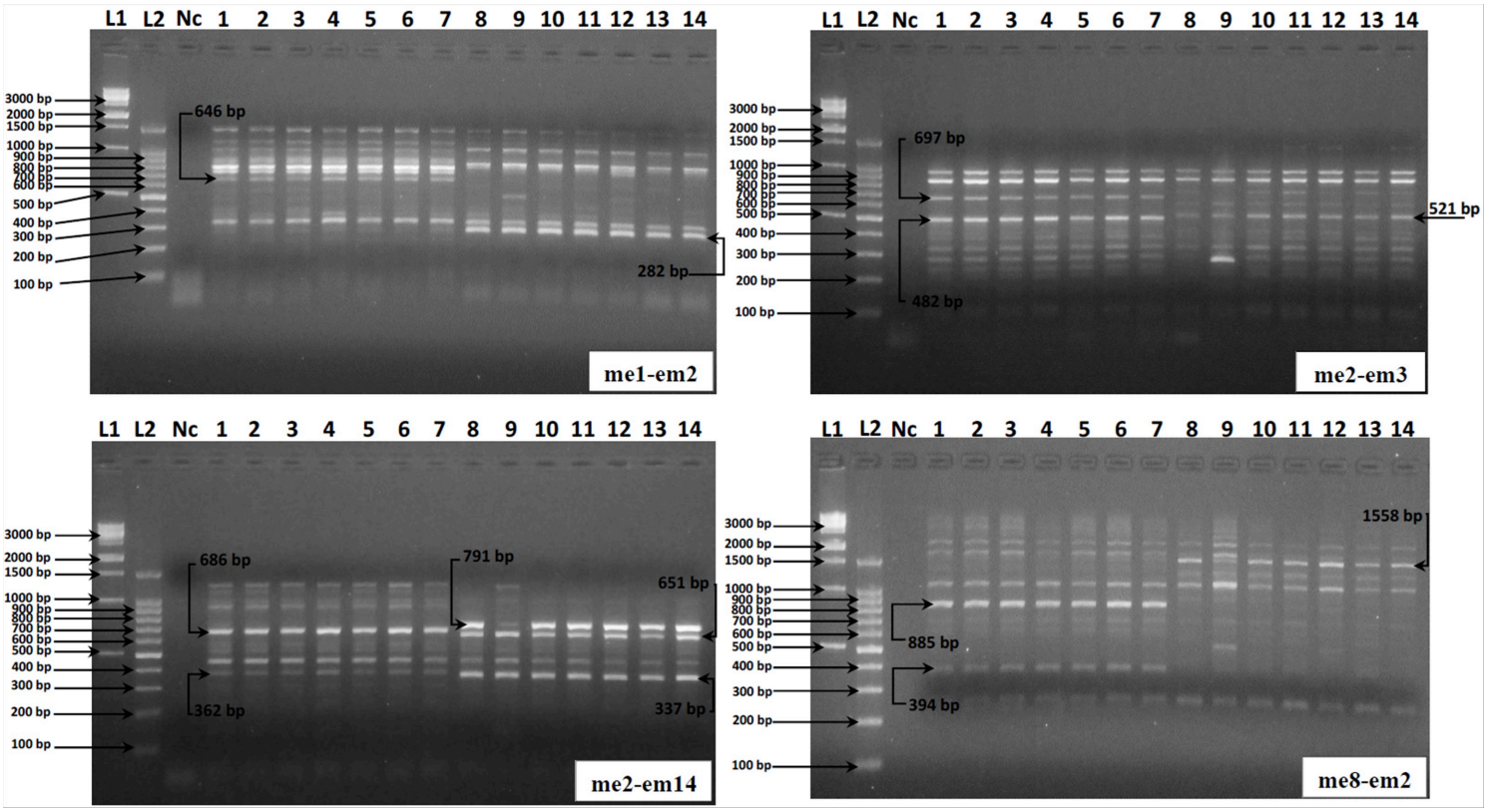

Figure 4

Amplification profiles of representative SRAP markers

Fig. 4 footnotes

L1: 1kb DNA Ladder (Biomatik, USA)

L2: 100 pb DNA Ladder (Biomatik, USA)

Nc: Negative control

Numbers 1 to 7 represent *Trifolium juliani* samples, and numbers 8 to 14 represent to *Trifolium squarrosum* samples. Polymorphic bands differentiating the two species are indicated on the gel images. Primer codes are provided in Table 1

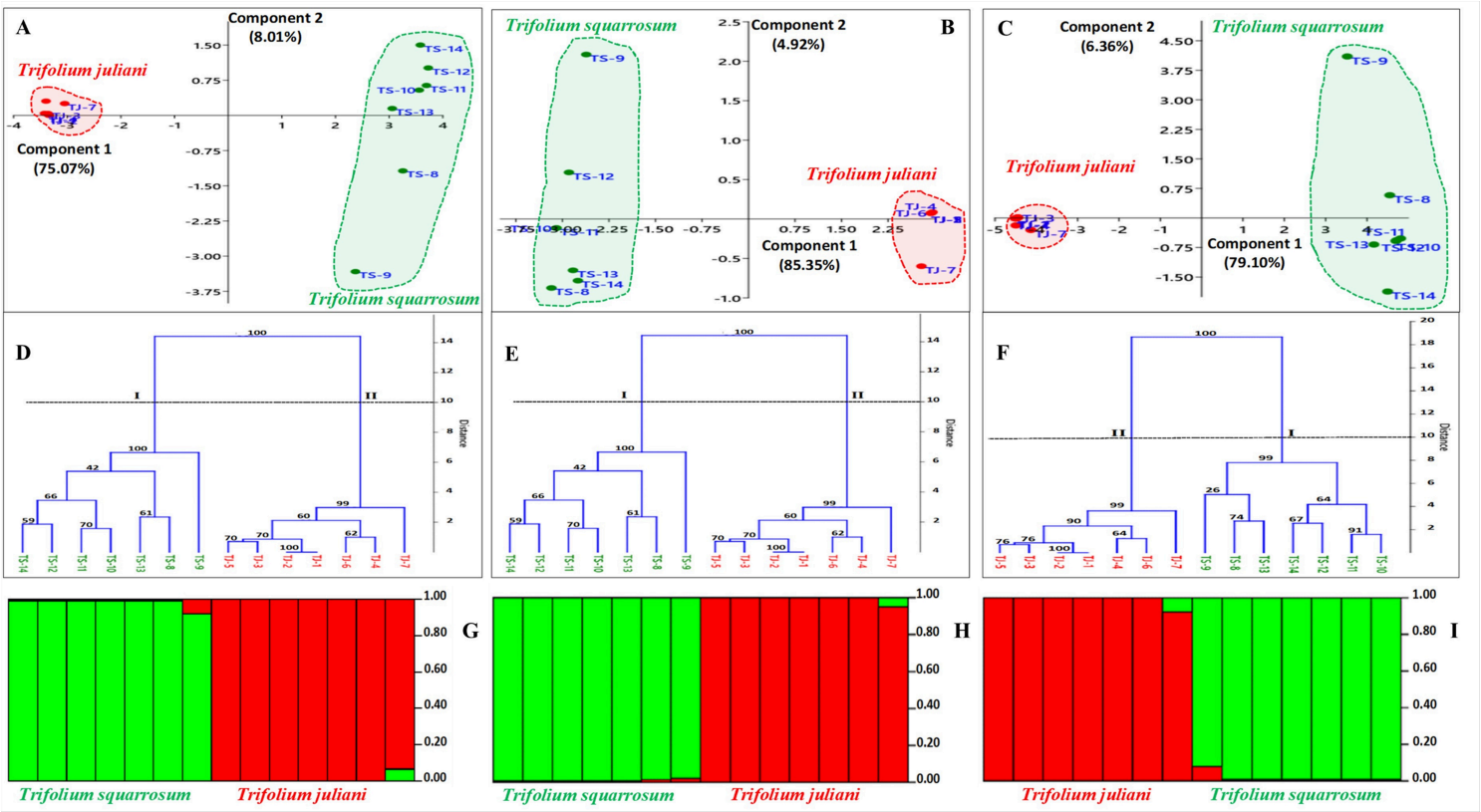

Figure 5

Genetic structure of two clover species, based on ISSR and SRAP molecular markers

Fig. 5 footnotes

TJ-1 to TJ-7 represent the seven individuals of *T. juliani*, and TS-8 to TS-13 represent the seven individuals of *T. squarrosum*.

A, B and C: Principal component analysis based on ISSR, SRAP, and combined marker data, respectively.

D, E and F: Hierarchical clustering based on ISSR, SRAP, and combined marker data, respectively.

G, H and I: Bayesian analysis implemented in *STRUCTURE*based on ISSR, SRAP and combined marker data, respectively