Journal of Life Science 2005, Vol. 15. No. 2. 273~278 ©JLS

Genetic Variation of Alien Invasive Red Clover (*Trifolium pratense*) in Korea

Man Kyu Huh*, Kyung-Tae Chung[1] and Yong-Kee Jeong[1]

Department of Molecular Biology, Dongeui University, 995 Eomgangno, Busabjin-gu, Busan 614-714, Korea
[1]Life Science and Biotechnology Major, Dongeui University, 995 Eomgangno, Busabjin-gu, Busan 614-714, Korea

Received March 3, 2005 / Accepted April 11, 2005

Trifolium pratense (red clover, Fabaceae) is a short-lived herbaceous species and the species is introduced from Europe or North America to Korea approximately 60 years ago. Allozyme variability was examined in populations representing this species. A high level of genetic variation was found in *T. pratense* populations. Ten of 19 loci (52.6%) showed detectable polymorphism. Genetic diversity was 0.220. The sexual reproduction, high fecundity, and colonization process are proposed as possible factors contributing to high genetic diversity. Genetic diversity (0.220) was lower than that (0.285) of North American red clover, *T. pratense*. Korean populations of red clover may be founded by a small sample of larger or moderate populations. An indirect estimate of the number of migrants per generation (Nm = 4.20) indicated that gene flow was extensive among Korean populations of this species.

Key words – Allozyme variability, red clover, *Trifolium pratense*

Many plant species introduced by humans to areas outside their natural ranges (hereafter 'alien plants') establish self-perpetuating populations (i.e. they become 'natuarlized'). Some of these species spread from sites on introduction, become integrated into native communities and disrupt their functioning. In many cases, invasive alien species suppress or eliminate native species, causing a loss of biodiversity[26].

The genus *Trifolium*, the clover, consists of approximately 300 species distributed throughout the world[27]. Although cosmopolitan, most species are distributed in temperate regions, with centers of diversity in Asia Minor and southeastern Europe [8]. This familiar short-lived perennial grows wild along roadsides, in meadows and in fields, and is extensively cultivated as a forage crop for cattle. It grows best in soils that are rich in calcium, potassium, and phosphorus[3,4].

The legume genera such as genus *Trifolium* including *Trifolium repens* and *T. pratense* are well known to have self-incompatibility problems and sterility complications. The genus *Trifolium* can reproduce either clonally or sexually via flowers. The edible blossoms are sweet tasting with a honey-like fragrance. Bees are attracted to clover blossoms. Rhizomes generally are horizontal, with shallow elongations or prostrate stem rooting at the nodes.

Red clover, *T. pratense* L., is introduced from Europe or North America to Korea approximately 60 years ago [14]. In Korea, the species usually grows on importable ports or waste places near ports which have been recently disturbed. Typical populations of red clover are small and distributed in patches. The species is diploid (2n=14, 28) with red flowers.

Although this alien species has been considered as an important species in ecology and agriculture in Korea, population structure of this species has not been studied. The objectives of our study were to estimate how much allozyme diversity is maintained in the species, and to describe how genetic variation is distributed within and among populations. In addition, we compared the genetic diversity and population structure of *T. pratense* and plant species having similar life-history characteristics.

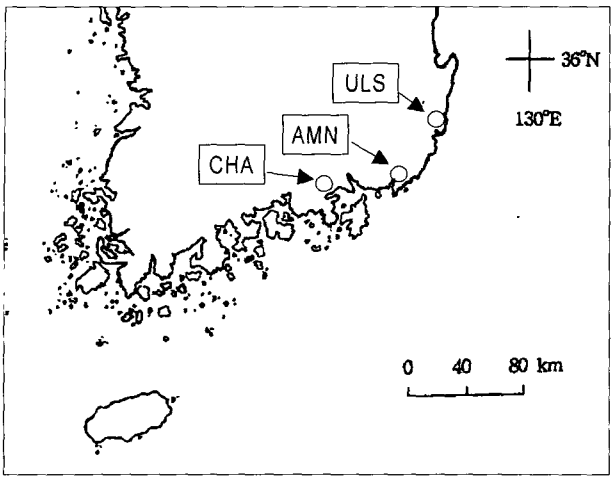
Materials and Methods

Sampling procedure and enzyme electrophoresis

Leaf tissues were collected from three populations of *T. pratense* in Korea (Fig. 1). More than 40 plants for red clover were sampled from each population. To avoid including individuals from the same rhizome, the distance between the selected individuals was about 2.0 m.

The procedures for homogenization, starch gel electrophoresis, and enzyme assay were those described by Soltis *et al.*[24]. Leaves were homogenized by mechanical grinding to release enzymes from cell and organellar membranes, using a Tris-HCl grinding buffer-PVP solution. Electro

*Corresponding author
Tel : +82-51-890-1529, Fax : +82-51-890-1521
E-mail : mkhuh@deu.ac.kr



CHA: Changwon-ci, Gyeongsangnam-do; AMN: Amnam-dong, Busan-ci; ULS: Ulsan-ci, Ulsan.

Fig. 1. Collection sites for populations of *T. pratense* for isozyme analysis.

phoresis was performed with an 11% starch gel. Ten enzyme systems were assayed: diaphorase (DIA), fluorescent esterase (EST), leucine aminopeptidase (LAP), and peroxidase (PER) were resolved on System 9 of Soltis *et al.* [24]; glucose phosphate isomerase (PGI), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), and shikimate dehydrogenase (SKD) on Soltis *et al.*'s System 10.

Data analysis

Statistics of enzyme data was based on allele and genotype frequencies in each population. The following genetic parameters were calculated using a POPGENE computer program (version 1.31) developed by Yeh *et al.* [29]: the percentage polymorphic loci (*Pp* for population level and Ps for species level), mean number of alleles per locus (*A*), effective number of alleles per locus (*Ae*), and gene diversity (He) [10]. Species (indicated with the subscript s) and mean population (indicated with the subscript p) levels of genetic diversity were calculated as in Hartl and Clark [11]. Observed heterozygosity (*Ho*) was compared with Hardy-Weinberg expected values using Wright's fixation index (*F*) or inbreeding coefficients[28]. Deviations from genotype frequencies expected under the Hardy-Weinberg equilibrium were tested using the GENEPOP ver. 3.1 program[22]. Multiple tests were performed using the sequential Bonferroni procedure[15].

To elucidate the organization of the variation in *T. repens*, genetic variation was examined by partitioning of the total genetic diversity (H_T) to within (H_S) and among (D_{ST}) population components using Nei's[21] genetic diversity statistics. A measure of differentiation among populations, relative to the total diversity was calculated at each locus as $G_{ST}=D_{ST} / H_T$. Weir and Cockerham's (1984) estimates of Wright's F_{ST} (G_{ST}) were computed for variable loci with FSTAT ver. 1.2[7].

To elucidate the extent of genetic departure of populations from each other, Nei's genetic identity (I) and genetic distance (D) were calculated for each pairwise combination of populations[20].

The genetic structure within and among populations was also evaluated using Wright's[28] *F*-statistics: F_{IT}, F_{IS}, and F_{ST}. F_{IT} and F_{IS} measure excesses of homozygotes or heterozygotes relative to panmictic expectations, within samples and within populations, respectively. Deviations of F_{IT} and F_{IS} from zero were tested using chi-square statistics[16]. Two indirect estimates of gene flow were calculated. Estimates of the number of migrants per generation (*Nm*) were based on G_{ST} or the average frequency of private alleles, found in only one population[23]. Genetic diversity was tested against regions by Spearman rank to seek any correlation between genetic variation in the populations and environmental factors[30].

Results

Genetic diversity

A high level of genetic variation was found in the *T. pratense* populations. Ten of the 19 loci (52.6%) were polymorphic in at least one population. Red clover was monomorphic at the *Est-3* locus, but white clover was polymorphic at the same locus. An average of 43.9% of the loci (*Pp*) was polymorphic within populations, with individual population values ranging from 42.1% to 47.3% (Table 1). The average number of alleles per locus (*A*) was 1.68 across populations. The average effective number of alleles per locus (*Ae*) was 1.47 across populations, ranging from 1.43 for the population with the lowest mean number of alleles to 1.53 for the population with the highest mean. The effective number of alleles per locus (*Ae*) was similar at the species (1.44) and the population level (1.47). The mean genetic diversity within population (*Hep*) was 0.225. Populations AMN had the highest expected diversity (0.242), while population CHA had the lowest (0.208). Genetic diversity at the species level was high (*Hes*=0.225), and the population level (*Hep*=0.225) was the same trend.

Table 1. Allozyme variation within three populations of *T. pratense*. Percentage of polymorphic loci (*P*), mean number of alleles per polymorphic population (*Ap*), mean number of alleles per locus (*A*), effective number of alleles per locus (*Ae*), observed heterozygosity (*Hop*), and Hardy-Weinberg expected heterozygosity or genetic diversity (*Hep*).

Population	*P*	*Ap*	*A*	*Ae*	*Hop* (SD)	*Hep* (SD)
CHA	42.1	2.63	1.68	1.43	0.145(0.013)	0.208(0.058)
AMN	42.1	2.63	1.68	1.53	0.126(0.012)	0.242(0.061)
ULS	47.4	2.44	1.68	1.46	0.138(0.012)	0.225(0.056)
Mean	43.9	2.56	1.68	1.47	0.136	0.225
Species	52.6	2.50	1.79	1.44	-	0.225

Genetic structure

On a per locus basis, the proportion of total genetic variation was found among populations (G_{ST}) ranged from 0.056 for *Dia-2* to 0.005 for *Skd* with a mean of 0.204, indicating most of the genetic variance (5.6%) resided within populations (Table 2). The correlation coefficients between genetic distance and geographical distance using Mantel's test for all populations were 0.31 ($r^2=0.35$). Most of the variation in genetic distance seemed to be caused by unknown factors other than geographic distance.

In *T. pratense* populations, deficiency of heterozygosity was substantial (Table 3). All fixation indices were positive, and 58.3% of those (14/24) were significant. None index was negative.

Total genetic diversity values (H_T) and interlocus variation in the within-population genetic diversity (H_S) were 0.417 and 0.400, respectively (Table 2).

The values of genetic distance (D) were below 0.100. Genetic identity (I) values among pairs of populations ranged from 0.924 to 0.997. Total populations cluster below genetic distance of 0.241 (Fig. 2). The indirect estimate of gene flow based on mean G_{ST} was high ($Nm=4.20$), and the estimated gene flow based on private alleles was 1.56.

Discussion

The most striking result emerging from this study is that *T. pratense* maintain high levels of genetic diversity in populations than does the average plant species. For example, their genetic diversity of 0.225 are higher than that for species with a sexual and asexual reproduction mode (0.138), temperate-zone species (0.146), and short-lived her-

Table 2. Estimates of genetic diversity statistics and 10 polymorphic loci in *T. pratense*. Total genetic diversity (H_T), genetic diversity within populations (H_S), among populations (D_{ST}), deviations of genotype frequencies from Hardy-Weinberg expectations over all populations (F_{IT}) and within individual populations (F_{IS}), and proportion of total genetic diversity partitioned among populations (G_{ST}).

Locus	H_T	H_S	D_{ST}	F_{IT}	F_{IS}	G_{ST}
Dia-1	0.566	0.559	0.008	0.474	0.467	0.014
Dia-2	0.629	0.626	0.003	0.530	0.527	0.005
Est-1	0.396	0.385	0.012	0.393	0.375	0.028
Est-2	0.499	0.439	0.061	0.566	0.506	0.121
Lap	0.068	0.064	0.005	0.482	0.444	0.067
Pgi	0.455	0.455	0.001	0.323	0.322	0.001
Idh	0.422	0.420	0.002	0.203	0.199	0.005
Skd	0.187	0.149	0.038	0.272	0.085	0.204
Per-2	0.547	0.543	0.003	0.437	0.433	0.006
Per-3	0.404	0.360	0.044	0.219	0.123	0.109
Mean	0.417	0.400	0.018	0.390	0.348	0.056

Table 3. Wright's fixation indices for three populations of red clover

Pop.	*Dia-1*	*Dia-2*	*Est-1*	*Est-2*	*Lap*	*Pgi*	*Idh*	*Per-2*	*Per-3*
CHA	0.407*	0.362*	0.250	0.439***	-	0.226	0.213	0.323*	0.169
AMN	0.564***	0.635***	0.523***	0.669***	-	0.450**	0.206	0.432***	0.081
ULS	0.444***	0.548***	0.218	0.389	0.451***	0.298	0.213	0.560***	-

Note: A dash indicates fixed loci. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

baceous perennials (0.116)[9]. The percentages of polymorphic loci at the species level were 52.6% for red clover. These values are also higher than average for species with a reproduction mode that is sexual and asexual (43.8%), short-lived herbaceous perennials (41.3%), and temperature-zone species (48.5%)[9]. In addition, among the *Trifolium* species (Table 3), *T. repens* in Korea had the highest H_T value. These comparisons suggest that genetic diversity levels of *T. repens* are higher than those of the North American *Trifolium* species.

The relatively high level of genetic variation found in red clover is consistent with several aspects of its biology. First, the breeding system of a species is an important determinant of variability at both the species and population levels. Red clover is bisexual and self-incompatible, apparently making it a species that is primarily animal-pollinated. Predominantly outcrossing species maintain higher levels of intrapopulation genetic variation than do predominantly inbreeding species[2,6]. Second, a perennial species such as clover generally maintains relatively higher levels of variation than do annuals[17]. Finally, the reproduction modes of clover play an important role in genetic variability. Vegetative reproduction and spread can affect the genetic structure of populations[19]. Cook[5] contended that clonal growth could retard the loss of genetic diversity within populations. White and red clovers can regenerate from fibrous roots when many plants are harvested by firewood or otherwise destroyed. Species with independent ramets could spread the risk of mortality, thereby reducing the probability of genet death and preserving genetic diversity [13]. Hartnett and Bazzaz[12] have also argued that physiological independence among ramets may maintain genetic diversity by buffering clones against localized, patch-specific selection forces. Sexual reproduction could enhance and maintain genetic variation[1]. Red clover reproduces by seed or by vegetative spread.

Trifolium fragiferum species in North America have much lower within-population genetic diversity and much higher genetic differentiation among populations (Table 4). That is at least partially due to their life form. For example, *T. repens* and *T. pratense* are perennials. In contrast, *T. fragiferum* is cultivated as an annual, winter annual, or biennial. As state above, a perennial species maintains relatively higher levels of variation than do annuals[17]. *T. hirtum* is an annual legume native to the Mediterranean region and Asia Minor. It was also introduced from Turkey into California in 1944[18]. Although they did not interpret why *T. hirtum* is low genetic diversity, the species itself is very low genetic diversity as an annual as well as cultivars.

Although Korean populations of *T. pratense* maintain high levels of genetic diversity than the average plant species, they show a lower genetic diversity that the North American red clover (*T. pratense*). Although the direct evidence of introduction from the same North American populations to Korea, it is considered that *T. pratense* are originated from North America because Korea imported most goods from North America at that time. Especially Korea was put under military administration of USA. for 1945-1948. Naturalized populations of cultivated species are ultimately a product of both their biological characteristics and historical cultivation practices[8]. Korean populations of red clover may be founded by a small sample of larger or moderate populations (i.e. founder effect). Thus red clover in Korea may exhibit reduced genetic diversity. In Korea, white clover is used as a meadow and white clover seeds of several tons are imported from Europe or North America. But red clover is a weed and farmers weed the red clover in the fields. Thus, viewing the distribution of red clover populations in Korea, red clover may be introduced by a chance into Korea with imports. Analysis of fixation indices, calculated for all polymorphic loci in red clover populations, showed a deficiency of heterozygotes relative to Hardy-Weinberg expectations (Table 3). As most of the variation is spread within populations, a small founding sample from a population may carry some proportions of the variation in the species.

The correlations between rainfall, altitude, latitude, and the shortest distance from an import port with genetic

Table 4. Measures of genetic variability for all previously studied *Trifolium species*

	P	*A*	*Ap*	*Hes*	H_T	H_S	G_{ST}	*Nm*	Data source
T. hirtum	23.8	1.18	1.97	-	0.082	0.055	0.300	-	Molina- and Jain(1992)
T. fragiferum	53.5	1.57	-	0.194	0.257	0.187	0.222	0.876	Bulinska-Rodomska(2000)
T. pratense (North America)	76.9	-	2.60	0.285	0.371	0.385	0.028	4.750	Hagen and Hamrick(1998)
T. pratense (Korea)	52.6	1.79	2.50	0.220	0.417	0.400	0.056	4.200	This study
T. repens	57.9	2.05	2.82	0.295	0.510	0.492	0.037	6.500	Unpublished data

diversity per population were examined (data not shown). Genetic diversity versus other factors except latitude and the distance from a port did not show a significant correlation. A patterns of decreasing genetic diversity are observed with increasing latitude and geographic distance from an import port. From the results, we suppose that white clover may be diffused from import ports into inland regions and from southern Korea to northern areas.

Most introduced plants arrive without the pollinator that serve them in their natural range, but thrive in the presence of generalist pollinator (native or introduced). Generalist pollinators abound in natural ecosystems and they readily visit white clover (author observation). The numbers of nodules containing nitrogen-fixing bacteria for white clover in Korea are 1.3~2.5 times for red clover in Korea (author observation). It is suggested that the weak ability of nitrogen-fixing associations between red clover and *rhizobia* may have played roles in shaping the narrow distribution and population structure of this species.

Occasionally, the alien plant, white clover, maintains high level of genetic diversity. The low levels of population differentiation are probably due to high rates of gene flow among populations as a result of seed and pollen movement. Mutualisms that facilitate invasions occur at all the main phases of the life cycle of invading white clover.

References

1. Bayer, R. J. 1990. Patterns of clonal diversity in the *Antennaria rosea* (Asteraceae) polyploid agamic complex. *Am. J. Bot.* **77**, 1313-1319.
2. Brown, A. H. D. 1989. Genetic characterization of plant mating systems, pp. 145-162. In Brown, A. H. D. (ed.), *Plant population genetics, breeding and genetic resources.* Sinauer, Sunderland, MA.
3. Bulinska-Raomska, Z. 2000. Enzyme polymorphism and adaption in strawberry clover (*Trifolium fragiferum* L.). Genet. Resour. *Crop Evol.* **47**, 197-205.
4. Caradus, J. R., D. R. Woodfield and A. V. Stewart. 1996. Overview and vision for white clover, pp. 1-6. In Woodfield, D. R. (ed.), *White clover, New Zealand's competitive edge, Christchurch,* Agronomy Society of New Zealand.
5. Cook, R.E. 1983. Clonal plant populations. *Am. Sci.* **71**, 244-253.
6. Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. *Prog. Phytochem.* **7**, 1-46.
7. Goudet, J. 1995. FSTAT v-1.2: a computer program to calculate F-statistics. *J. Hered.* **86**, 485-486.
8. Hagen, M. J. and J. L. Hamrick. 1998. Genetic variation and population genetic structure in *Trifolium pratense. J. Hered.* **89**, 178-181.
9. Hamrick, J. L. and M. J. W. Godt. 1989. Allozyme diversity in plant species, pp. 304-319. In Brown, A.H.D. (ed.), *Plant population genetics, breeding and genetic resources.* Sinauer, Sunderland, MA.
10. Hamrick, J. L., M. J. W. Godt and S. L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. *New For.* **6**, 95-124.
11. Hartl, D. L. and A. G. Clark. 1989. *Principles of population genetics,* pp. 1-682, 2nd ed, Sinauer associates, Inc., MA.
12. Hartnett, D. C. and F. A. Bazzaz. 1985. The regulation of leaf, ramet, and genet densities in experimental populations of the rhizomatous perennial, *Solidago canadensis. J. Ecol.* **73**, 429-443.
13. Huh, M. K. and H. W. Huh. 2001. Genetic diversity and population structure of wild lentil tare. *Crop Sci.* **41**, 1940-1946.
14. Lee, Y. N. 1997. *Flora of Korea.* Kyo-Hak Publishing Co, Seoul, Korea.
15. Lessios, H. A. 1992. Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Marine Biol.* **112**, 517-523.
16. Li, C. C. and D. G. Horvitz. 1953. Some methods of estimating the inbreeding coefficient. *Am. J. Hum. Genet.* **5**, 107-117.
17. Loveless, M. D. and J. L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. *Ann. Rev. Ecol. Syst.* **15**, 65-95.
18. Molina-Freaner, F. and S. K. Jain. 1992. Isozyme variation in California and Turkish populations of the colonizing species *Trifolium hirtum. J. Hered.* **83**, 423-430.
19. Murawski, D. A. and J. L. Hamrick. 1990. Local genetic and clonal structure in the tropical terrestrial bromeliad, *Aechmea magdalenae. Am. J. Bot.* **77**, 1201-1208.
20. Nei, M. 1972. Genetic distance between populations. *Am. Nat.* **106**, 282-292.
21. Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA.* **70**, 3321-3323.
22. Raymond, M. and F. Rouset. 1995. GENEPOP version 1.2: a population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248-249.
23. Slatkin, M. 1985. Rare alleles as indicators of gene flow. *Evolution* **39**, 53-65.
24. Soltis, D. E., C. H. Haufler, D. C. Darrow and G. J. Gastony. 1983. Starch gel electrophoresis of ferns: A compilation of grinding buffers, gel and electrode buffers, and staining schedules. *Am. Fern J.* **73**, 9-27.
25. Weir, B. S. and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370.
26. Williamson, M. 1996. *Biological Invasions.* Chapman & Hall, London.
27. Woodland, D. W. 1991. *Contemporary Plant Systematics.* Prentice Hall, Inc, Englewood cliffs, New Jersey.
28. Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* **19**, 395-420.
29. Yeh, F. C., R. C. Yang and T. Boyle. 1999. *POPGENE*

version 1.31, Microsoft Windows-based Freeware for Population Genetic Analysis.

30. Zar, J. H. 1984. *Biostatistical Analysis*. 2nd ed. Prentice-Hall, Inc, NJ.

초록 : 붉은토끼풀의 유전적 변이와 집단구조

허만규* · 정경태[1] · 정영기[1]

(동의대학교 분자생물학과, [1]응용생명과학전공)

붉은토끼풀(*Trifolium pratense*)은 전세계적으로 분포하는 근연성 초본종이다. 붉은토끼풀은 유럽 또는 북미에서 약 60년전 한국으로 도입되었다. 전분 젤 전기영동을 사용하여 한국내 분포하는 붉은토끼풀의 유전적 변이와 집단구조를 분석하기 위해 사용되었다. 붉은토끼풀에서 높은 유전적 다양성이 발견되었다. 19개 대립유전좌위중 10개(52.6%)는 다형현상을 나타내었다. 유전적 다양성은 0.220이었다. 유성생식, 높은 다산성, 집단형성화 과정이 높은 유전적 다양성을 유지하는데 기여하는 것으로 제시하였다. 한국의 붉은토끼풀의 다양도(0.220)는 북미의 붉은토끼풀 다양도(0.285)보다 낮았다. 이는 북미의 일부 집단에서 유입되었거나 창시자효과 때문일 것으로 사료된다. 간접적 평가를 통한 세대당 이주하는 수는 4.20이였고, 유전자 유동은 한국집단에서 높게 유지되고 있음을 시사한다.