Bangladesh J. Plant Taxon. **23**(2): 167-173, 2016 (December)
© 2016 Bangladesh Association of Plant Taxonomists

GENETIC DIVERSITY AND PHENETIC RELATIONSHIPS OF FIVE *TRIFOLIUM* L. SPECIES (FABACEAE) BY INTER SIMPLE SEQUENCE REPEATS MARKERS

YOURANG HWANG AND MAN KYU HUH[1]

Department of Molecular Biology, Dong-eui University, 995 Eomgwangno, Busanjin-gu, Busan 614-714, Korea

Keywords: Genetic variation; Inter simple sequence repeats; *Trifolium*.

Abstract

Five species of *Trifolium* L. (*T. repens* L., *T. pretense* L., *T. hybridum* L., *T. campestre* Schreb., and *T. dubium* Sibth.) were analyzed used to evaluate the genetic diversity and their phenetic relationships using inter-simple sequence repeats (ISSR) markers. Overall, *T. pratense* exhibited higher variation than other species. 114 amplicons were produced by ISSR markers, of which 77 (67.5%) bands were polymorphic. *T. dubium* showed the low genetic variation. Total genetic diversity values (H_T) varied between 0.333 and 0.487, for an average over all polymorphic loci of 0.282. On a per-locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) was 0.380. This indicated that about 38.0% of the total variation was among species. The estimate of gene flow, based on G_{ST}, was very low among species of genus *Trifolium* (N_m = 0.816). An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 95.8% the total genetic diversity was within species. *T. pratense* and *T. hybridum* were grouped together and this clade was sister with *T. repens*. Two remainder species with yellow flowers were grouped together. Information on genetic diversity for *Trifolium* is valued for the management of germplasm and for evolving conservation strategies.

Introduction

Trifolium L., the clover genus, is one of the largest genera in Fabaceae family. 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). *Trifolium repens*, also known as White or Dutch clover, originated in the Mediterranean region and quickly spread throughout Europe (Baker and Williams, 1987; Lane *et al*., 1997). White clover is adapted to a wide climate range from the Arctic to the subtropics and has a wide altitudinal ranges. It is found up as 6000 m in the Himalaya Range (Baker and Williams, 1987). It has also become naturalized in China, Mongolia, Korea, and Japan.

The genus *Trifolium* includes more than 20 clover cultivated species as forages (Hirano, 2005). White clover (*T. repens*) in Korea has been introduced from Europe about two hundred years ago. Most species belonging to genus *Trifolium* can tolerate wide variations in temperature, sunlight, and pH of soil. With the recent development of organic farming, legumes have been considered candidates of fertilizer (Paplauskiene and Dabkeviciene, 2012). 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). However, *Trifolium* is one of major weeds for lawns, farming fields, and golf courses in Korea. Especially many plants of *Trifolium* are also considered to cause

[1] Corresponding author. E-mail: mkhuh@deu.ac.kr

damage to the environment and have gradually the superior competitive ability on golf courses to create fairways and teeing areas.

Alsike clover (*T. hybridum*), field clover (*T. campestre*), suckling clover (*T. dubium*), are European grassland legumes that have spread to many parts of the world. Recently they have been also introduced to Korea. These non-native clovers can rapidly invade and dominate vegetated and bare areas in Korea (Huh *et al.*, 2005).

Many molecular marker techniques have been developed and they have been extensively used in plant systematic studies, measurement of variation to establish evolutionary relationships within or among species, and population genetic research (Hu and Vick, 2003; Gupta and Rustgi, 2004; Rizza *et al*., 2007). Inter simple sequence repeats (ISSR) markers have the advantage over randomly amplified polymorphic DNA (RAPD) in that the primers are longer, allowing for more stringent annealing temperatures (Wolfe and Liston, 1998). These higher temperatures apparently provide a higher reproducibility of bands than in RAPD (Nagaoka and Ogihara, 1997). Tsumura *et al.* (1996) found that most of their ISSR bands (96%) segregated according to Mendelian expectations.

The aim of this study was the estimation of population structure, genetic diversity, and genetic relationships of five clover species in Korea.

Materials and Methods

Plant materials

Five clover species, *T. repens* L., *T. pratense* L., *T. hybridum* L., *T. campestre* Schreb., and *T. dubium* Sibth. were used for ISSR analysis (Table 1). Thirty plants were collected for each species. Within populations, plants are genetically subdivided by micro-environmental heterogeneity (Sackville and Chorlton, 1995). Clover has a creeping growth habit and spreads with stolons or runners above the soil with adventitious roots forming at each node. The geographic distance between the selected individuals was about 1.0 m to avoid inclusion of individuals emanating from the same rhizome. *Medicago sativa* L. was used as an outgroup species in this study.

DNA extraction

Total genomic DNA was extracted from a fresh young leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol. Briefly, Approximately 1.2 g fresh leaves per individual was ground to fine powder in liquid nitrogen with a mortar and pestle. The pulverized material was transferred to a micro-tube and Plant DNA Zol solution was added. The sample was shaken gently at room temperature for 10 min. After adding 24:1 chloroform/isoamyl alcohol, the sample was centrifuged at 12,000 g. The DNA precipitate was recovered with 70% ethanol, dried, and dissolved in TE buffer. The extracted DNA concentrations were calculated with a fluorometer (DyNA Quant 200, Hoefler, Amersham Biosciences, USA) using bisbenzimide (Amersham Biosciences, USA) as the fluorescent dye.

ISSR analysis

The ISSR amplification assay developed by Zietkiewicz *et al.* (1994) using primers listed in Table 1. PCR was performed within a total volume of 25 □ using a PTC-100 DNA Engine Dyad Peltier thermal cycler (MJ Research, Watertown, MA, USA). Each PCR mixture contained PCR buffer (Promega; 20 mM This-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.24 mM of each dNTP, 12.5 pmol of each primer, 0.25 units of BIOTAQ DNA polymerase (Bioline), and 25 ng of genomic

DNA. An initial denaturation step of 5 min at 94ºC was followed by 30 cycles of amplification (1 min sec at 94ºC, 1 min at 50ºC, 1.5 min at 72ºC) and a final elongation step of 10 min at 72ºC.

The amplification products were separated by electrophoresis on 2.0% agarose gels in Tris-Borate buffer, and stained with ethidium bromide. A 100 bp ladder DNA marker (Pharmacia) was used in the end of for the estimation of fragment size.

Statistical analyses

PCR-amplified ISSR fragments detected on gels were scored absent (0) or present (1). Only unambiguously reproducible bands were scored and used in the analyses. The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh *et al*. (1999): the percentage of polymorphic loci (P_p), mean numbers of alleles per locus (*A*), effective number of alleles per locus (A_e), and gene diversity (*H*) (Nei, 1973) and Shannon's index (*I*) of phenotypic diversity. Shannon–Weaver index of diversity (Shannon and Weaver, 1963): the formula for calculating the Shannon diversity index (H') is:

$$H' = -\Sigma\, pi \ln pi$$

*p*i is the proportion of important value of the *i*th species (*p*i = *n*i / N, *n*i is the important value index of *i*th species and N is the important value index of all the species).

Polymorphism information content (PIC) value was calculated using the formula PIC, PIC = 1 - p^2- q^2, where p = band frequency and q = no-band frequency (Rizza *et al*., 2007).

Nei's gene diversity formulae (H_T, H_S, and G_{ST}) were used to evaluate genetic diversity within and among cultivars (Nei, 1973). H_T is the expected heterozygosity of an individual in an equivalent random mating total interspecies. H_S is the expected heterozygosity of an individual in an equivalent random mating total intraspecies. The G_{ST} coefficient corresponds to the relative amount of differentiation among cultivars. Furthermore, gene flow (*Nm*) between the pairs of species was calculated from G_{ST} values by $Nm = 0.5(1/G_{ST} - 1)$ (McDermott and McDonald, 1993).

Shannon's index of genotypic diversity (H_O) for ISSR was estimated to quantity the degree of within species diversity following the formula (Bowman *et al*., 1971): $H_O = -\sum pi \log pi$, where *pi* is the frequency of a particular phenotype *i*.

A phenetic relationship was constructed by the neighbor-joining (NJ) method in PHYLIP version 3.57 using MEGA5 program (Tamura *et al*., 2011). Parsimony analyses were conducted using PAUP* *4.0b3a* (Swofford, 1999). Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data.

Results and Discussion

From the 20 decamer primers used for a primary ISSR analysis, thirteen primers produced good amplification products both in quality and variability (Table 1). The remaining primers either did not amplify or showed unclear amplification across all genotypes. 114 amplicons were produced by ISSR marker, of which 77 (67.5%) bands were polymorphic. Polymorphism information content (PIC) for ISSR primers ranged from 0.244 to 0.498 with an average of 0.287 per primer.

In a simple measure of inter-cultivars variability i.e. the percentage of polymorphic bands, *T. pratense* exhibited the highest variation (49.1%) among clovers and *T. dubium* the lowest (36.0%) (Table 2). The average number of alleles per locus (*A*) was 1.423 across species, varying from 1.360 to 1.491. The effective numbers of alleles per locus (A_E) was 1.311 across species, varying from 1.251 to 1.374. The mean genetic diversity within species was 0.175. Shannon's index of phenotypic diversity (*I*) of *T. pratense* (0.302) was highest of all taxa and *T. hybridum* was the

second (0.286). Overall, *T. pratense* exhibited higher variation than other species. Two species (*T. campestre* and *T. dubium*) with yellow flowers were shown the low genetic variation.

The first fragment (ISSR-06-01) of primer ISSR-01 was specific band for *T. repens* which did not show at other species. The ISSR-01-04 fragment of primer ISSR-01 was also specific band for *T. pratense*. These specific fragments seemed to be useful markers to discriminate among species.

Table 1. List of decamer oligonucleotide utilized as primers, their sequences, and associated polymorphic fragments.

No. of Primer	Sequence(5' to 3')	No. of fragments detected	Percentage of polymorphism bands	PIC
ISSR-01	$(AG)_8G$	11	9	0.489
ISSR-02	$(CA)_8RG$	8	8	0.498
ISSR-03	$(GA)_8GT$	10	7	0.458
ISSR-04	$(GA)_8CG$	7	4	0.452
ISSR-05	$(GA)_8GT$	10	7	0.328
ISSR-06	$(GA)_8CG$	11	7	0.418
ISSR-07	$(GA)_8TC$	9	6	0.452
ISSR-08	$(GA)_8TC$	7	5	0.408
ISSR-09	$GCGA(AC)_8$	9	7	0.285
ISSR-10	$GCGA(CA)_8$	7	4	0.328
ISSR-11	$CCGG(AC)_8$	10	5	0.275
ISSR-12	AGAGTTGGTAGCTCTTG ATC	8	4	0.244
ISSR-13	$(AC)_8T$	7	4	0.310
Total	-	114	77	0.287

Table 2. Measurements of genetic variation for five clover species used in this study. The number of polymorphic loci (*N*p), percentage of polymorphism (*P*p), mean number of alleles per locus (*A*), effective number of alleles per locus (A_E), gene diversity (*H*), and Shannon's information index (*I*).

Species	N_P	P_P	*A*	*Ae*	*H*	*I*
Trifolium repens	48	42.1	1.421	1.309	0.173	0.252
Trifolium hybridum	53	46.5	1.465	1.360	0.198	0.286
Trifolium pratense	56	49.1	1.491	1.374	0.209	0.302
Trifolium campestre	43	37.7	1.377	1.251	0.147	0.217
Trifolium dubium	41	36.0	1.360	1.259	0.147	0.215
Mean	48.2	42.3	1.423	0.311	0.175	0.254

Total genetic diversity values (H_T) for polymorphic loci varied between 0.333 (ISSR-04) and 0.487 (ISSR-12) (Table 3). An average (H_T) over all 114 loci for five species with 13 ISSR primers was 0.282. In interlocus variation in the within-species, mean genetic diversity (H_S) was low (0.175). On a per-locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) ranged from 0.216 for ISSR-08 to 0.547 for ISSR-02, with a mean of 0.380. This indicated that about 38.0% of the total variation was among species. Thus, about genetic variation (62.0%) resided within species. The estimate of gene flow, based on G_{ST}, was very low

among species (N_m = 0.816). Values of genetic distance (D) were ≤ 0.233 (Table 4). Genetic identity values among pairs of species ranged from 0.508 to 0.956.

Table 3. Estimates of genetic diversity of five selected clover species in Korea. Total genetic diversity (H_T), genetic diversity within populations (H_S), the proportion of total genetic diversity partitioned among populations (G_{ST}), and gene flow (Nm).

Primer	H_T	H_S	G_{ST}	Nm
ISSR-01	0.405	0.231	0.422	1.821
ISSR-02	0.428	0.186	0.547	0.778
ISSR-03	0.380	0.217	0.420	1.097
ISSR-04	0.333	0.199	0.304	5.631
ISSR-05	0.409	0.197	0.527	3.061
ISSR-06	0.395	0.264	0.358	7.133
ISSR-07	0.432	0.294	0.324	4.266
ISSR-08	0.474	0.368	0.216	5.405
ISSR-09	0.386	0.265	0.331	2.279
ISSR-10	0.453	0.338	0.242	2.880
ISSR-11	0.474	0.317	0.328	2.791
ISSR-12	0.487	0.367	0.248	2.917
ISSR-13	0.427	0.247	0.413	0.871
Total mean	0.282	0.175	0.380	0.816

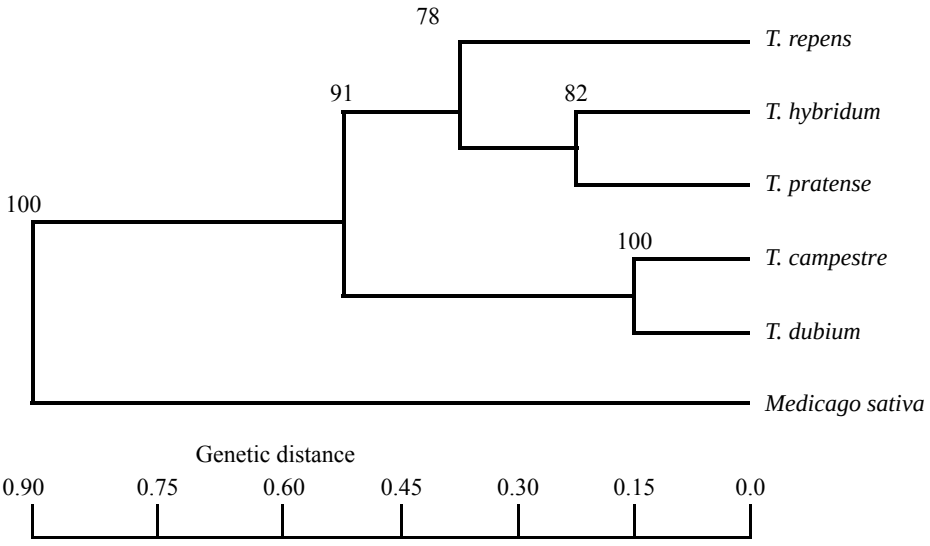

Fig. 1. A phentic tree for five selected species with one outgroup based on ISSR analysis. Numbers above branches are jackknife values derived from heuriatic-based searches on sequences data.

Table 4. Genetic identity (upper diagonal) among five selected clover species and genetic distances (low diagonal) based on ISSR analysis.

Species	*T. repens*	*T. hybridum*	*T. pratense*	*T. campestre*	*T. dubium*
T. repens	-	0.815	0.849	0.815	0.807
T. hybridum	0.205	-	0.866	0.508	0.792
T. pratense	0.164	0.144	-	0.833	0.836
T. campestre	0.205	0.217	0.183	-	0.956
T. dubium	0.214	0.233	0.179	0.045	-

Table 5. Partitioning of the genetic diversity into within and among genus *Trifolium* in Korea.

Primer	H_{VAR}	H_{SP}	H_{VAR} / H_{SP}	(H_{SP} - H_{VAR})/ H_{SP}
ISSR-01	2.110	2.306	0.915	0.085
ISSR-02	1.874	1.901	0.986	0.014
ISSR-03	2.057	2.207	0.932	0.068
ISSR-04	1.660	1.806	0.919	0.081
ISSR-05	2.209	2.277	0.970	0.030
ISSR-06	2.187	2.317	0.944	0.056
ISSR-07	2.043	2.138	0.955	0.045
ISSR-08	1.886	1.914	0.986	0.014
ISSR-09	2.109	2.182	0.967	0.033
ISSR-10	1.771	1.912	0.926	0.074
ISSR-11	2.263	2.279	0.993	0.007
ISSR-12	2.052	2.064	0.994	0.006
ISSR-13	1.848	1.907	0.969	0.031
Total mean	2.005	2.093	0.958	0.042

An assessment of the proportion of diversity present within species, H_{VAR}/H_{SP}, indicated that about 95.8% the total genetic diversity was within species. Thus, the other portion of genetic variation (4.2%) resided within genus (Table 5). The result was lower than that (G_{ST}) of *F*-statistics.

Clustering of five cultivars, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). Five species were well separated each other. *T. pratense* and *T. hybridum* were grouped together and this clade was sister with *T. repens*. Two remainder species, *T. campestre* and *T. dubium* with yellow flowers were grouped together.

References

Baker, M.J. and Williams, W.M. 1987. White Clover. CAB International, Wallingford, UK, pp. 299-322.

Bowman, K.D., Hutcheson, K., Odum, E.P. and Shenton, L.R. 1971. Comments on the distribution of indices of diversity. Stat. Ecol. **3**: 315-359.

Ellison, N.E., Liston, A., Steiner, J.J., Williams, W.M. and Taylor, N.L. 2006. Molecular phylogenetics of the clover genus (*Trifolium-Leguminosae*). Mol. Phylogenet. Evol. **39**: 688-705.

Gillet, J.M., Collins, M. and Taylor, N.J. 2001. The World of Clovers. Iowa State University Press, Ames., pp. 457.

Gupta, P.K. and Rustgi, S. 2004. Molecular markers from the transcribed/expressed region of the genome in higher plants. Funct. Integr. Genomics **4**: 139-162.

Hirano, R. 2005. Ecogeographic and genetic survey of white clover (*Trifolium repens* L.) on St Kilda. Thesis of Master, University of Birmingham, UK, pp. 1-85.

Hu, J. and Vick, B.A. 2003. Target region amplification polymorphism: a novel marker technique for plant genotyping. Plant Mol. Biol. Report **21**: 289-294.

Huh, M.K., Chung, K.T., and Jeong, Y.K. 2005. Genetic variation of alien invasive red clover (*Trifolium pratense*) in Korea. J. Life Sci. **15**: 273-278.

Lane, L.A., Ayes, J.F. and Lovett, J.V. 1997. A review of the introduction and use of white clover (*Trifolium repens* L.) in Australia – significance for breeding objectives. Australian Journal of Experimental Agriculture **37**: 831-839.

Nagaoka, T. and Ogihara, Y. 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. **94**: 597-602.

McDermott, J.M. and McDonald, B.A. 1993. Gene flow in plant pathosystems. Ann. Rev. Phytopathy. **31**: 353-373.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA **701**: 3321-3323.

Paplauskienė, V. and Dabkevičienė, G. 2012. A study of genetic diversity in *Trifolium hybridum* varieties using morphological characters and ISSR markers. Žemdirbystė-Agriculture, **99**: 313-318.

Rizza, M.D., Real, D., Reyno, R., Porro, V., Burgueno, J., Errico, E. and Quesenberry, K.H. 2007. Genetic diversity and DNA content of three South American and three Eurasiatic *Trifolium* species. Genetics and Molecular Biology **30**: 1118-1124.

Sackville, H. and Chorlton, N.R. 1995. Collecting Plant Genetic Diversity. CAB International, Wallingford, Oxford, pp. 467-483.

Shannon, C. E. and Weaver, W. 1963. The Measurement Theory of Communication. Univ. of Illinois Press, Urbana, 1-132.

Swofford, D. L. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Ver. 4.0b3a. Sinauer Associates,Sunderland, Massachusetts.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**: 2731-2739.

Taylor, N.L. and Quesenberry, K.H. 1996. Red Clover Science, Kluwer, Boston, MA. pp. 11-24.

Tsumura, Y., Ohba, K. and Strauss, S.H. 1996. Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theor. Appl. Genet. **93**: 40-45.

Wolfe, A.D. and Liston, A. 1998. Molecular Systematics of Plants: DNA Sequencing, Kluwer, New York, USA, pp. 43-86.

Yeh, F.C., Yang, R.C. and Boyle, T. 1999. POPGENE Version 1.31, Microsoft Windows-based Freeware for Population Genetic Analysis. University of Alberta, Alberta, pp. 1-228.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics **20**: 176-183.

(Manuscript received on 26 April 2016; revised on 1 September 2016)