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Inter Simple Sequence Repeat (ISSR) Markers for Genetic Diversity Studies in Trifolium Species

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Abstract
Trfiolium species are economically important livestock forage and green manure crops and they can fix nitrogen through root nodulation by the bacterium *Rhizobium* leguminosarum. The development and utilization of molecular markers for the exploitation and identification of plant genetic diversity is one of the most key developments in the field of molecular genetics studies. Research on genetic diversity of trifolium species using molecular markers has been increasing over time for the management of germplasm and for evolving conservation strategies. DNA based markers are widely used, since their availability in potentially unlimited number and the property that they generally are not affected by developmental differences or environmental influences. This review discusses the principles of Inter simple sequence repeat (ISSR) marker and its application to genetic diversity studies of different Trifolium species.
Key words: Genetic diversity, ISSR markers, Trifolium

Introduction

Clover (Trifolium) is one of the largest genera belonging to the tribe Trifolieae under family Fabaceae (Nick *et al*., 2006).This genus consists about 250-300 species with a wide distribution and adaption to different agro-ecological regions (Gillet *et al*., 2001; Ellison *et al*., 2006). The three primary diversity centers for the genus Trifolium are located approximately in Turkey (Mediterranean centre), Ethiopia (African centre), and northern California, USA (American centre) (Zohary and Heller, 1984). Recent work supports the Mediterranean origin of the genus in the Early Miocene period, 16-23 million years ago (Ellison *et al*., 2006). It is one of economically important genus in the family of Fabaceae due to the wide growing of at least 16 species as livestock forage and green manure crops (Gillett and Taylor, 2001) and the capacity of over 125 species to fix nitrogen through root nodulation by the bacterium Rhizobium leguminosarum (Sprent, 2001). Most species belonging to genus Trifolium can tolerate wide variations in temperature, sunlight, and pH of soil. Many species of Trifolium are known to have been cultivated on a commercial scale including white and red clover (T. repens and T. pratense), the two most economically important pasture legumes in the UK (Taylor and Quesenberry, 1996).

In order to conserve genetic resources for plant improvement, it is necessary to preserve, maintain and document genetic diversity (Lane *et al*., 2000). Studies on DNA polymorphism are of great relevance in plant breeding since they give a deeper insight into genetic diversity. A number of PCR-based DNA markers have been developed in recent years to evaluate genetic variation at the intraspecific and interspecific levels (Wolfe and Liston, 1998). It is important, however, to understand that different markers have different properties and will reflect different aspects of genetic diversity (Karp and Edwards, 1995).

In Trifolium, primarily RAPDs have been utilized for the evaluation of genetic diversity in *T. pratense* (Ulloa *et al*., 2003), *T. resupinatum* (Arzani and Samei, 2004). However, the ISSR-PCR method (Wolfe and Liston, 1998) using primers based on di, tri, tetra, penta nucleotide repeats have the advantage over randomly amplified polymorphic DNA (RAPD) in that the primers are longer, allowing for more stringent annealing temperatures. These higher temperatures apparently provide a higher reproducibility of bands than in RAPD (Nagaoka and Ogihara, 1997). For the Trifolium species studied, the ISSR markers were used for the first time to study genetic diversity of three South American and three Eurasiatic species (Rizza *et al*., 2007). Research on genetic diversity of trifolium species using ISSR marker has been increasing over time. The research findings suggest that ISSR marker system applied are suitable for clover DNA polymorphism studies. This review discusses the principles of Inter simple sequence repeat (ISSR) marker and its application to genetic diversity studies of different Trifolium species.

Principles of Inter Simple Sequence Repeat (ISSR) markers

ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes. The primer can be based on any of the SSR motifs (di-, tri-, tetra- or penta-nucleotides) found at microsatellite loci giving a wide array of possible amplification products (Blair *et al*., 1999). The potential use of ISSR markers depends on the variety and frequency of microsatellites, which changes with the species and with the targeted SSR motifs (Morgante and Olivieri, 1993). In addition, the number of bands produced by an ISSR

primer with a given microsatellite repeat should reflect the relative frequency of that motif in the genome and would provide an estimate of the motif's abundance as an alternative for library hybridization (Blair *et al*., 1999). The amplification and data-scoring protocols used for ISSR markers are similar to those used for random amplified polymorphic DNA (RAPD) markers with the exception that ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher stringency (Wolfe and Liston, 1998). The annealing temperature depends on the GC content of the primer used and ranges from 45 to 65℃. The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. ISSR is a dominant marker like RAPD in which visible bands are assigned to genetic loci with two alleles: 1= presence and 0= absent but greater robustness in repeatability and generates larger number of polymorphisms per primer because variable regions in the genome are targeted (Ila *et al*., 2003; Kafkas *et al*., 2006). The sources of variation in ISSR markers could be: (1) mutations at the priming site (SSR), which could prevent Amplification of a fragment as in RAPD markers and thus give a presence/absence polymorphism. (2) An insertion/deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism depending on the amplifiability of the resulting fragment size. This method has a wide range of uses, including the characterization of genetic relatedness among populations (Ammiraju *et al*., 2001), genetic fingerprinting (Blair *et al*., 1999), gene tagging (Ammiraju *et al*., 2001), detection of clonal variation (Leroy and Leon, 2000), cultivar identification (Wang *et al*., 2009), phylogenetic analysis (Gupta *et al*., 2008), detection of genomic instability (for example, it was used in human quantification of genomic instability to estimate prognosis in colorectal cancer (Brenner, 2011), and assessment of hybridization (Wolfe *et al*., 1998).

Among molecular markers, ISSRs are widely used in genetic diversity studies because amplification in this technique does not require genome sequence information for designing the primer, and the primers are not proprietary and can be synthesized by any one, their development costs are low, and the laboratory procedures can easily be applied to any plant species (Aga *et al*., 2005; Tesfaye *et al*., 2013; Zietkiewicz *et al*., 1994). The method provides highly reproducible results and generates abundant polymorphisms in many systems. This technique can rapidly differentiate closely related individuals (Zietkiewicz *et al*., 1994) and have been successfully used to assess genetic diversity among closely related cultivars which were difficult to distinguish with other molecular marker (Dagani *et al*., 2003; Salhi-Hannachi *et al*., 2005; Okpul *et al*., 2005).

Application of Inter Simple Sequence Repeat (ISSR) markers in Trifolium genetic diversity study

ISSRs have been used to study genetic diversity among several species of Trifolium. Paplauskienė and Dabkevičienė, (2008) used ISSR markers to study red clover varieties genetic diversity using seven ISSR primers and scored 39–43 fragments were generated in the DNA profiles of the red clover varieties, of which 52.5–60.4% of the fragments were polymorphic. Comparison of the varieties revealed the lowest number of fragments in the individuals of the variety ‘Vyliai’ (39), of which 57.4% were polymorphic. In the DNA profiles of the individuals of the varieties ‘Liepsna’ and ‘Arimaičiai’ 43 fragments were identified. Research on the intravarietal genetic diversity suggests that plants of the variety ‘Lieps na’ are characterised by the greatest polymorphism (0.313), while ‘Arimaičiai’ has been found to be the most homogeneous variety (0.241). The genetic distance between the varieties varied from 0.010 to 0.458. According to Nei’s coefficient value, the greatest genetic distance was noted between the varieties ‘Vyliai’ and ‘Arimaičiai’ (0.177) and between ‘Liepsna’ and ‘Arimaičiai’ (0.181). Variety-specific DNA fragments were identified in some red clover varieties. Their experimental results showed that it is expedient to apply the ISSR method in red clover breeding schemes for revealing genetic diversity.

Dabkevičienė *et al*., (2011) used ISSR marker to assess the level and pattern of genetic diversity in four Trifolium species represented by two varieties, one breeding sample and two wild population of T. pratense, four wild populations of T. medium, two varieties and one population of T. resupinatum, and two varieties and three wild populations of T. repense. They observed 69.5% polymorphism in T. medium, 68.9% in T. resupinatum, 76.2% in T. pretense and 73.6% in T. repense. Dabkevičienė *et al*., (2012) used ISSR markers for genetic diversity study of T. hybridum varieties (‘Daubiai’, ‘Lomiai’, ‘Poliai’,‘Skriveru’). A total of 37 fragments were amplified, of which 62.2% were polymorphic. Genetically most distant were individuals of ‘Lomiai’ and ‘Daubiai’ cultivars.

Rizza *et al*., (2007) studied the genetic diversity of 34 genotypes from six Trifolium species (T. polymorphum Poir., T. riograndense Burkart , T. argentinense Speg, T. medium L., T. pretense L and T. repens L.) using ISSR markers. Six selected primers generated 186 polymerase chain reaction (PCR) products exploring 112 loci. These primers were able to discriminate among and within species, with the PCR products being on average 41.6% species-specific and 59.9% polymorphic at the within species level. The highest level of intra-species polymorphisms was detected in T. argentinense (92.9%), followed by T. medium (89.5%).

Aryanegad *et al*., (2013) characterized genetic variation of 14 accessions of three Trifolium species from Iran. By ten ISSR primer combinations resulting in 75 bands in total, all of which were polymorphic. They

found 60% polymorphism in T. fragiferum, 58.67% in T. hybridum and 77.32% in T. pratense. Their study revealed that the variation within species accounted for 71% of the total molecular variance. Cluster analysis and Scatter plot based on first and second axis from principal coordinate analysis for accessions, showed that the primers ISSR could clearly separate species and accessions of each species were placed to each other.

ISSR markers have been used in recent times for genetic diversity studies of trifolium species: Hwang and Huh, 2016 studied the genetic diversity of five species of Trifolium L. (*T. repens L.*, *T. pretense L.*, *T. hybridum L.*, *T. campestre* Schreb., and *T. dubium* Sibth.) by using inter-simple sequence repeats (ISSR) markers with 13 ISSR primers. A total of 114 bands were produced by ISSR markers, of which 77 (67.5%) bands were polymorphic. Overall, T. pratense exhibited higher variation than other species. T. dubium showed the low genetic variation. Tadesse *et al*., (2017) studied the genetic diversity of 48 accessions of *T. steudneri*, representing two populations from Ethiopia. Four selected ISSR primer combinations generated a total of 65 bands, of which 63 (96.92%) were polymorphic. Dendrograms based on ISSR analyses of 48 accessions of *T.steudneri* species revealed a clear assignment of all the accessions according to the geographical origin where they were sampled. Tadesse *et al*., 2017 also studied the genetic diversity of 24 accessions of Trifolium quartinianum, representing three populations from Ethiopia by four ISSR primers. A total of 84 bands were amplified by the four di-nucleotid ISSR primers in the overall experimental materials. Genetic diversity was high at the species level (PPL = 100%, h = 0.29, I = 0.44). Comparison of population-based genetic diversity showed that Gojam population was the most diverse. Analysis of molecular variance (MOVA) revealed high level of within-population variation with 83.13%. This could be caused by high pollen and seed flow among-populations.

Conclusion

Information on genetic diversity for Trifolium is valued for the management of germplasm and for evolving conservation strategies. The research findings suggest that ISSR marker systems applied are suitable for clover DNA polymorphism studies. The studies clearly indicated the presence of variable genotypes with their unique identity that deserve conservation attention. ISSR marker will increasingly be important enabling genetic studies of these and other trifolium species addressing questions regarding the evolutionary origin, centers of diversity, domestication, genetic structure of populations, characterization of germplasm and establishing markers for important agronomic traits. Despite this, the publications involving genetic diversity measurement using ISSR markers are still rare in trifolium species, especially due to the high number of species of this genus. The results will considerably support germplasm collection and maintenance strategies and enable the development of improved breeding methods.

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