ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Characterization of Genetic Structure of *Trifolium repens, T. pratense,* and *T. hybridum* Using Inter Simple Sequence Repeats (ISSR) Markers

Youna Lee, and Man Kyu Huh*.

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

ABSTRACT

Inter simple sequence repeats (ISSR) markers were used to evaluation of genetic diversity of three clover species, *Trifolium repens, T. pratense,* and *T. hybridum*, from the South Korea. 71 amplicons were produced by ISSR markers, of which 56 (78.9%) bands were polymorphic. Polymorphism information content (PIC) for ISSR primers ranged from 0.815 to 0.906 with an average of 0.878 per primer. Overall, *T. pratense* exhibited higher variation than other species. *T. repens* was shown the low genetic variation. Total genetic diversity values (HT) varied between 0.358 (ISSR-04) and 0.450 (ISSR-02), for an average over all polymorphic loci of 0.320. On a per-locus basis, the proportion of total genetic variation due to differences among species (GST) was 0.450. This indicated that about 45.0% of the total variation was among species. The estimate of gene flow, based on GST, was very low among species of genus Trifolium (Nm = 0.610). An assessment of the proportion of diversity present within species, HPOP/HSP, indicated that about 91.1% the total genetic diversity was among species. Thus, the other portion of genetic variation (8.9%) resided within species. Three species were well separated each other by neighbor-joining (NJ) method. *T. pratense and T. hybridum* were grouped together and this clade was sister with T. repens.

Keywords: inter simple sequence repeats, clover species, genetic variation

Corresponding author

INTRODUCTION

The genus *Trifolium* consists about 250-300 species with a wide distribution and adaption to different agro-ecological regions [1, 2]. The origin of white clover is assuming the Mediterranean region [3]. 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 [3]. It has also become naturalized in China, Mongolia, Korea, and Japan.

The genus *Trifolium* includes more than 20 clover cultivated species as forages [4]. White clover (*T. repens* L.) in Korea has been introduced from Europe about two hundreds ago. Most species belonging 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 [5]. 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 [6]. However, *Trifolium* is one of major weeds for lawns in Korea. Many plants of *Trifolium* are also considered to cause damage to the environment and have gradually the superior competitive ability on golf courses to create fairways and teeing areas.

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 [7, 8, 9]. 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 [10]. These higher temperatures apparently provide a higher reproducibility of bands than in RAPD [11]. Tsumura et al. [12] 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 three clover species in Korea.

MATERIALS AND METHODS

Plant materials and preparation of DNA

Three clover species, *T. repens* L., *T. pratense* L., and *T. hybridum* L., were used for ISSR analysis (Table 1). Thirty plants were collected from each species. Within populations, plants are genetically subdivided by microenvironmental heterogeneity [13]. Collection of ramets from the same linage, plants were collect as far as possible from each another. *Medicago sativa* L. was used as an outgroup species in this study.

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 leaf tissue 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. [14] using primers listed in Table 1. PCR was performed within a total volume of 25 $\mu\ell$ 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 94°C was followed by 30 cycles of amplification (1 min sec at 94°C, 1 min at 50°C, 1.5 min at 72°C) and a final elongation step of 10 min at 72°C.

The amplification products were separated by electrophoresis on 2.0% agarose gels in Tris-Borate

ISSN: 0975-8585

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*. [15]: 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) [16] and Shannon's index (I) of phenotypic diversity. Polymorphism information content (PIC) value was calculated using the formula PIC, $1 - \sum pi^2$, where pi is the frequency of the ith allele detected in all individuals of the species; for ISSR values PIC = $1 - p^2 - q^2$, where p = band frequency and q = no-band frequency [17].

Nei's gene diversity formulae (H_T, H_S, and G_{ST}) were used to evaluate genetic diversity within and among cultivars [16]. 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 $Nm = 0.5(1/G_{ST} - 1)$ [18].

Nei's genetic identity and genetic distance between genotypes were based on the probability that an amplified fragment from one individual will also be present in another [16].

Shannon's index of genotypic diversity (H_O) for ISSR was estimated to quantity the degree of within species diversity following the formula [19]: $H_O = -\sum pi \log pi$, where pi is the frequency of a particular phenotype i.

H_O can be calculated and compared for different populations [20]. Let

$$H_{POP} = 1/n\ H_O$$

be the average diversity over the n different populations and let

$$H_{SP} = -\sum p \log p$$

be the diversity of species calculated from the phenotypic frequencies p in all the species considered together (Paul et al., 1997). Then the proportion of diversity within species, H_{POP}/H_{SP}, can compared with that between species $(H_{SP}-H_{POP})/H_{SP}$.

A phenetic relationship was constructed by the neighbor-joining (NJ) method in PHYLIP version 3.57 using MEGA5 program [21].

RESULTS

From the 20 decamer primers used for a primary ISSR analysis, seven 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. 71 amplicons were produced by ISSR marker, of which 56 (78.9%) bands were polymorphic.

Polymorphism information content (PIC) for ISSR primers ranged from 0.815 to 0.906 with an average of 0.878 per primer.

In a simple measure of inter-cultivars variability i.e. the percentage of polymorphic bands, *T. pratense* exhibited the highest variation (50.7%) among clovers and *T. repens* the lowest (33.6%) (Table 2). The average number of alleles per locus (A) was 1.446 across species, varying from 1.507 to 1.366. The effective numbers of

alleles per locus (A_E) was 1.312 across species, varying from 1.347 to 1.248. The mean genetic diversity within species was 0.176. Shannon's index of phenotypic diversity (*I*) of *T. pratense* (0.267) was highest of all taxa and *T. repens* was the second (0.210). Overall, *T. pratense* exhibited higher variation than other species. *T. repens* was shown the low genetic variation.

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	Pp. of bands	PIC
ISSR-01	$-(AG)_8G-$	12	11	0.906
ISSR-02	$-(CA)_8RG-$	10	8	0.888
ISSR-03	$-(GA)_8GT-$	12	10	0.903
ISSR-04	$-(GA)_8CG-$	7	5	0.815
ISSR-05	$-(GA)_8GT-$	10	7	0.885
ISSR-06	$-(GA)_8CG-$	11	7	0.897
ISSR-07	$-(GA)_8TC-$	9	8	0.855
Mean	-	10.1	8.0	0.878

Table 2: Measurements of genetic variation for three selected 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 pratense	36	50.7	1.507	1.347	0.195	0.267
T. hybridum	33	46.5	1.465	1.341	0.190	0.275
T. repens	26	36.6	1.366	1.248	0.143	0.210
Mean	31.7	44.6	1.446	1.312	0.176	0.251

The ISSR-01-06 fragment of primer ISSR-01, ISSR-03-01 fragment of primer ISSR-03, and ISSR-06-01 fragment of primer ISSR-06 had specific band for *T. hybridum* which did not show at other species. The ISSR-01-04 fragment of primer ISSR-01 and ISSR-03-09 fragment of primer ISSR-03 had specific band for *T. pratense*. These specific fragments seemed to be useful markers to discriminate among species. However, there was not found in unique band to *T. repens*.

Total genetic diversity values (H_T) varied between 0.358 (ISSR-04) and 0.450 (ISSR-02), for an average over all polymorphic loci of 0.320 (Table 3). In interlocus variation in the within-species, mean genetic diversity (H_S) was low (0.176). On a per-locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) ranged from 0.222 for ISSR-01 to 0.461 for ISSR-02, with a mean of 0.450. This indicated that about 45.0% of the total variation was among species. Thus, about genetic variation (55.0%) resided within species. The estimate of gene flow, based on G_{ST}, was very low among species (N_m = 0.610). Values of genetic distance (D) were ≤ 0.342 (Table 4). Genetic identity values among pairs of species ranged from 0.710 to 0.759.

Table 3: Estimates of genetic diversity of three 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	0.399	0.301	0.222	4.685
ISSR-02	0.450	0.233	0.461	1.211
ISSR-03	0.420	0.247	0.405	4.469
ISSR-04	0.358	0.162	0.447	1.614
ISSR-05	0.401	0.246	0.378	1.285
ISSR-06	0.385	0.232	0.394	2.850
ISSR-07	0.361	0.195	0.414	1.917
Total mean	0.320	0.176	0.450	0.610

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

Cultivars	*T. pratense*	*T. hybridum*	*T. repens*
T. pratense	-	0.710	0.759
T. hybridum	0.342	-	0.745
T. repens	0.276	0.295	-

An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 87.5% the total genetic diversity was among species. Thus, the other portion of genetic variation (91.1%) resided within species (Table 5). The result was lower than that (G_{ST}) of *F*-statistics.

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	2.140	2.419	0.884	0.116
ISSR-02	2.021	2.246	0.900	0.100
ISSR-03	2.196	2.404	0.914	0.086
ISSR-04	1.522	1.799	0.863	0.137
ISSR-05	2.119	2.222	0.953	0.047
ISSR-06	2.230	2.318	0.962	0.038
ISSR-07	1.803	1.998	0.902	0.098
Total mean	2.008	2.201	0.911	0.089

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

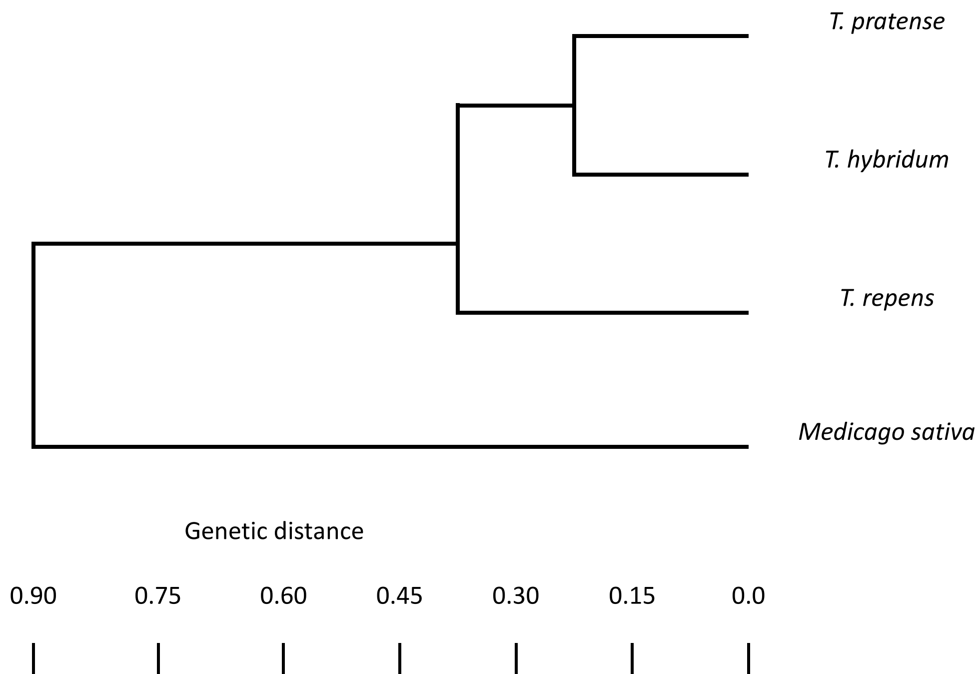

Figure 1: A phentic tree for three selected species with one outgroup based on ISSR analysis.

DISCUSSION

The problem of biological invasion has been recognized by SCOPE (Scientific Committee on the Problems of Environment) as a central problem in the conservation of biological communities. The invasive alien plants have serious ecological implications for the conservation of native biodiversity, maintenance of

plant community structure, plant succession and ecosystem processes in the areas invaded by them [13].

Weeds in the South Korea are still evolving hundreds of years after their introduction and are unlikely to have yet reached their full potential as invaders. The weeds are getting better and better adapted to life in their new environment, so they will presumably become even more problematic invaders as time goes on. White clover originated from the Mediterranean region of Europe and was spread through Europe and Western Asia with migrating animals before recorded history [3]. It is generally accepted that European accidently introduced white clover (*T. repens*) into Korea in the late 19th century and widely distributed. Although the species has not used in a mixes sward with grasses in many countries, the plants are treated as weed in Korea. Recently (maybe 3~5 years ago) with new white clover (*T. hybridum*) and red clover (*T. pratense*) were invaded Korea and they grow in damp, swampy soils, lawns, and grassy places as weeds. After traditional old white clover (*T. repens* L.) in Korea has been introduced from Europe about two hundreds ago, the species has changed the composition of Korean communities in seedling roots of native grassland species. Recent introduced clovers, *T. pratense* and *T. hybridum* exhibited higher variation than *T. repens* (Table 2). The formers can inhibit the regrowth of native plant species as the later has done. Although white clovers are persistent, widely adapted perennial nitrogen producers with tough stems and a dense shallow root mass that protects soil from erosion and suppresses weeds, they provide invaders opportunities for luxuriant growth and more prolific reproduction, which allows them to out-compete native species (for example, genus *Zoysia*; *Zoysia japonica* Steud., *Z. macrostachya* Franch. et Savat., *Z. sinica* Hance, and *Z. tenuifolia* Willd. *Arundinella hirta* (Thunb.) Koidz), and expand their range of distribution. The invasive and aggressive plant species, *T. pratense* and *T. hybridum* which have high genetic diversity can be more problems on many parts of the Korea than *T. repens*, as for many aspects of biology, is unusually distinctive.

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