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Full Length Research Paper

Genetic diversity in Chinese natural zoysiagrass based on inter-simple sequence repeat (ISSR) analysis

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Zoysiagrass (*Zoysia* sp.) is extensively used in turf establishment and livestock herbage due to its many outstanding characters. Native *Zoysia* sp. are widely distributed in China. Inter-simple sequence repeat (ISSR) markers were used to investigate the genetic diversity and genetic relationships of 81 Chinese wild zoysiagrass accessions and three commercial cultivars. The results show that 33 ISSR primers produced 388 clear bands, among which 375 were polymorphic. The genetic similarity coefficients (GSCs) among 84 zoysiagrass accessions or cultivars ranged from 0.644 to 0.866 with an average of 0.751. The GSCs within species were significantly higher than that among species. Cluster analysis using an unweighted pair group method with arithmetic mean (UPGMA) method showed that the 84 zoysiagrass accessions could be classified into 10 major groups. Accessions from similar geographic regions were generally clustered together, which indicated a correlation between molecular groupings and the geographical origin. The investigation demonstrated the genetic diversity of different germplasm, and that ISSR markers are an effective tool for the study of genetic variation in zoysiagrass.

Key words: Chinese accessions, genetic diversity, inter-simple sequence repeat (ISSR) markers, zoysiagrass.

INTRODUCTION

Zoysiagrass *(Zoysia* sp.), with well-developed stolon and short culm, is able to form a dense swards (Weng et al., 2007). It was extensively used in turf establishment and livestock herbage. The genus zoysia consists of 16 species that are naturally distributed on sea coasts and grasslands around the East Asia. Five species have been identified from southern Hokkaido to the southwest islands in Japan (Kitamura, 1989). Of these, *Zoysia japonica* Steud., *Zoysia matrella* Merr., and *Zoysia tenuifolia* Wild are utilized as turfgrass. In addition, *Z. japonica* is also used as forage grass in Japan and other countries in East Asia (Shoji, 1983; Fukuoka, 1989). In China, *Zoysia* sp. are distributed from north eastern area of Liaoning province in the north to Fujian province in the south (Jin et al., 2004), with a variety of ecological types. These wild resources survived through long-term natural selection, and thereby had strong environmental suitability and stress resistance (Jin and Han, 2004).

Previous researchers investigated the genetic variation of some zoysiagrass germplasm. The *Zoysia* sp. grown in various environments of coastal areas in Tanwan had a great variation in morphology, isozyme pattern, and salt tolerance (Weng et al., 1995; Weng and Chen, 2001; Weng, 2002). Kitamura (1989) and Choi et al. (1997a, b) evaluated the morphology and isozyme pattern of *Zoysia* sp. collected from Japan and Korea, respectively. However, morphological characteristics are not adequate to reveal genetic differences among cultivars because phenotypic traits are easily influenced by environment. Kitamura (1970) investigated morphological characteristics of natural zoysiagrass populations and found that the classification criteria of *Zoysia* sp. should be reconsidered because morphological characteristics varied continuously among species. With the development of molecular techinique, molecular marker has been considered as a preferred method for evaluating the genetic diversity of plant germplasm because it could even distinguish closely related genotypes (Nybom, 1994). Molecular markers are not easily affected by

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environmental factors or by development stages (Bebeli and Kaltsikes, 1993). Molecular data can contribute to a more holistic picture of genetic diversity within a collection of populations (Curley and Jung, 2004). Yaneshite et al. (1997) employed restriction fragment length polymorphism (RFLP) markers to study the genetic diversity within 17 zoysiagrass accessions from Japan. Choi and Yang, (1996) and Weng (2007) found extensive diversity of wild zoysiagrass accessions collected from Korea and Taiwan based on randomly amplified polymorphic DNA (RAPD) technique by investigating morphological characteristics in natural populations. Guo et al. (2007, 2009) evaluated genetic diversity and interspecific relationship of 96 China *Zoysia* sp. wild germplasm by applying simple sequence repeat (SSR) and sequence related amplified polymorphism (SRAP) markers. However, compared to its wide distribution, the research of molecular variation in Chinese wild zoysiagrass is limited.

The ISSR marker is a widely used molecular marker technique, in terms of its high reproducibility, low cost, and less complexity (Reddy et al., 2002). It has been used in identification and genetic relationship estimation of many plant species. However, there are limited reports on the genetic diversity among zoysiagrass species based on ISSR markers.

In China, wild zoysiagrass is abundant and extensively distributed. However, there is very limited information on the general genetic variation among indigenous Chinese zoysiagrass germplasm. In this study, we used ISSR markers; (i) to estimate the genetic relationship among 81 Chinese natural zoysiagrass accessions and three cultivars, and (ii) to classify them and provide the basic information for conservation and breeding strategies for zoysiagrass.

MATERIALS AND METHODS

Plant materials

Eighty-four accessions of four species of zoysiagrass [81 were natural zoysiagrass accessions collected from seven provinces of China (Table 1), and three commercial cultivars (Zenith, Meyer and Grif16454)] were used in this study. Of these materials, there were 50 Z. *japonica*, 21 *Zoysia sinica*, six *Zoysia macrostachya*, and seven *Z. matrella* (L.) based on morphology identification (Table 1). According to their provinces of collection, these natural zoysiagrass accessions were classified into seven groups. All the accessions were propagated asexually in Wuhan Botanical Garden, Chinese Academy of Sciences. They were grown in a mixture of 9 sand: 1 organic material in pots (15 cm in diametre and 20 cm deep). The pots were kept in a greenhouse with a daily maximum/ minimum temperature of 30/25°C, a 12 h photoperiod.

Genomic DNA extraction

Total DNA was isolated from young fresh zoysiagrass leaves (0.1 g) using the cetyl trimethylammonium bromide (CTAB) method as described by Doyle (1991) with slight modification. Leaf tissues were directly ground in liquid nitrogen with a mortar and pestle. The powder was transferred into 2 ml centrifuge tubes with 0.9 ml of CTAB extraction buffer (containing 2% CTAB, 5 M NaCl, 0.5 M EDTA pH 8.0, 1 M Tris–HCl pH 8.0). After 30 min of incubation at 65°C, equal volume of chloroform/isoamyl alcohol (24:1) was added into each tube. After being vortexed gently for three min, the mixtures were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred to new tubes and cold isopropanol was added to ⅔ volume of supernatant. After 30 min on ice, DNA was precipitated by centrifugation at 12,000 rpm for 10 min at 4°C. The pellets were washed with 70% ethanol, and dissolved in TE buffer. DNA concentration was quantified using UV spectrophotometer, and the integrity was examined on 0.8% agarose gel electrophoresis.

ISSR analysis

According to previous reports (Zeng et al., 2006; Fan et al., 2007; Liu et al., 2007; Xiao et al., 2007), 60 ISSR primers were synthesized. These primers were screened with six accessions for polymorphism and reproducibility. 33 primers producing clear, stable and polymorphic fragments were used for ISSR analysis. PCR amplification was performed in a total volume of 25 µl. The reaction mixture included 40 ng DNA template, 0.5 µM primer, 0.2 mM dNTP (Pharmacia, America), 1.5 µM $MgCl_2$ (Fermentas, EU), 1×Tap buffer (with $(NH_4)_2SO_4$) (Fermentas, EU), and 1.0 U Taq DNA polymerase (Fermentas, EU). The PCR was carried out in a Mastercycler gradient PCR machine (Eastwin, China). All the PCRs were performed using a programme for denaturing at 94°C for 5 min; 5 cycles at 94°C for 45 s, 60°C for 45 s, 72°C for 1.5 min decreasing by increments of 1°C for annealing with each cycle; 38 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 1.5 min; and then extending at 72°C for 7 min.

After amplification, 2 µL loading buffer was added to the PCR products. The mixture was then analysed on 1.8% agarose gel in 1×Tris-aceticacid-EDTA (TAE) buffer and stained with ethidium bromide (0.5 µg/ml). The image bands were acquired through UV light using Gel Doc XR system (Bio-rad, America). DL2000 molecular marker was used to estimate the size of the fragments amplification. All testing was repeated at least twice.

Data analysis

Distinct and reproducible bands produced by ISSR primers were scored in terms of a binary code [present (1) or absent (0)] among all accessions.

Jaccard's coefficient of genetic similarity was calculated based on the binary data (matrix) (Sneath and Sokal, 1973) between all possible pairs of accessions. Each of the seven geographical groups was subjected to the following analyses: the actual number (n_a) of alleles was counted for each amplified locus. The effective number of alleles was estimated as $n_e=1 + 4N_eu$ for each locus, where N_e is the effective population size and u is the average mutation rate (Kimura and Crow, 1964). The Shannon diversity index (I) is a common diversity index used to account for both abundance and evenness of the alleles present, and is useful for understanding allele structure at an ISSR locus (Shannon, 1949; Cai et al., 2010). Shannon's information index was estimated for each locus using the formula $I = -\sum PiLnPi$ $=1-S$, where S is the total number of alleles in the locus, and Pi is the proportion of S made up of the ith allele. Nei's gene diversity (He) is another common diversity index in population genetics (Nei, 1973). In this study, gene diversity was estimated according to the formula of Nei (1973) for each locus, $He = 1 - \sum P_{ij}^2$, where P_{ij} is the frequency of the jth allele for ith locus summed across all alleles of the locus.

Table 1. Details of 84 germplasm accessions used in this study.

Sample number	Origin	Habitat	Species	Latitude (H)	Longitude (E)	Altitude (m)
1	Rizhao,Shandong	Wilderness	*Z. japonica* Steud.	35°17′914″	119°26′164″	6
2	Juxian,Shandong	Mountain	*Z. japonica* Steud.	35°29′244″	119°17′954″	106
3	Jiaozhou,Shandong	Ditch	*Z. japonica* Steud.	36°12′647″	120°00′611″	44
4	Jiaonan,Shandong	Hillside	*Z. japonica* Steud.	36°06′258″	119°59′635″	35
5	Jiaonan,Shandong	Roadside	*Z. japonica* Steud.	35°59′002″	119°59′109″	72
6	Qingdao,Shandong	Hillside	*Z. japonica* Steud.	36°18′436″	120°30′786″	99
7	Jiaonan,Shandong	Alkaline land	*Z. macrostachya* Franch. Et Sav	/	/	/
8	Jiaonan,Shandong	Alkaline land	*Z. matrella (L.)* Merr.	/	/	/
9	Jimo,Shandong	Roadside	*Z. japonica* Steud.	36°18′976″	120°37′826″	72
10	Jimo,Shandong	Alkaline land	*Z. sinica* Hance	36°24′286″	120°41′783″	7
11	Jimo,Shandong	Hillside	*Z. japonica* Steud.	36°32′324″	120°38′846″	50
12	Jimo,Shandong	Roadside,ditch	*Z. japonica* Steud.	36°34′123″	120°38′755″	66
13	Rushan,Shandong	Cliff,rock tunnels	*Z. japonica* Steud.	36°47′931″	121°21′305″	61
14	Rushan,Shandong	Ridge,hillside	*Z. japonica* Steud.	37°00′255″	121°29′930″	59
15	Muping,Shandong	Woodland	*Z. japonica* Steud.	37°08′806″	121°29′807″	75
16	Muping,Shandong	Hillside	*Z. sinica* Hance	37°15′789″	121°31′784″	71
17	Penglai,Shangdong	Hillside	*Z. sinica* Hance	37°43′298″	120°49′870″	71
18	Penglai,Shangdong	Hillside	*Z. japonica* Steud.	37°38′765″	120°50′783″	131
19	Yantai,Shandong	Roadside	*Z. japonica* Steud.	37°23′861″	121°21′640″	20
20	Chizhou,Anhui	Roadside,ditch	*Z. japonica* Steud.	/	/	/
21	Chizhou,Anhui	Foot of a hill	*Z. japonica* Steud.	30°32′073″	117°25′352″	54
22	Nanlin,Anhui	Hirst	*Z. japonica* Steud.	30°48′488″	118°16′485″	30
23	Nanlin,Anhui	Hillside	*Z. sinica* Hance	30°48′490″	118°16′487″	30
24	Nanlin,Anhui	Hirst	*Z. japonica* Steud.	30°50′774″	118°18′456″	22
25	Hefei,Anhui	Roadside	*Z. japonica* Steud.	31°52′075″	117°29′923″	17
26	Hefei,Anhui	Nature meadow	*Z. japonica* Steud.	31°49′644″	117°35′332″	66
27	Feidong,Anhui	Hillside	*Z. sinica* Hance	31°48′320″	117°38′690″	50
28	Chaohu,Anhui	Hillside	*Z. japonica* Steud.	31°45′423″	117°47′304″	26
29	Chaohu,Anhui	Roadside	*Z. japonica* Steud.	31°40′382″	117°51′863″	75
30	Chaohu,Anhui	Nature meadow	*Z. japonica* Steud.	31°45′326″	118°09′619″	21
31	Jurong,Jiangsu	Nature meadow	*Z. japonica* Steud.	32°00′620″	119°06′023″	39
32	Jurong,Jiangsu	Country road	*Z. japonica* Steud.	31°58′605″	119°13′462″	30
33	Zhengjiang,Jiangsu	Country road	*Z. japonica* Steud.	32°08′361″	119°20′756″	30
34	Lianyungang,Jiangsu	Mountain road	*Z. japonica* Steud.	34°41′819″	119°24′382″	16
35	Lianyungang,Jiangsu	Mountain road	*Z. japonica* Steud.	34°41′944″	119°24′627″	84
36	Lianyungang,Jiangsu	Roadside	*Z. japonica* Steud.	34°41′944″	119°24′627″	84
37	Guanyun,Jiangsu	Hillside	*Z. japonica* Steud.	34°18′305″	119°14′240″	31
38	Dongtai,Jiangsu	Country road	*Z. sinica* Hance	32°53′608″	120°34′650″	7
39	Dongtai,Jiangsu	Beside the pond	*Z. japonica* Steud.	32°54′723″	120°53′368″	10
40	Dongtai,Jiangsu	Beside the pond	*Z. sinica* Hance	32°53′797″	120°54′004″	11
41	Dongtai,Jiangsu	Benches	*Z. macrostachya* Franch. Et Sav	32°51′785″	120°34′039″	4
42	Dongtai,Jiangsu	Benches	*Z. macrostachya* Franch. Et Sav	32°51′785″	120°34′039″	4
43	Gongjinggang,Jiangsu	Alkaline land	*Z. macrostachya* Franch. Et Sav	32°44′586″	120°51′878″	9
44	Gongjinggang,Jiangsu	Alkaline land	*Z. macrostachya* Franch. Et Sav	32°45′552″	120°51′928″	7

All these analyses were conducted using the Popgene 1.32. A clustering analysis of all accessions was performed using UPGMA method, and then principal coordinate analysis (PCA) was carried out using the software package NTSYSpc 2.1. The confidence limits for the dendrogram groupings were performed by bootstrapping using the Win Boot programme.

Table 1. Contd.

Sample number	Origin	Habitat	Species	Latitude (H)	Longitude (E)	Altitude (m)
45	Huzhou,Zhejiang	Roadside	*Z. sinica* Hance	30º36′562″	119º53′077″	146
46	Xiaofeng,Zhejiang	Tea garden	*Z. sinica* Hance	30º34′730″	119º31′661″	74
47	Xiaofeng,Zhejiang	Tea garden	*Z. japonica* Steud.	30º34′731″	119º31′662″	-70
48	Huzhou,Zhejiang	Roadside	*Z. sinica* Hance	30º36′563″	119º53′078″	-142
49	Jinhua,Zhejiang	Hillside	*Z. macrostachya* Franch. Et Sav	29º03′782″	119º44′885″	39
50	Fuzhou,Fujian	Rock tunnels	*Z. japonica* Steud.	26º05′180″	119º14′194″	30
51	Fuzhou,Fujian	Botanical garden	*Z. sinica* Hance	26º05′171″	119º14′334″	68
52	Fuzhou,Fujian	Botanical garden	*Z. matrella* (L.) Merr.	26º05′171″	119º14′334″	68
53	Changle,Fujian	Seaside	*Z. sinica* Hance	25º48′794″	119º36′642″	13
54	Shantou,Guangdong	Seaside	*Z. sinica* Hance	23º13′662″	116º41′094″	23
55	Shantou,Guangdong	Seaside	*Z. sinica* Hance	23º25′145″	116º59′325″	9
56	Shantou,Guangdong	Seaside	*Z. matrella* (L.) Merr.	23º25′172″	117º00′364″	9
57	Shantou,Guangdong	Seaside	*Z. sinica* Hance	23º25′128″	116º58′126″	9
58	Gaoyao,Guangdong	Hillside	*Z. matrella* (L.) Merr.	23º02′660″	112º24′819″	12
59	Yunfu,Guangdong	Coentry road	*Z. matrella* (L.) Merr.	22º53′435″	112º16′945″	18
60	Donghai,Guangdong	Nature meadow	*Z. japonica* Steud.	21º01′209″	110º27′251″	19
61	Shenyang,Liaoning	Dike	*Z. sinica* Hance	41º33′580″	123º19′045″	36
62	Anshan,Liaoning	Foot of hill	*Z. japonica* Steud.	41º03′100″	123º08′334″	77
63	Xiuyan,Liaoning	Hillside	*Z. japonica* Steud.	40º12′400″	123º17′194″	88
64	Xiuyan,Liaoning	Hillside	*Z. japonica* Steud.	40º12′400″	123º17′194″	88
65	Xiuyan,Liaoning	Hirst	*Z. japonica* Steud.	40º16′203″	123º21′222″	71
66	Xiuyan,Liaoning	Hillside	*Z. japonica* Steud.	40º19′047″	123º25′535″	92
67	Xiuyan,Liaoning	Hillside	*Z. japonica* Steud.	40º18′457″	123º34′602″	80
68	Fengcheng,Liaoning	Roadside	*Z. japonica* Steud.	40º19′194″	123º43′597″	96
69	Fengcheng,Liaoning	Hirst	*Z. japonica* Steud.	40º22′867″	123º79′141″	119
70	Fengcheng,Liaoning	Rangeland with spare forest	*Z. japonica* Steud.	40º24′984″	124º03′142″	112
71	Dandong,Liaoning	Wilderness	*Z. sinica* Hance	40º02′080″	124º21′450″	16
72	Dandong,Liaoning	Roadside	*Z. sinica* Hance	39º56′000″	124º16′040″	16
73	Dandong,Liaoning	Mountain road	*Z. sinica* Hance	39º52′035″	123º54′143″	20
74	Dandong,Liaoning	Roadside	*Z. sinica* Hance	39º52′889″	123º42′961″	17
75	Dandong,Liaoning	Beside the pond	*Z. japonica* Steud.	39º51′766″	123º31′779″	8
76	Dalian,Liaoning	Mountain	*Z. sinica* Hance	39º41′770″	122º55′848″	22
77	Dalian,Liaoning	Corn field	*Z. japonica* Steud.	39º31′989″	122º28′737″	38
78	Dalian,Liaoning	Hillside	*Z. japonica* Steud.	39º27′574″	122º24′911″	20
79	Dalian,Liaoning	Roadside	*Z. japonica* Steud.	39º07′559″	121º43′544″	22
80	Dalian,Liaoning	Hillside	*Z. japonica* Steud.	38º57′771″	121º19′762″	65
81	Dalian,Liaoning	Seaside	*Z. sinica* Hance	39º58′178″	121º19′650″	14
82	Zenith	cultivar	*Z. japonica* Steud.	/	/	/
83	Meyer	cultivar	*Z. japonica* Steud.	/	/	/
84	Grif16454	cultivar	*Z. matrella* (L.) Merr.	/	/	/

RESULTS

ISSR analysis

33 primers generated 388 bands ranging from approximately 100 to 2000 bp in size, of which, 375 bands were polymorphic (96.65%) (Table 2). Figure 1 shows a typical PCR amplification patterns by primer I3 in 84 Zoysia accessions. Each primer produced five to 18 polymorphic bands, and the largest amount of bands was

Table 2. ISSR primers used in this study.

Primer	Primer sequence (5'-3')	Tm (°C)	Total band	Phlymorphism		Bands size (bp)
				Band	%	
P2	$(TG)_8RA$	61.1	18	18	100	250 - 2000
P3	$(CA)_8A$	61.1	15	15	100	140 - 1000
P5	$(GT)_8T$	61.1	16	16	100	250 - 2000
P8	$(GGGGT)_3$	69.3	11	11	100	250 - 1200
P9	$(AC)_8YT$	61.1	17	17	100	190 - 1500
P10	$(AC)_8YA$	61.1	14	13	92.9	250 - 2000
P12	$(AC)_8YG$	63.5	8	6	75	250 - 1500
P13	$(CCCT)_4$	70.1	8	8	100	500 - 1700
P14	$HVH(TG)_7$	53.8	16	16	100	250 - 2000
P20	$(AC)_8TG$	64.5	13	13	100	250 - 2000
P21	$(AG)_8GCC$	69.7	9	8	88.9	250 - 1000
P22	$(GACA)_4$	59.8	18	18	100	170 - 1900
P23	$(CA)_8TA$	62.2	9	9	100	250 - 500
P25	$(AC)_8GA$	64.5	17	17	100	210 - 1750
P26	$(AC)_8C$	63.5	7	6	85.7	250 - 1700
P32	$(AG)_8GC$	66.8	14	14	100	190 - 1500
P36	$(AC)_8GT$	64.5	7	7	100	250 - 750
P39	$(GA)_8GCC$	69.7	11	11	100	250 - 1000
P42	$ACTCGTACT(AG)_7$	71.8	7	6	85.7	250 - 1500
P43	$CGTAGTCGT(CA)_7$	73.5	15	15	100	270 - 1750
P45	$AGTCGTAGT(AC)_7$	71.8	11	10	90.9	150 - 1500
N1	$(GA)_8C$	63.5	9	9	100	350 - 1600
N11	$(AG)_8YC$	63.5	9	8	88.9	250 - 900
N14	$(AG)_8YA$	61.1	11	11	100	210 - 1700
N17	$(GA)_8RC$	63.5	10	9	90	250 - 2000
N20	$(GA)_8YG$	63.5	13	12	92.3	110 - 1500
N22	$(GA)_8YA$	61.1	8	8	100	340 - 1600
N23	$(GA)_8YT$	61.1	10	10	100	250 - 1000
N24	$(GT)_8YC$	63,5	15	15	100	200 - 2000
N25	$(GT)_8YG$	63.5	14	13	92.9	430 - 2000
N26	$(GT)_8YA$	61.1	8	7	87.5	250 - 1700
I1	$(CT)_8G$	63.5	5	4	80	300 - 1600
I3	$(AC)_8CG$	66.8	15	15	100	250 - 2000
Total			388	375	11.36	
Mean			11.76	96.65		

produced by P2 and P22. 21 of the 33 primers showed 100% polymorphism. None of the pair of accessions exhibited identical band patterns, indicating that these ISSR primers could discriminate all the 84 accessions. 17 bands were unique to a single accession. The primer P23 amplified three unique bands, and the primer P22 produced two unique bands.

Genetic similarities

Jaccard's genetic similarity coefficients (GSCs) were caculated based on the original matrix data. Pair-wise comparison of accessions indicated GSCs between accessions ranged from a minimum of 0.644 (between 2 and 56) to a maximum of 0.866 (between 35 and 36), with a mean of 0.751. The GSCs within or among the species are shown in Table 3. The mean GSCs within the species of *Z*. *japonica*, *Z*. *sinica*, *Z*. *macrostachya* and *Z*. *matrella* (L.) was 0.760, 0.745, 0.778 and 0.749, respectively. The species *Z*. *japonica* had the most widely GSCs range (from 0.649 to 0.866). The GSCs within the *Z*. *sinica*, *Z*. *macrostachya* and *Z*. *matrella* (L.) species were changed from 0.649 to 0.845, 0.727 to 0.840, and 0.691 to 0.835, respectively. Among the species, the maximum mean GSCs (0.751) was between *Z*. *japonica* and *Z*. *sinica* (*Z*.

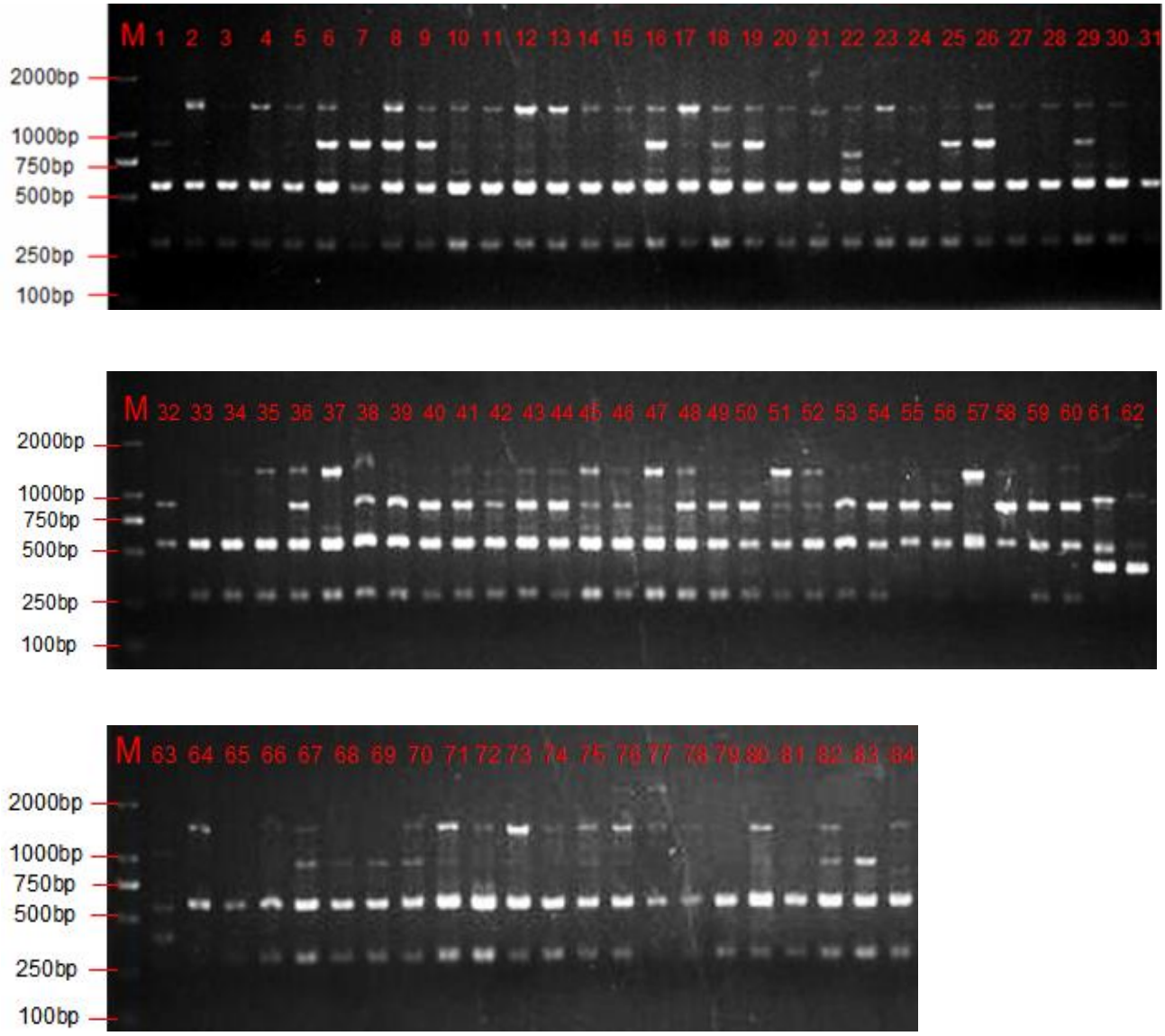

Figure 1. PCR amplification patterns by primer I3 in 84 Zoysia accessions.

japonica vs. *Z*. *sinica*). The mean GSCs of *Z*. *japonica* vs. *Z*. *macrostachya* (0.746) was similar to that of *Z*. *sinica* vs *Z*. *macrostachya* (0.748). Meanwhile, the mean GSCs of *Z*. *japonica* vs *Z*. *matrella* (0.739) was similar to that of *Z*. *sinica* vs. *Z*. *matrella* (0.738). The mean GSCs of *Z*. *macrostachya* vs. *Z*. *matrella* (0.732) was the minimum.

Genetic diversity among different geographic groups

Based on different geographic origin, the 81 accessions were divided into seven groups. Genetic parameters among the seven groups were analyzed by ISSR marker (Table 4). As shown in Table 4, polymorphism rate significantly varied with geographical groups, from 40.22% in Zhejiang to 75.98% in Liaoning. The observed number of alleles per locus (n_a) varied from 1.40 in Zhejiang group to 1.76 in liaonign group. Consequently, it was shown that there was also variation for the effective number of alleles (n_e), ranging from 1.22 in Anhui, Zhejiang, Fujian to 1.28 in Liaoning (Table 4). There existed varitions for I within and among the geographical groups, ranging from 0.13 in Zhejiang to 0.18 in Liaoning with an average of 0.15. H_e did also vary with the geographical groups, ranging from 0.20 in Zhejiang to 0.30 in Liaoning, with an average of 0.25 (Table 4).

According to the polymorphism rate and gene diversity index (H_e and I), the trend of genetic diversity among the seven groups was as follows: Liaoning group > Shandong group > Jiangsu and Guangdong group > Anhui group > Fujian group > Zhejiang group. The similar results were also obtained by other genetic parameters.

Table 3. Variance range of genetic similarity coefficient between different species of zoysiagrass.

Species comparison		Genetic similarity coefficient		
		Mean	Minimum	Maximum
Within species	*Z. japonica* vs *Z. japonica*	0.760	0.649	0.866
	Z. sinica vs *Z. sinica*	0.745	0.649	0.845
	Z. macrostachya vs *Z. macrostachya*	0.778	0.727	0.840
	Z. matrella (L.) vs *Z. matrella* (L.)	0.749	0.691	0.835
Between species	*Z. japonica* vs *Z. sinica*	0.751	0.664	0.851
	Z. japonica vs *Z. macrostachya*	0.746	0.662	0.832
	Z. japonica vs *Z. matrella* (L.)	0.739	0.644	0.827
	Z. sinica vs *Z. macrostachya*	0.748	0.686	0.825
	Z. sinica vs *Z. matrella* (L.)	0.738	0.662	0.802
	Z. macrostachya vs *Z. matrella* (L.)	0.732	0.686	0.804

Table 4. Population genetic parameters of *Zoysia* sp. germplasm in China.

Parameter	Geographical group						
	Shandong	Liaoning	Anhui	Zhejiang	Jiangsu	Fujian	Guangdong
GS	19	21	11	5	14	4	7
NPL	126	136	99	72	114	74	103
PR (%)	70.39	75.98	55.31	40.22	63.69	41.34	57.54
n_a	1.70	1.76	1.55	1.40	1.64	1.41	1.58
n_e	1.26	1.28	1.22	1.22	1.25	1.22	1.26
I	0.17	0.18	0.14	0.13	0.16	0.14	0.16
He	0.27	0.30	0.22	0.20	0.26	0.21	0.26

GS, Group size; NPL, number of polymorphic loci; PR, polymorphism rate; n_a, observed number of alleles; n_e, effective number of alleles; I, Shannon's information index; He, average Nei's gene diversity.

Cluster analysis

An UPGMA dendrogram was constructed based on the ISSR data (Figure 2). As a result, all the zoysiagrass accessions could be grouped into ten groups (A to J) and some of these groups (A and C) could be further clustered into subgroups. The results show that the accessions from the same geographic regions were generally, but not completely clusted in the same cluster, indicating a correlation between molecular groupings and the geographical origin.

Most of these accessions (52/84=61.9%) were clustered into the group A, which can be futher divided into two sub-groups, A1 and A2. These 52 accessions were collected from Shandong, Jiangsu, Anhui and Liaoning province, respectively (Figure 2). Accessions from the same province or neighboring regions were generally clustered together in the same subgroup. For example, Subgroups A1 comprised 13 accessions from Shandong, 10 from Anhui and six from Jiangsu province. Actually, these three provinces are adjacent in geography. Subgroup A2 included all the 21 accessions (61 to 81) collected from Liaoning province, one accession from Shandong province (3) and one commercial cultivar (Grif16454). Also, all of these 52 accessions are *Z. japonica* and *Z. sinica*, except one accession of *Z. matrella*. As for group B, it contained eight accessions collected from Jiangsu province (37 to 44), and two accessions from Zhejiang province. Two of these accessions are *Z. japonica*, four are *Z. sinica* and the other four are *Z. macrostachya*. There were 11 accessions in the group C. Cluster C could be further separated into two subgroups. Subgroup C1 included the seven accessions from Zhejiang, Fujian, Guangdong province, and these accessions belong to four species. Subgroup C2 comprised four accessions and all of these are *Z. sinica*. The group D was composed of only two accessions collected from Shandong province. Cluster E, G, H, and J

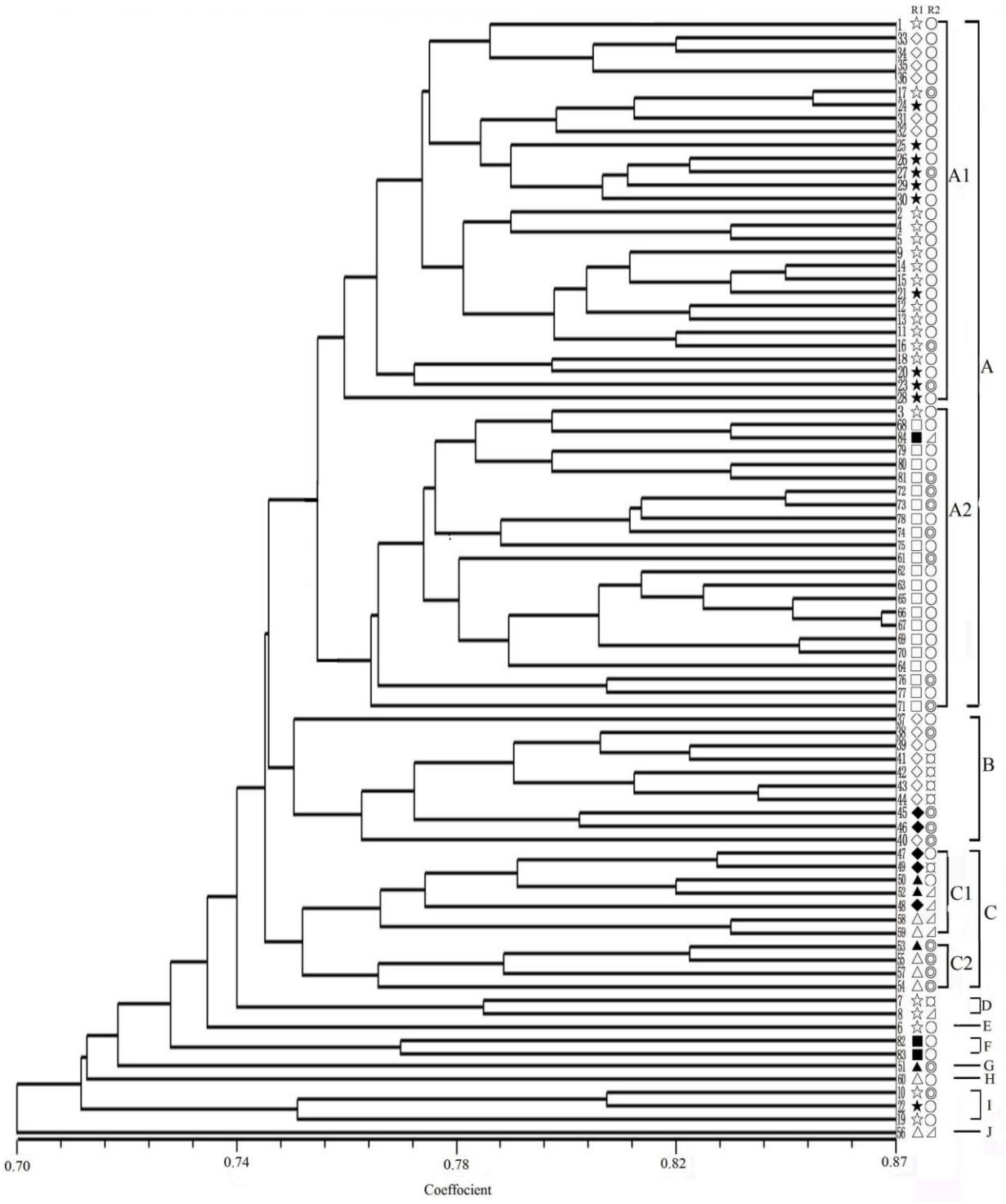

Figure 2. Dendrogram of 84 accessions of zoysia derived from an UPGMA cluster analysis based on Jaccard's similarity coefficient matrix. R1, Province including: ☆, ShanDong; ★, AnHui; ◇, JiangSu; ◆, ZheJiang; □, LiaoNing; △, GuangDong; ▲, FuJian; ■, cultivated species; R2: species including ○, *Z. japonica* Steud.; ◎, *Z. sinica* Hance; ¤, *Z. macrostachya* Franch. Et Sav; ⊿, *Z. matrella*(L.) Merr.

all had only one accession from Shandong (6), Fujian (51), and Guangdong (60 and 56) province, respectively. Cluster F was composed of two commercial cultivars and both of these cultivars are *Z. japonica*, while cluster I contained three accessions from Shandong and Anhui province.

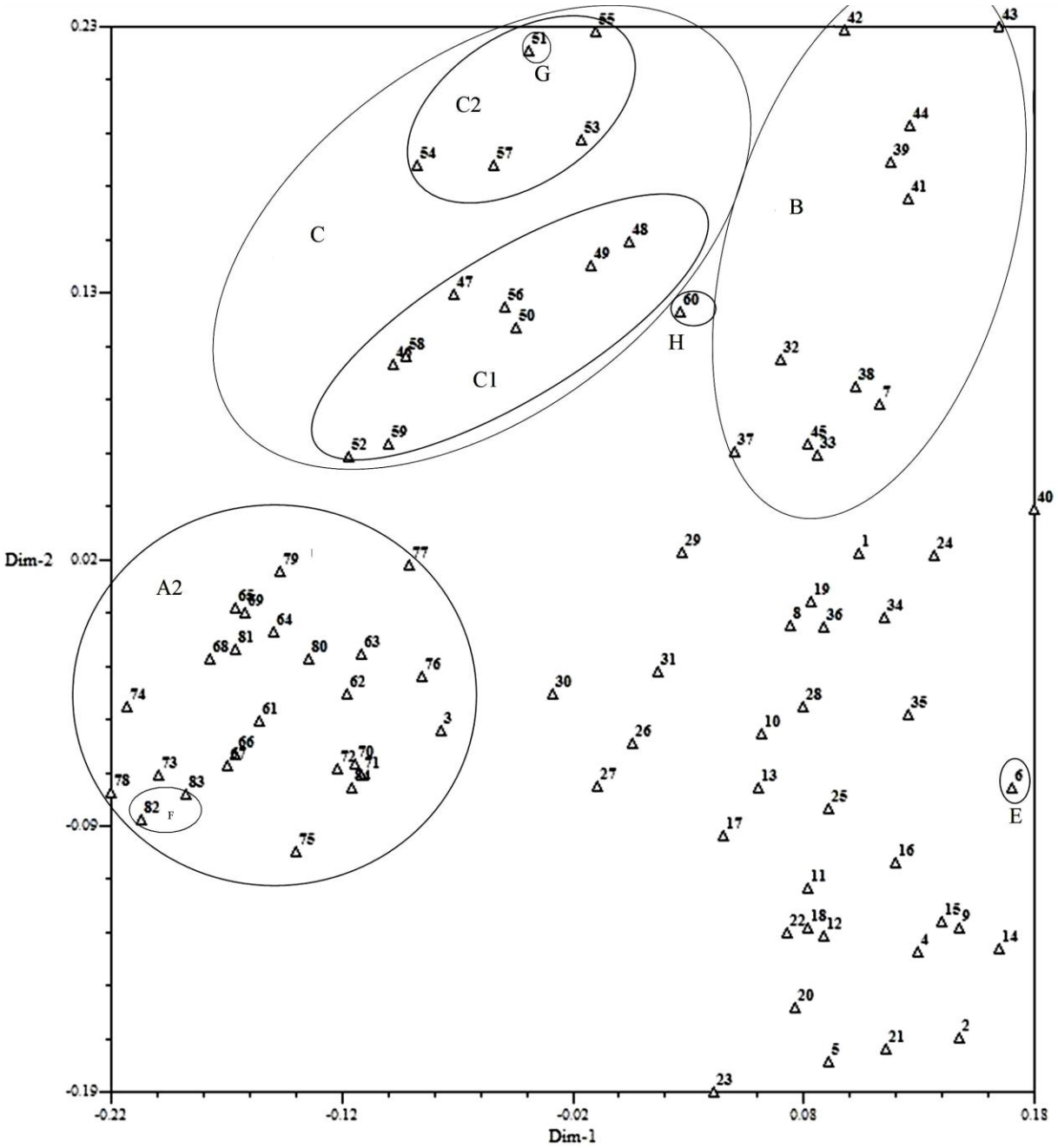

Figure 3. Principle coordinate analysis (PCA) based on the genetic similarity coefficients derived from the polymorphic ISSR results for the 84 zoysiagrass accessions.

PCA was also performed to display the relationship among the 84 zoysiagrass types on two coordinate axes (Figure 3). The PCA revealed the similar grouping of accessions as the dendrogram constructed by UPGMA and placed the 84 genotypes into four distinct groups. Those accessions in subgroup A2 and cluster F were gathered together. Among the other Chinese natural accessions, those in cluster B and C were separated from others. Both 6 and 60 were separated from the other accessions, which were located in genetic cluster E and H in the dendrogram, respectively. The cluster D, F, I and the sub cluster A1 of similar origin were grouped together.

DISCUSSION

In this study, ISSR marker was successfully used to differentiate the 81 Chinese wild zoysia accessions and three commercial cultivars. The 33 selected primers generated 388 bands with an average of 11.76 bands per

primer. The polymorphic bands (PPB) accounted for 96.65% of total bands. The high PPB was in agreement with many investigations using ISSR technique in other plant species (Hess et al., 2000; Belaid et al., 2006; Terzopoulos and Bebeli, 2008). Therefore, it suggested that ISSR molecular markers could be effectively used to assess the genetic diversity of wild zoysia genus accessions.

In this study, GSCs between each pair of the 84 accessions ranged from 0.644 to 0.866, with a mean of 0.751, suggesting a great level of genetic diversity among Chinese wild zoysiagrass accessions. Guo et al. (2007) also found a wide GSCs ranging from 0.592 to 0.936 among 96 zoysiagrass accessions collected from 12 provinces in China by SSR analysis. The difference in GSCs between these two studies could be due to the difference in accessions amount, the sampling sites, the molecular marker method, and variety of species. Moreover, similar to our findings, Guo et al. (2009) also observed that GSCs within species were higher than that among species by using SRAR markers. In this study, *Z. japonica* had the most widely GSCs range, followed by *Z. sinica*. Among the species, the maximum mean GSCs was between *Z. japonica* and *Z. sinica*. The mean GSCs of *Z. japonica* vs *Z. macrostachya* was similar to that of *Z. sinica* vs *Z. macrostachya* and the mean GSCs of *Z. japonica* vs *Z. matrella* was similar to that of *Z. sinica* vs *Z. matrella*. This was consistent with the distribution range of these four species (Li et al., 2004). Our study indicate that the mean GSCs between the species of *Z. japonica* and *Z. sinica* was significantly higher than the other five combinations, which is in agreement with the results of previous studies (Choi et al., 1997a, b; Guo et al., 2007; 2009). Both previous and our results show that zoysiagrass accessions had a great genetic variation regardless of their origination. This might be attributed to the wide distribution of zoysia, north-south across about 20 degrees in latitude (43°22 N to 23°30 N), east-west across about 34 degrees in longitude (109°E to 143°E). Therefore, with the long-term evolution, zoysiagrass formed great genetic variation in order to adapt to the different environment and weather conditions.

The study indicates that there was great genetic diversity within and among the geographical groups. The genetic diversity level of seven groups is related to the sample size, the group with more samples which generally had higher genetic diversity level (Sankar and Moore, 2001). The investigations showed that Liaoning and Shandong population possessed richer genetic diversity than other populations. In China, wild zoysiagrass germplasm were distributed mainly in Liaoning and Shandong province. However, the genetic diversity of these two populations was destroyed gradually by human activities. Thereby, it was no surprise that the genetic diversity within these two populations is decreasing.

The clustering results demonstrated also that the accessions belonging to the same species were not completely clustered in the same cluster. For instance, the mean GSCs between *Z. japonica* and *Z. sinica* was higher than others, so the majority accessions of these two species were classified together. Cluster A comprised of most of these two species. Cluster B comprised two *Z. japonica*, four *Z. macrostachya* and four *Z. matrella* (*L.*) accessions. Both clusters D and F comprised two species whereas cluster E, G, H and J had only a single accessions belonging to *Z. japonica*, *Z. sinica*, *Z. japonica*, *Z. matrella*, respectively. This result indicates that the genetic differentiation in *Zoysia* sp. in China is less related to the taxonomic status. The same tendency was found by RAPD analysis (Lin, 2000), isozyme analysis (Weng, 2002), and SSR and SRAP analysis (Guo, 2007, 2009). Weng et al. (2007) also reported that UPGMA analysis result was inconsistent with the morphological classification of zoysia in conventional taxonomy. This phenomenon may be related with specific adaptation, flowering habit and pollination system. Probably, due to the high ability of *zoysia* sp. to hybridize interspecifically, the gene flow might have occurred among species. Numerous previous studies indicated that there might be certain mechanisms to promote cross-pollination in zoysia species (Hong and Yean, 1985). Thus, the outcrossing breeding system perhaps accounted for high levels of genetic variation within species and high levels of genetic similarity coefficient among species.

The UPGMA clustering analysis indicated that the zoysiagrass acessions from same or adjacent regions were inclined to be classified together. This indicated that those accessions grown in a similar environment also tended to be classified together. It seems that there is some correlation between the molecular groups and geographic origins. Similar results were also found by Weng et al. (2007) in zoysia accessions collected in Taiwan, Penghu Islands and Lanyu using RAPD markers. However, there are some exceptions; the 6, 51 and 56 accessions which originated from Shandong, Fujian and Guangdong province respectively were separated from the main groups. Those accessions perhaps have some special genetic feature that is distinct from other zoysia accessions. On the other side, those exceptions might attribute to gene mutation or asexual propagation in regions other than origin area through human activities of river run-off (Yi et al, 2008). Surprisingly, three commercial cultivars were not clustered together although they were all introduced from America. Accession Grif16454 is *Z. matrella* (L.) which was collected originally from China while the others are *Z. japonica* gathered from North Korea (Meyer) and other country (Zenith) (Xu et al., 2004). The result shows that Grif16454 was clustered togther with those accessions from Liaoning Province. Therefore, Grif16454 might have been collected from Liaoning province by previous American scholars.

To conclude, this study indicates abundant genetic variation among Chinese wild zoyiagrass germplasm. The majority of Chinese natural accessions from the adjacent

regions were clustered into one group, showing a correlation between molecular groups and geographic origins but differentiated into species. The results might provide valuable information for the conservation of Chinese natural zoyiagrass resources. In addition, this study may also provide useful information for the selection of parental combinations in the zoysia breeding program.

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