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DNA Polymorphism and Assessments of Genetic Relationships in genus *Zoysia* Based on Simple Sequence Repeat Markers

Man Kyu Huh*

Department of Molecular Biology, Dong-eui University, Busan 614-714, Korea

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The genetic variability of four species of the genus *Zoysia* collected from South Korea was analyzed using an inter-simple sequence repeat (ISSR) marker system. Polymerase chain reactions (PCR) with eight ISSR primers generated 86 amplicons, 76 (87.1%) of which were polymorphisms. The polymorphism information content (PIC) value of the ISSR marker system was 0.848. The percentage of polymorphic loci (*P*p) ranged from 41.2% to 44.7%. Nei's gene diversity (*H*) ranged from 0.149 to 0.186, with an average overall value of 0.170. The mean of Shannon's information index (*I*) value was 0.250. Total genetic diversity values (H_T) varied between 0.356 (ISSR-1) and 0.418 (ISSR-16), for an average overall polymorphic loci of 0.345. Interlocus variation in within-species genetic diversity (H_S) was low (0.170). On a per-locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) was 0.601. This indicated that about 60.1% of the total variation was among species. Thus, about 39.9 of genetic variation was within species. The estimate of gene flow, based on G_{ST}, was very low among species of the genus *Zoysia* (N_m = 0.332). The phylogenic tree showed three distinct groups: *Z. macrostachya* and *Z. tenuifolia* clades and other species were formed the separated clusters. In conclusion, the ISSR assay was useful for detecting genetic variation in the genus *Zoysia,* and its discriminatory power was comparable to that of other genotyping tools.

Key words : Genetic variability, genus *Zoysia*, polymorphism information content (PIC), phylogenic tree, simple sequence repeat markers (ISSR)

Introduction

The genus *Zoysia* Willd. (Family *Poaceae*, subfamily *Chloridoideae*, tribe *Zoysiece*) includes about ten species [7] and is commonly known as zoysiagrass (*Zoysia* sp.) or lawn. Zoysiagrass is found throughout East Asia (China, Korea and Japan) and Australasia, grown especially in warm regions [17, 27].

Wild species belonging genus *Zoysia* have been survived though long-term natural selection [13], and thereby can tolerate wide variations in temperature, sunlight, and water. Zoysiagrass is widely used for turfgass and forage grass in as Korea and other countries in East Asia. *Zoysia* grasses can diminish soil erosion on slopes, and are excellent at repelling weeds throughout the year. Some types of *Zoysia* are available commercially as making lawn ground, park grass, and garden grass. In addition, they are used on golf courses to create fairways and teeing areas after several hybridization processes.

DNA markers have numerous applications in plant molecular genetic research [6]. The two most common uses of DNA markers have been the assessment of genetic diversity within plant germplasm and evolutionary relationship within or among species [12, 19]. One major use of DNA techniques is to reveal genetic diversity within and between populations or species.

Inter simple sequence repeat (ISSR) technique is a polymerase chain reaction (PCR) based technique, reported by Zietkiewicz et al. [34]. ISSR technique involves PCR amplification of DNA using a single primer composed of a microsatellite sequence. ISSR technique involves amplification of DNA segments between two identical microsatellite repeat regions oriented in opposite direction using primers designed from microsatellite core regions [29]. The ISSR has mild technical difficulty, good reproducibility and reasonable cost, permitting its use for genetic studies of population. PCR products were resolved by performing agarose gel electrophoresis. Compared to arbitrary markers such as random amplified polymorphic DNA (RAPD), ISSR markers are

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

highly reproducible due to the use of longer primers.

Considering the potentials of the ISSR marker based genetic diversity analysis, the present study aimed to evaluate the extent of genetic diversity and phylogenetic relationships among four species of genus *Zoysia* collected from Korea.

Materials and Methods

Sample materials

Four species within genus *Zoysia* Willd. occur in Korea. *Zoysia japonica* Steud., *Z. macrostachya* Franch. et Savat., *Z. sinica* Hance, and *Z. tenuifolia* Willd. were selected. Plant materials collected from four large natural populations per species. To avoid including individuals from the same lineage by clonal reproduction, the distance between the selected individuals was about 5.0 m. *Arundinella hirta* (Thunb.) Koidz. was used as an outgroup species in this study.

Genomic DNA isolation and ISSR analysis

The total genomic DNA was extracted from the leaf tissues using the plant DNA Zol Reagent (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's protocol. DNA quantity was checked using a mini fluorometer (TKO 100 Mini-Fluorometer, Hoefer Scientific Instruments).

ISSR primers (University of British Columbia, Canada) synthesized by Sigma Aldrich Inc., were used for the polymorphism survey (Table 1).

PCR (polymerase chain reaction) amplification was carried out in 25 µl reaction containing 25 ng of genomic DNA, 1 µM of primer, 10 mM of dNTPs (2.5 mM each), 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.3 units (U) of *Taq* DNA polymerase (Promega, USA). PCR conditions were programmed for initial denaturation at 94°C for 5 min, 36 cycles of 1 min denaturation at 94°C, 45 sec annealing at 42-50°C, 2 min extension at 72°C, and final extension for 10 min at 72°C. The annealing temperature for PCR amplification was maintained based on the specificity of the primer pair used. PCR amplified products of ISSR primers were subject to horizontal gel electrophoresis using 2.0% agarose gel and stained with ethidium bromide. The bands on gels were documented using Alpha Imager 1200TM (Alpha Innotech Co., USA). All the reactions were repeated twice and only reproducible bands were scored for analyses.

Table 1. Lists of decamer oligonucleotide utilized as primers, their sequences, and associated bands for Korean genus *Zoysia*

No. of Primer	Sequence (5′ to 3′)	No. of fragments detected	Pp. of bands	PIC
ISSR-01	-(AG) $_8$G-	11	10	0.849
ISSR-02	-(CA)$_8$RG-	9	9	0.791
ISSR-03	-(GA)$_8$GT-	10	9	0.871
ISSR-05	-(GA)$_8$CG-	8	7	0.806
ISSR-06	-(GA)$_8$CG-	12	10	0.878
ISSR-10	-(AC)$_8$T-	9	7	0.844
ISSR-12	-(GT)$_8$GT-	15	15	0.848
ISSR-16	-CCGG(AC)$_8$-	11	9	0.896

Data analysis

The unambiguous ISSR bands were scored visually as present (1) or absent (0) and the binary data matrix was constructed. Several standard genetic parameters, the percentage of polymorphic loci (*P*p), mean number of alleles per locus (*A*), effective number of alleles per locus (A_E), Nei's [21] gene diversity (*H*), and Shannon's Information index (*I*) were estimated using the computer program, POPGENE ver. 1.31 [33]. Polymorphism information content (PIC) value was calculated using the formula PIC, $1 - \sum pi^2$, where *pi* is the frequency of the *i*th allele [25].

The estimation of genetic similarity (GS) between genotypes was based on the probability that an amplified fragment from one individual will also be present in another [23]. GS was converted to genetic distance (1-GS) [15].

Species differentiation analyses have been assessed within and among according to Nei's gene diversity formulae (H_T, H_S, and G_{ST}) [22]. The G_{ST} coefficient corresponds to the relative amount of differentiation among populations or species. Furthermore, gene flow (*Nm*) between the pairs of species was calculated from G_{ST} values by $Nm = 0.5(1/G_{ST}-1)$ [20].

Shannon's index of genotypic diversity (H_O) for ISSR was estimated to quantity the degree of within species diversity following the formula [3]: $H_O = -\sum pi \log pi$, where *pi* is the frequency of a particular phenotype *i*.

H_O can be calculated and compared for different populations [24]. 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 [24]. Then the proportion of diversity within species, H_{POP}/H_{SP},

can compared with that between species (H_{SP}-H_{POP})/H_{SP}.

Cluster analyses

A phenetic relationship was constructed by the neighbor joining (NJ) method using the NEIGHBOR program in MEGA5 [28].

Results

For analysis of variability of genus *Zoysia*, eight primers were used for studying the ISSR banding patterns across the entire samples. The eight primers generated a total of 85 consistently scorable bands, 76 of which were polymorphic (89.4% polymorphism) (Table 1). The average number of amplification products was 10.6 per primer; the maximum was 15 with $(GT)_8YT$ (ISSR-12), whereas the minimum was 8 with $(GA)_8CG$ (ISSR-5).

Polymorphism information content (PIC) for ISSR primers ranged from 0.791 to 0.896 with an average of 0.848 per primer (Table 1).

In a simple measure of inter-populations variability i.e. the percentage of polymorphic bands (*P*p), *Z. japonica* exhibited the highest variation (47.1%) and *Z. tenuifolia* the lowest (41.2%) (Table 2). The average number of alleles per locus (*A*) was 1.439 across species, varying from 1.412 to 1.471. The effective numbers of alleles per locus (A_E) at the lowest species and the highest species level were 1.253 and 1.334, respectively. The mean genetic diversity within species was 0.170. Overall, *Z. sinica* and *Z. japonica* exhibited high variation among species. *Z. macrostachya* and *Z. tenuifolia* was shown the low genetic variation.

Total genetic diversity values (H_T) varied between 0.356 (ISSR-1) and 0.418 (ISSR-16), for an average over all polymorphic loci of 0.345 (Table 3). Interlocus variation in the within-species genetic diversity (H_S) was low (0.170). On a per-locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) ranged from 0.273 for ISSR-1 to 0.660 for ISSR-6, with a mean of 0.506. This indicated that about 50.6% of the total variation was among species. Thus, about genetic variation (40.4%) resided within species. The estimate of gene flow, based on G_{ST}, was very low among species of genus *Zoysia* (N_m = 0.488). Values of genetic distance (D) were <0.470 (Table 4). Genetic identity values among pairs of populations ranged from 0.625 to 0.813. Genetic distances between species were high. There was not shown significant difference among four species.

An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 71.0% the total genetic diversity was among species. Thus, the other portion of genetic variation (29.0%) resided within species (Table 5).

Table 2. Codes of countries and measurements of genetic variation for genus *Zoysia*

Code	Np	*P*p	*A*	A_E	*H*	*I*
Zoysia sinica	38	44.7	1.447	1.334	0.186	0.269
Z. macrostachya	35	41.2	1.412	1.253	0.149	0.222
Z. japonica	40	47.1	1.471	1.325	0.184	0.270
Z. tenuifolia	36	42.4	1.424	1.280	0.162	0.240
Mean	37.3	43.9	1.439	1.298	0.170	0.250

Table 3. Estimates of genetic diversity of genus *Zoysia* in Korea

Locus	H_T	H_S	G_{ST}	*Nm*
ISSR-01	0.356	0.220	0.273	1.670
ISSR-02	0.372	0.207	0.377	1.730
ISSR-03	0.383	0.217	0.399	2.433
ISSR-05	0.374	0.186	0.507	0.886
ISSR-06	0.385	0.121	0.660	0.711
ISSR-10	0.383	0.216	0.438	3.267
ISSR-12	0.403	0.171	0.572	0.870
ISSR-16	0.418	0.205	0.526	1.723
Total mean	0.352	0.141	0.601	0.332

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*).

Table 4. Genetic identity (above diagonal) and genetic distances (below diagonal) of genus *Zoysia* based on ISSR

Code	*sinica*	*macrostachya*	*japonica*	*tenuifolia*
Z. sinica	-	0.6252	0.7132	0.6693
Z. macrostachya	0.4697	-	0.7749	0.7233
Z. japonica	0.3381	0.2550	-	0.8132
Z. tenuifolia	0.4015	0.3239	0.2067	-

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

Primer	H_{VAR}	H_{SP}	H_{VAR} / H_{SP}	(H_{SP} - H_{VAR}) / H_{SP}
ISSR-01	1.552	2.373	0.654	0.346
ISSR-02	1.663	2.245	0.741	0.259
ISSR-03	1.693	2.213	0.765	0.235
ISSR-05	1.769	2.131	0.830	0.170
ISSR-06	1.125	2.423	0.464	0.536
ISSR-10	1.732	2.123	0.816	0.184
ISSR-12	1.588	3.194	0.497	0.503
ISSR-16	2.128	2.338	0.910	0.090
Total mean	1.706	2.380	0.710	0.290

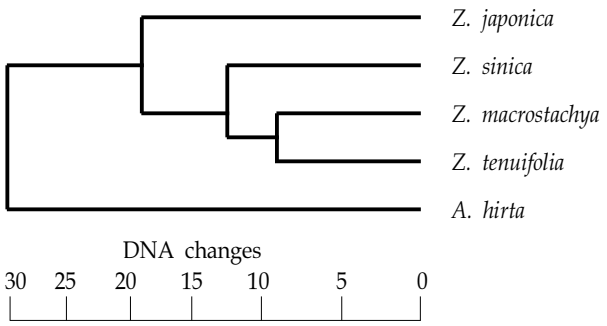

Fig. 1. The tree for Korean genus *Zoysia* based on ISSR analysis using MEGA5.

Clustering of four species of genus *Zoysia*, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). Four species of genus *Zoysia* were well separated each other. The phylogenic tree showed three distinct groups; *Z. macrostachya* and *Z. tenuifolia* clade and the other species.

Discussion

Genetic diversity of Korean *Zoysia* species is comparable with other species, although the use of different methods (e.g., isozyme [co-dominant marker] and RAPD [dominant marker], ISSR [sequence based marker system) may preclude meaningful direct comparisons.

In ISSR analysis, nine species belonging to genus *Zoysia* maintain moderate or higher than mean level of genetic diversity compared with other plant species [10]. For example, the percentage of polymorphic loci at the species level for *Zoysia* is 43.9%, which is similar to species with temperature-zone distributions (48.5%), species with a sexual and asexual reproduction mode (43.8%), and those with a short-lived perennial herbaceous (41.3%) (Hamrick and Godt 1989). Its genetic diversity of 0.170 is higher than that for temperate-zone species (0.146), species with a sexual and asexual reproduction mode (0.138), and those with a short-lived perennial herbaceous (0.116) [10].

The results of other research for *Zoysia* were similar to the results of this study. Choi et al. [5] analyzed 93 native zoysiagrass lines collected from the southern and western coastal regions of South Korea. They also estimated genetic diversity of zoysiagrass using RAPD [4]. Weng et al. [30] also analyzed genetic diversity of 131 clones of Taiwan *Zoysia* species by using isozyme and RAPD. Li and Tong [16] reported genetic diversity of 105 plants from seven *Z. sinica* populations in different regions of China using ten RAPD primers. About 4.8, 30, and 70% of the genetic variation was detected among groups, populations, and within populations, respectively. In ISSR literature, polymorphism and genetic diversity (*H*) within species in Chinese zoysiagrass were 96.7% and 0.250, respectively [31].

The relatively high level of genetic variation found in genus *Zoysia* is consistent with several aspects of its biology. First, the particular breeding system that a plant possesses is an important determinant of variability at both the species and population levels. Flowering plants exhibit a spectacular diversity in reproductive systems, and this can have important effects on the amount and structuring of genetic variation within and among populations [9]. Species of the genus *Zoysia* are rarely reproduced sexually and pertetuates theyselves clonally by rhizomes [1]. Whereas sexual reproduction might initially act to enhance genetic variation, asexual reproduction can maintain this enhanced variability [2]. Second, a perennial and/or long-lived species generally maintains relatively higher levels of variation than do annuals [18]. Because individuals of genus *Zoysia* are so long-lived, opportunities for the accumulation of mutations should be high [14].

Genetic differentiation among populations is principally a function of natural selection, genetic drift, and gene flow among populations via pollen and seed dispersal [26]. Of the total variation observed in *Zoysia* species about 20.0-27.7% was due to differences among species (G_{ST} = 0.601, $H_{VAR})/H_{SP}$ = 0.710). Predominantly wind-pollinated outcrossing species have on an average less than 10 % of the genetic variation between populations [11]. This high level of genetic differentiation also suggests that gene flow among species is low (Nm = 0.332), though no chromosomal barrier of interspecific crossing [8, 32].

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초록 : ISSR에 의한 잔디속 식물의 DNA 다형성과 유전적 관계 평가

허만규*
(동의대학교 자연생활과학대학 분자생물학과)

한국에서 채집한 잔디속(genus *Zoysia*) 식물 종의 유전적 변이를 단순 서열 반복(Inter-Simple Sequence Repeat Markers, ISSR) 마커 시스템으로 조사하였다. 8개의 ISSR 시발체를 이용한 중합효소 사슬 증폭반응에서 86개의 분절의 증폭물을 얻었으며 이 중 76(87.1%)개 분절이 다형성을 나타내었다. ISSR 마커 시스템에서 다형성 정보 지수(PIC)는 0.848이었다. 다형성 대립유전자좌위의 퍼센트(*P*p)는 41.2%에서 44.7%까지 나타내었다. 네이(Nei)의 유전자 다양성(*H*)은 0.149에서 0.186까지 이며 평균은 0.170이었다. 샤논(Shannon)의 정보 지수(*I*)의 평균값은 0.250이었다. 대립유전자좌위에 근거하여 전체 변이에서 종 간 차이를 나타내는 변이의 몫(G_{ST})은 0.601였다. 이는 전체변이의 약 60.1%는 종 간에 있음을 의미한다. 따라서 변이의 약 39.9%는 종 내에 있었다. G_{ST}에 근거한 유전자 흐름(이동)은 잔디속 간에는 대단히 낮았다(N_m = 0.332). 계통도는 3개의 뚜렷한 분지군으로 분리되었다. 왕잔디(Zoysia macrostachya)와 금잔디(Z. tenuifolia) 분지군, 갯잔디(Z. sinica) 단독 분지군, 잔디(Z .japonica) 단독 분지군이었다. 결론적으로 잔디속 식물에 대한 ISSR 분석은 유전적 변이를 탐지하는데 유용하며, 종을 구분하는 유전자형의 대한 식별력을 주었다.