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Genetic diversity analysis and fingerprint construction for 45 Chinese *Zoysia* germplasm collections

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Abstract

Zoysia spp. germplasm exhibit genetic variation between and within species. A comprehension of the genetic diversity of *Zoysia* germplasm could enable the effective utilization of these germplasms in future breeding endeavors. Ten simple sequence repeats (SSR) primer pairs and nine sequence-related amplified polymorphism (SRAP) primer pairs were used to analyze genetic diversity and construct DNA fingerprints for 45 Chinese *Zoysia* germplasm collections. We detected 231 SSR polymorphic bands and 149 SRAP polymorphic bands with 97.18% and 93.43% polymorphism ratios, respectively. The genetic similarity coefficient of the 45 germplasm collections ranged from 0.623 to 0.856, with an average of 0.727. Forty-five germplasm collections were divided into six major clusters when the genetic similarity coefficient was 0.71 based on the unweighted pair group method with the arithmetic averaging (UPGMA) method. Both SSR and SRAP molecular marker systems can be used to identify all germplasm collections, the SSR primer pair (Xgwm234-5B) and SRAP primer pairs (Me3-Em1 and Me3-Em2) can effectively distinguish 45 *Zoysia* spp. accessions. Collectively, we utilized both SSR and SRAP molecular markers to generate DNA fingerprints in this study providing a theoretical foundation for germplasm conservation and assisting in selecting and breeding new varieties of *Zoysia*.

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Introduction

As a perennial warm-season grass, *Zoysia* (*Zoysia* spp.) is recognized for its low maintenance requirements as well as relatively high tolerance to drought, disease, and traffic, and is widely cultivated for athletic fields, home lawns, and other recreational sites, particularly in east Asia[1]. With the rapid development of the turf industry, it is extensively recognized that germplasm resources are hugely important. Therefore, a large number of researches are focussed on the collection[2–5], evaluation[6–8], and breeding[9–11] of *Zoysia* spp.

Zoysia species exhibit a high rate of outcrossing and are prone to interspecific hybridization, resulting in a wide range of genetic variation among *Zoysia* plants[12]. Kimball et al.[13] identified interspecific hybridization and hypothesized that these hybrids were the result of introgression between species through common breeding methods of *Zoysia*, including directed hybridization of selected parents and cross-pollination in open cross areas. Genetic background analysis of abundant germplasm is an important prerequisite for identifying parents and breeding *Zoysia* varieties[14]. Traditionally morphological identification has several drawbacks due to the susceptibility to environmental factors and plant growth period as well as the limited morphological indexes. Furthermore, some germplasm are difficult to differentiate based on phenotype alone[15]. Molecular markers are an effective means to grasp the genetic information of germplasm. Among several common molecular markers, simple sequence repeats (SSRs) are widely used molecular markers in plant genetics and breeding, due to their multiallelic, codominant inheritance and extensive genome coverage[16]. Sequence-related amplified polymorphism (SRAP) was developed by Li & Quiros[17] in *Brassica*. This method is advantageous due to its simplicity, reasonable throughput rate, disclosure of numerous codominant markers, and targeting of open reading frames (ORFs)[18]. Both marker systems have been applied to a range of fields, including the analysis of genetic diversity of germplasm[19–22], the identification of cultivar and marker-trait association[23]. SSR and SRAP have been reported to be effectively utilized to examine the genetic diversity[4], analyze genetic similarity among cultivars[24], and identify molecular markers linked with quantitative trait loci for biotic and abiotic stress tolerance in *Zoysia*[3,25]. These markers also serve as a potent tool for constructing DNA fingerprints[7,26,27].

The evaluation and improvement of germplasm are of great significance for the effective utilization of these resources. Previous researchers investigated the genetic variation of some *Zoysia* germplasm. Anderson[28] measured inflorescence traits, morphological characteristics, and restricted fragment length polymorphisms (RFLPs) to evaluate the genetic and morphological variations in 11 species of *Zoysia*. Li et al.[29] reported a linkage map of *Zoysia matrella* based on SSR markers, combined the previously reported SSR linkage maps of different *Zoysia* species (*Z. japonica* and *Z. matrella*) and different mapping populations (F1, S2, and F2)[30] to construct a complete SSR genetic linkage map. Xie et al.[31] conducted a study to analyze the genetic diversity and relationships of 84 *Zoysia* germplasms using inter-simple sequence repeat (ISSR) markers. Moore et al.[32] analyzed changes in levels of allelic diversity at the gene and population levels in 40 *Zoysia* cultivars released between 1910 and 2016 using SSR markers.

In this study, 45 Chinese *Zoysia* germplasm collections were analyzed by SSR and SRAP markers. The results of these analyses were used to clarify the genetic relationship, analyze the genetic diversity of 45 Chinese *Zoysia* germplasm collections, and construct the fingerprint to distinguish breeding lines from the two commercially available cultivars at the molecular level. The aim of this study was to provide a theoretical basis for the development, evaluation, and breeding of *Zoysia* germplasm.

Materials and methods

Plant material

Forty-five Chinese *Zoysia* germplasm collections were used for the study, among which 38 were collected from the provinces of Liaoning, Anhui, Zhejiang, and Hainan in China, five were breeding lines from radiation mutagenesis and two commercial *Z. japonica* cultivars, 'Lanyin No. 3' and 'Qingdao' (Table 1). Forty-five *Zoysia* germplasm collections were grown in the resource nursery of Turf Experiment Station of Nanjing Agricultural University, located at 119°14′38″ east longitude and 31°49′46″ north latitude, with a subtropical monsoon climate and an average annual temperature of 15.2 °C. A randomized block design was utilized with each germplasm planted in an area of 3 m × 3 m with three replicates and plots spaced 2 m apart in August, 2020. About 100 stolons were planted evenly in each plot with 2–3 stem nodes for each stolon. Plants were watered twice weekly and fertilized once a month. Plants were not trimmed in 2021 in order to measure morphological indexes, and trimmed once a week in 2022 to evaluate turf density.

Morphological characteristic data collection

Morphological data were collected on the 15th in June and September 2021. Six morphological characteristics of 45 Chinese *Zoysia* germplasm collections were determined as follows: leaf length and leaf width were obtained by randomly measuring the length and middle width of the third fully expanded leaf from the top, respectively. Measurements were repeated 10 times and the average was calculated as the final value for each replicate. Ten healthy stolons were randomly selected and the length and diameter of the fourth section were measured as internode length and stem diameter, respectively. The turf height, which is the natural height of plant growth, was measured using the five-point method[33]. Turf density was determined by counting the number of tillers in a 5 cm × 5 cm wire frame and each plot was counted three times.

Genomic DNA extraction

Genomic DNA was extracted from young *Zoysia* leaves (0.1 g) using the modified CTAB method[34]. The quality of DNA was verified by 0.8% agarose gel electrophoresis, and the DNA concentration was determined by NanoReady. The DNA of 45 samples was diluted to 50 ng·μL^{-1} using purified water and stored in the refrigerator at 4 °C or −20 °C for later use.

SSR and SRAP analysis

Forty SSR primer pairs from Röder et al.[35] and Tsuruta et al.[36] were used in this study. SSR-PCR and SRAP-PCR was performed in a total volume of 20 μL containing 1 μL genomic DNA, 10 μL of 2 × Mix (Yeasen Biotechnology Co., Ltd., Shanghai, China), 1 μL of 10 μmol·L^{-1} each PCR primer and 7 μL purified water. SSR-PCR reactions were performed in a Bio-Rad thermal cycler (Bio-Rad Inc., Hercules, CA, USA), DNA amplifications were performed with an initial step at 94 °C for 3 min, followed by 35 cycles of 50 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and a 10 min final extension step at 72 °C.

According to the SRAP primer design method published by Li & Quiros[17], 10 forward primers and five reverse primers were

Table 1. List of Chinese *Zoysia* germplasm collections used in this study.

No.	Germplasm ID	Type	Species	Source
1	ZG003	Breeding material	*Z.* sp.	Liaoning
2	ZG004	Breeding material	*Z.* sp.	Anhui
3	ZG007	Breeding material	*Z. sinica*	Anhui
4	ZG008	Breeding material	*Z. sinica*	Radiation mutagenesis (parent from Anhui)
5	ZG009	Breeding material	*Z. matrella*	Jiangsu
6	ZG011	Breeding material	*Z. pacifica*	Zhejiang
7	ZG013	Breeding material	*Z.* sp.	Anhui
8	ZG015	Breeding material	*Z. sinica*	Anhui
9	ZG017	Breeding material	*Z.* sp.	Anhui
10	ZG018	Breeding material	*Z.* sp.	Anhui
11	ZG021	Breeding material	*Z.* sp.	Anhui
12	ZG022	Breeding material	*Z.* sp.	Anhui
13	ZG023	Breeding material	*Z. matrella*	Anhui
14	ZG025	Breeding material	*Z.* sp.	Anhui
15	ZG026	Breeding material	*Z. matrella*	Anhui
16	ZG028	Breeding material	*Z.* sp.	Anhui
17	ZG029	Breeding material	*Z.* sp.	Zhejiang
18	ZG030	Breeding material	*Z. matrella*	Zhejiang
19	ZG032	Breeding material	*Z.* sp.	Zhejiang
20	ZG035	Breeding material	*Z.* sp.	Zhejiang
21	ZG037	Breeding material	*Z.* sp.	Zhejiang
22	ZG038	Breeding material	*Z.* sp.	Zhejiang
23	ZG040	Breeding material	*Z.* sp.	Zhejiang
24	ZG041	Breeding material	*Z.* sp.	Anhui
25	ZG043	Breeding material	*Z.* sp.	Anhui
26	ZG044	Breeding material	*Z.* sp.	Anhui
27	ZG046	Breeding material	*Z. sinica*	Hainan
28	ZG047	Breeding material	*Z.* sp.	Hainan
29	ZG048	Breeding material	*Z. pacifica*	Hainan
30	ZG049	Breeding material	*Z.* sp.	Hainan
31	ZG050	Breeding material	*Z. matrella*	Hainan
32	ZG053	Breeding material	*Z. pacifica*	Hainan
33	ZG056	Breeding material	*Z. pacifica*	Anhui
34	ZG057	Breeding material	*Z.* sp.	Anhui
35	ZG058	Breeding material	*Z. matrella*	Anhui
36	ZG059	Breeding material	*Z. sinica*	Radiation mutagenesis (parent from Anhui)
37	ZG060	Breeding material	*Z.* sp.	Anhui
38	ZG061	Breeding material	*Z.* sp.	Anhui
39	ZG062	Breeding material	*Z.* sp.	Anhui
40	ZG063	Breeding material	*Z.* sp.	Anhui
41	ZG081	Breeding material	*Z. japonica*	Radiation mutagenesis (parent from Anhui)
42	ZG082	Breeding material	*Z. japonica*	Radiation mutagenesis (parent from Anhui)
43	ZG083	Breeding material	*Z. japonica*	Radiation mutagenesis (parent from Anhui)
44	Lanyin No. 3	Cultivar	*Z. japonica*	Gansu
45	Qingdao	Cultivar	*Z. japonica*	Shandong

Table 2. Simple sequence repeats (SSR) primer sequences used for studying genetic diversity of 45 Chinese *Zoysia* germplasm collections.

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')
M3A10	CGAACGCGACATGACAATC	TCATGATGTTGGCAACCAC
Xgwm37-7D	ACTTCATTGTTGATCTTGCATG	CGACGAATTCCCAGCTAAAC
Xgwm102-2D	TCTCCCATCCAACGCCTC	TGTTGGTGGCTTGACTATTG
Xgwm111-7D	TCTGTAGGCTCTCTCCGACTG	ACCTGATCAGATCCCACTCG
Xgwm120-2B	GATCCACCTTCCTCTCTCTC	GATTATACTGGTGCCGAAAC
Xgwm169-6A	ACCACTGCAGAGAACACATACG	GTGCTCTGCTCTAAGTGTGGG
Xgwm445-2D	GTTGAGCTTTTCAGTTCGGC	ACGGAGAGCAACCTGCC
Xgwm46-7B	GCACGTGAATGGATTGGAC	TGACCCAATAGTGGTGGTCA
Xgwm52-3D	CTATGAGGCGGAGGTTGAAG	TGCGGTGCTCTTCCATTT
Xgwm234-5B	GAGTCCTGATGTGAAGCTGTTG	CTCATTGGGGTGTGTACGTG

randomly selected. SRAP-PCR reactions were performed with an initial step at 94 °C for 4 min and five cycles of 60 s at 37 °C and 60 min at 72 °C. Then 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 10 s; and then a final extension at 72 °C for 10 min.

The amplifications were performed in Applied Biosystems Veriti® thermal cycler. Amplification products were stored at 4 °C before being electrophoresed through 10% non-denatured polyacrylamide gels in 1 × TBE (pH 8.0) buffer running at 120 V constant voltage for 1.5 h and then the gels were stained with fast silver stain[37,38]. According to the silver staining results, 10 SSR primer pairs and nine SRAP primer pairs were initially screened against 45 Chinese *Zoysia* germplasm collections (Tables 2 & 3).

Data analysis

Gel images from all accessions were visually evaluated and coded as either a '1' for the presence or '0' for the absence of a band for each marker. Based on this method, matrices of '0' and '1' were obtained for 45 germplasm collections based on the amplification of each pair of primers. Popgene 3.2 software was used to calculate the number of alleles (Na), effective number of alleles (Ne), Shannon information index (I) and Nei's gene diversity (H). Genetic distances were calculated for the 45 Chinese *Zoysia* germplasm collections according to Dice[39] using NTSYS-pc. Genetic similarity coefficient (GS) was calculated, and dendrograms was constructed using unweighted pair-group method with arithmetic averages (UPGMA) by the NTSYS-pc computer program package. The confidence probability of the fingerprint was calculated based on the probability formula $P = 1/2^n$, where n is the number of alleles, i.e., the number of polymorphic bands for each primer pair.

Table 3. Sequence-related amplified polymorphism (SRAP) primer sequences used for studying genetic diversity of 45 Chinese *Zoysia* germplasm collections.

Forward primer (5'-3')	
Me1	TGAGTCCAAACCGGATA
Me2	TGAGTCCAAACCGGAGC
Me3	TGAGTCCAAACCGGACC
Me4	TGAGTCCAAACCGGACA
Me5	TGAGTCCAAACCGGTGC
Me6	TGAGTCCAAACCGGAGA
Me7	TGAGTCCAAACCGGACG
Me8	TGAGTCCAAACCGGAAA
Me9	TGAGTCCAAACCGGAAC
Me10	TGAGTCCAAACCGGAAT
Reverse primer (5'-3')	
Em1	GACTGCGTACGAATTCAA
Em2	GACTGCGTACGAATTCTG
Em3	GACTGCGTACGAATTGAC
Em4	GACTGCGTACGAATTTGA
Em5	GACTGCGTACGAATTAAC

Results

Results of SSR and SRAP primers amplification

A total of 395 bands were amplified with 19 pairs of primers, among which 380 were polymorphic bands, with a polymorphism ratio of 96.20% (Table 4). Ten SSR primer combinations amplified a total of 231 polymorphic bands with a polymorphism ratio of 97.18%. The number of bands scored per SSR primer combination ranged from 16 to 36, with a mean of 23.70. A total of 149 polymorphic bands were amplified by nine pairs of SRAP primers, and the polymorphism ratio was 93.43%. The number of bands scored per SRAP primer combination ranged from 13 to 30, with a mean of 17.56. Based on the amplification of SSR and SRAP primers, the effective alleles ranged from 1.192 to 1.556 with an average of 1.332, Shannon information index ranged from 0.236 to 0.510 with an average of 0.350, and Nei's gene diversity index ranged from 0.146 to 0.338, and the mean value is 0.219. The results showed that there was a high level of genetic diversity among the tested germplasm collections, and the SSR and SRAP primers screened were suitable for amplification detection of *Zoysia*.

Genetic similarities

NTSYS-pc software was used to calculate the value of GS between any two materials according to Dice's coefficient, which was based on the combined amplification results of SSR and SRAP (Table 5). The GS of the 45 germplasm collections ranged from 0.623 to 0.856, with an average value of 0.727 and a variation of 0.233. ZG048 was most genetically similar to ZG053 (GS = 0.856), and ZG025 was most genetically dissimilar to ZG032 (GS = 0.632). Furthermore, two commercial cultivars, Lanyin No. 3 and Qingdao, were found to be highly genetically similar (GS = 0.815). The least genetically similar to Lanyin No. 3 was ZG040 (GS = 0.651), while the least genetically similar to Qingdao was ZG008 (GS = 0.661).

Cluster analysis

Based on the results of SSR and SRAP molecular markers, the Dice genetic similarity coefficients were used to cluster 45 Chinese *Zoysia* germplasm collections. The cophenetic correlation for the UPGMA clustering was high (r = 0.75), suggesting that the cluster analysis strongly represented the similarity matrix. A UPGMA cluster was constructed based on combining data from both markers, which divided the 45 accessions into

Table 4. Polymorphism results from amplification by simple sequence repeats (SSR) and sequence-related amplified polymorphism (SRAP) primers in 45 Chinese *Zoysia* germplasm collections.

Primer	Total number of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Effective number of alleles	Shannon information index	Nei's diversity index
M3C06	27	27	100.00	1.443	0.426	0.272
Xgwm37-7D	17	16	94.12	1.201	0.259	0.146
Xgwm102-2D	24	22	91.67	1.350	0.352	0.224
Xgwm111-7D	28	28	100.00	1.254	0.295	0.172
Xgwm120-2B	36	35	97.22	1.556	0.510	0.338
Xgwm169-6A	16	16	100.00	1.276	0.333	0.197
Xgwm445-2D	32	32	100.00	1.396	0.398	0.250
Xgwm46-7B	16	15	93.75	1.394	0.399	0.255
Xgwm52-3D	20	19	95.00	1.409	0.413	0.262
Xgwm234-5B	21	21	100.00	1.249	0.305	0.179
SSR marker average	23.70	23.10	97.18	1.353	0.369	0.229
Me3-Em1	30	30	100.00	1.275	0.337	0.199
Me2-Em2	17	16	94.12	1.314	0.333	0.204
Me3-Em2	13	12	92.31	1.448	0.389	0.258
Me4-Em4	20	20	100.00	1.422	0.417	0.264
Me6-Em4	14	13	92.86	1.192	0.236	0.185
Me5-Em2	13	11	84.62	1.252	0.292	0.174
Me3-Em3	19	17	89.47	1.410	0.388	0.251
Me6-Em2	17	16	94.12	1.217	0.286	0.163
Me7-Em1	15	14	93.33	1.254	0.284	0.171
SRAP marker average	17.56	116.56	93.43	1.309	0.329	0.208
Average of all markers	20.79	20	96.20	1.332	0.350	0.219
Total	395	380	—	—	—	—

six major clusters at a similarity index value of 0.71 (Fig. 1). Cluster I contained 32 germplasm collections, comprised of 17 germplasm collections from Anhui, six germplasm collections from Zhejiang, five germplasm collections from Hainan, one material from Liaoning, one radiation mutagenic material and two commercial cultivars. Cluster II comprised of three germplasm collections from Anhui. Cluster III consisted of four accessions, comprising of one from Jiangsu, two from Anhui and one breeding germplasm collections. Cluster IV included two germplasm collections from Zhejiang. Cluster V included only one germplasm from Hainan, ZG046, indicating that ZG046 had some genetic differences from other germplasm collections from the same region. Cluster VI contained three radiation mutagenic materials. Using a genetic similarity coefficient of 0.73, the samples of Cluster I were differentiated into three subgroups: A, B, and C. Subgroup A consisted of Lanyin No. 3, Qingdao, and five germplasm collections from Anhui and Liaoning. Subgroup B contained 10 from Zhejiang and Anhui and one from Hainan (ZG050). Subgroup C included one radiation mutagenic material, nine germplasm collections from Zhejiang and Anhui, and four germplasm collections from Hainan.

Cluster I contained most of the germplasm collections and had the most diverse source locations, including four sources, indicating that there was genetic similarity among the germplasm from different sources. For example, ZG003 from Liaoning and ZG004 from Anhui preferentially clustered together and then aggregated with the rest of the germplasm collections in Cluster I. The genetic similarity coefficient of the two germplasm collections was 0.853, which was higher than the average. Aside from Cluster I, the other accessions preferentially clustered together according to their source location, such as Clusters II, IV, and VI.

In terms of morphological characteristics of the major clusters (Table 6), germplasm collections in subgroup A of Cluster Ⅰ were mainly presented with long and wide leaves, long internodes, large diameter of above ground stems and low density. Germplasm collections of subgroup B was tall and dense, making them suitable for vegetative propagation in ecological restoration or green space construction in parks. Germplasm collections in Subgroup C and Clusters II and III exhibited intermediate morphological characteristics, belonging to the intermediate type germplasm collections. Germplasm collections from Cluster IV (ZG032 and ZG040) were tall with low density which may not be suitable for utilizing as turf. ZG046 was the sole material in Cluster V with short leaves and short internodes, superior traits for sports field turf. Germplasm collections in Cluster VI were mainly characterized by narrow leaves and fine texture, suggesting potential to be used for ornamental lawn in urban green spaces.

Fingerprints of 45 *Zoysia* germplasms

The selected SSR and SRAP primers were used to amplify the DNA from 45 Chinese *Zoysia* germplasm collections, and the results of amplified bands were stable and reproducible. Fewer primers are preferred if they are able to distinguish among varieties. In this study, one SSR primer set (Xgwm234-5B) and two SRAP primer sets (Me3-Em1 and Me3-Em2) were selected to construct SSR and SRAP fingerprints of 45 germplasm collections, respectively, by considering the clarity and percentage of polymorphic of amplified bands for each primer pair (Table 7). The primer pairs Xgwm234-5B, Me3-Em1, and Me3-Em2 amplified 21, 30, and 12 polymorphic bands, respectively. The fingerprint detection probability formula $P = 1/2^n$ was used of which n was 21 and 42 for SSR and SRAP fingerprints, respectively. The confidence probability of both SSR and SRAP fingerprints were

Table 5. Genetic similarity coefficients (GS) among the 45 Chinese *Zoysia* germplasm collections.

Germplasm ID	Highest similarity (germplasm ID compared to)	Lowest similarity (germplasm ID compared to)	Average
ZG003	0.8532 (ZG004)	0.6228 (ZG032)	0.738
ZG004	0.8532 (ZG003)	0.6582 (ZG032)	0.756
ZG007	0.8127 (ZG009)	0.6532 (ZG029)	0.733
ZG008	0.7772 (ZG015)	0.6278 (ZG081)	0.703
ZG009	0.8127 (ZG007)	0.6633 (ZG081)	0.738
ZG011	0.7873 (ZG021)	0.6456 (ZG081)	0.716
ZG013	0.8152 (ZG021)	0.6684 (ZG081)	0.742
ZG015	0.7772 (ZG008)	0.6608 (ZG060)	0.719
ZG017	0.7722 (ZG025)	0.6582 (ZG032)	0.715
ZG018	0.8101 (ZG021)	0.6557 (ZG032)	0.733
ZG021	0.8304 (ZG022)	0.6658 (ZG046)	0.748
ZG022	0.8304 (ZG021)	0.6759 (ZG032)	0.753
ZG023	0.7848 (ZG026)	0.6532 (ZG081)	0.719
ZG025	0.7722 (ZG017)	0.6228 (ZG032)	0.698
ZG026	0.7848 (ZG023)	0.6430 (ZG032)	0.714
ZG028	0.7772 (ZG021)	0.6684 (ZG060)	0.723
ZG029	0.7646 (ZG035)	0.6506 (ZG081)	0.708
ZG030	0.7823 (ZG043)	0.6582 (ZG081)	0.720
ZG032	0.7418 (ZG037)	0.6228 (ZG025)	0.682
ZG035	0.8329 (ZG037)	0.6684 (ZG081)	0.751
ZG037	0.8329 (ZG035)	0.6532 (ZG081)	0.743
ZG038	0.8304 (ZG037)	0.6658 (ZG081)	0.748
ZG040	0.7316 (ZG047)	0.6456 (ZG003)	0.689
ZG041	0.7873 (ZG043)	0.6633 (ZG082)	0.725
ZG043	0.7873 (ZG041)	0.6658 (ZG032)	0.727
ZG044	0.7722 (ZG035)	0.6481 (ZG082)	0.710
ZG046	0.7519 (ZG047)	0.6456 (ZG081)	0.699
ZG047	0.7899 (ZG048)	0.6759 (ZG081)	0.733
ZG048	0.8557 (ZG053)	0.6633 (ZG081)	0.760
ZG049	0.7873 (ZG047)	0.6810 (ZG026)	0.734
ZG050	0.7595 (ZG058)	0.6709 (ZG004)	0.715
ZG053	0.8557 (ZG048)	0.6684 (ZG032)	0.762
ZG056	0.7873 (ZG057)	0.6684 (ZG103)	0.728
ZG057	0.8051 (ZG059)	0.6633 (ZG082)	0.734
ZG058	0.7873 (ZG013)	0.6532 (ZG082)	0.720
ZG059	0.8051 (ZG057)	0.6785 (ZG032)	0.742
ZG060	0.7646 (ZG049)	0.6354 (ZG032)	0.700
ZG061	0.8152 (ZG062)	0.6506 (ZG025)	0.733
ZG062	0.8253 (ZG063)	0.6557 (ZG032)	0.741
ZG063	0.8253 (ZG062)	0.6608 (ZG046)	0.743
ZG081	0.7949 (ZG083)	0.6278 (ZG032)	0.711
ZG082	0.7899 (ZG083)	0.6354 (ZG025)	0.713
ZG083	0.7949 (ZG081)	0.6481 (ZG040)	0.722
Lanyin No. 3	0.8152 (ZG105)	0.6506 (ZG040)	0.733
Qingdao	0.8152 (ZG103)	0.6608 (ZG008)	0.738

more than 99.999%. These results indicated that the fingerprint obtained in this study could be used to distinguish among the 45 Chinese *Zoysia* germplasm collections.

Discussion

Many *Zoysia* species, such as *Z. sinica*, *Z. japonica*, and *Z. matrella*, were utilized as turfgrass with relatively substantial genetic diversity. Weng et al.[40] analyzed 131 *Zoysia* plants collected from Taiwan with random amplified polymorphic DNA (RAPD) and isoenzymes and determined that these germplasms exhibit high genetic variation at the DNA level. Kimball et al.[41] selected 50 pairs of SSR primers to amplify 62 DNA samples from *Zoysia* cultivars and accessions, and the genetic similarity obtained from their analysis ranged from 0.29 to 0.51. In our study, the genetic similarity coefficient of 45 germplasm collections ranged from 0.623 to 0.856 indicating that the diversity of 45 germplasm collections was lower. The level of genetic diversity and similarity within and between natural populations is determined by the interaction of climate differences and gene flow, with climate being a major driving force for organisms to adapt to the environment and generate heritable mutations[42]. *Zoysia* has gained popularity and been widely utilized around the world due to its remarkable heat tolerance, cold tolerance, and saline-alkali tolerance, which results in the complex genetic variation of *Zoysia* plants.

In this study, the 45 germplasm collections were divided into six clusters by UPGMA. The geographic origin of Cluster I was relatively complex, comprising of germplasm collections from Anhui (17 samples), Zhejiang (six samples), Hainan (five samples), Liaoning (one sample), one radiation mutagenic material, and two commercial cultivars. Notably, the two control cultivars of Lanyin No. 3 and Qingdao are both in subgroup A of cluster I, yet their origins are quite distinct. Lanyin No. 3 was introduced from the United States by Gansu Ecology Institute in 1988, while Qingdao was collected in Jiaozhou Bay, Shandong Province in 1990, and subsequently cultivated and domesticated. Similarly, it has been reported that the *Zoysia* cultivars Empire, JaMur, and Atlantic were difficult to be differentiated by 40 SSR markers[24]. Numerous warm-season turfgrass cultivars are highly genetically similar despite reported pedigrees or place of collection[43,44]. Utilizing other types of markers such as single nucleotide polymorphisms (SNPs) or whole genome sequencing of these cultivars might be able to provide additional information to uncover genotypic diversity. Cluster V was comprised of a single ZG046 from Hainan, while the other five (ZG047, ZG048, ZG049, ZG050, and ZG053) from Hainan were placed in Cluster I. Additionally, these five germplasm collections exhibited significant differences from ZG046 in leaf length and leaf width. Results showed that the genetic distance between ZG046 and the other five germplasm collections was significant. Therefore, ZG046 can be used as a parent material to expand the genetic basis of the breeding population and increase the genetic diversity of breeding material, which would be beneficial for further *Zoysia* breeding improvement.

In this study, 45 Chinese *Zoysia* germplasm collections were characterized and analyzed based on their morphological traits and molecular clustering results. The morphological traits of *Zoysia* were found to be influenced by various factors, such as origin and genetic inheritance, as well as environmental aspects such as adaptability to the planting environment, centralized management of breeders and extensive cultivation, which have led to increased gene exchange among the *Zoysia* populations[45]. Liu et al.[46] conducted genetic diversity analysis of 42 seashore paspalum (*Paspalum vaginatum* Sw.) based on morphology and SRAP molecular markers, and the results of the molecular marker clustering showed that germplasms from the same region were more likely to be clustered together, while the morphology clustering showed that the germplasms clustered together often originated from distinct geographical locations.

DNA fingerprints generated using molecular markers can offer more precise genetic information[47]. Molecular markers have been utilized to construct fingerprints to identify plant

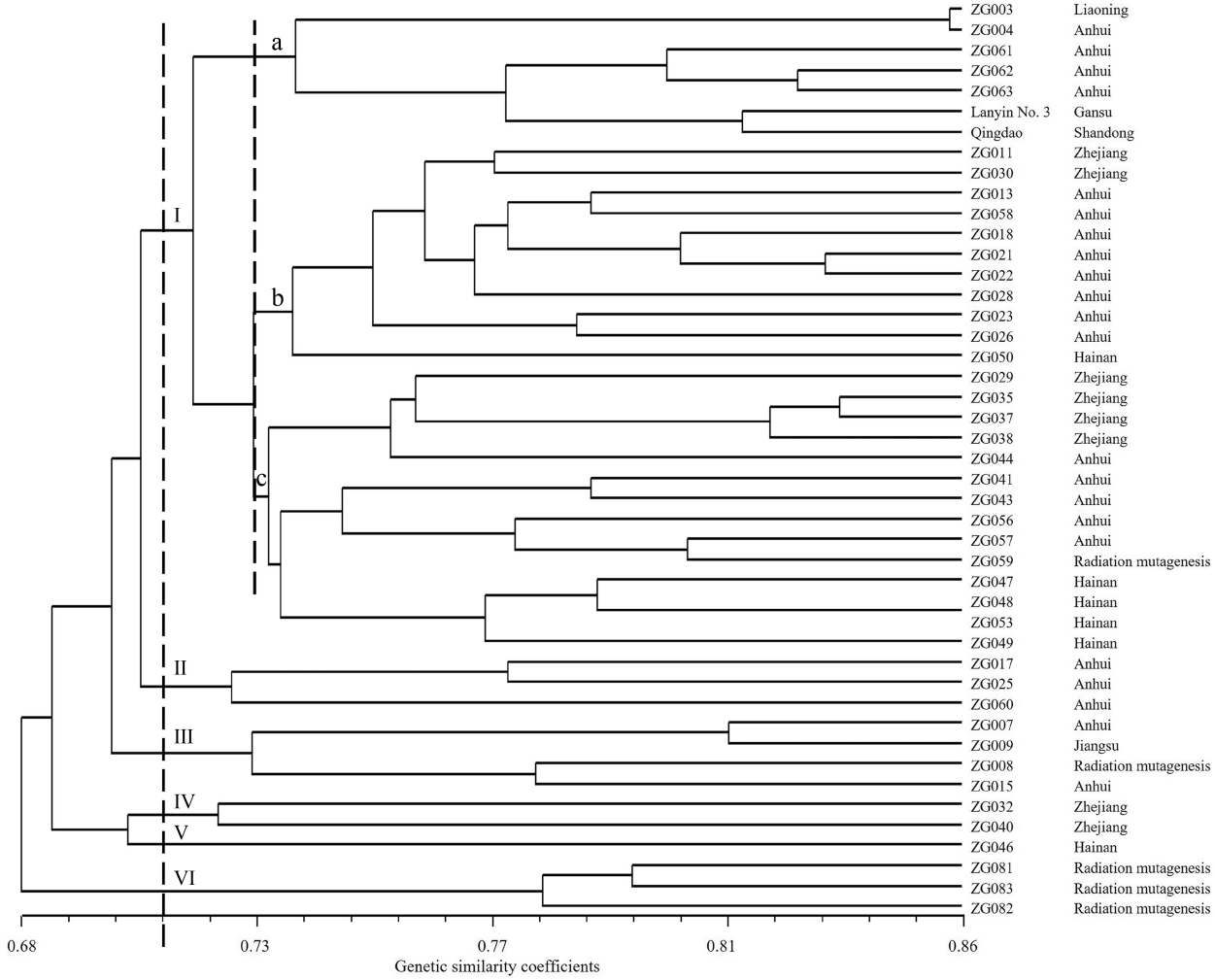

Fig. 1 Unweighted pair-group method with arithmetic averages dendrogram of the 45 Chinese *Zoysia* germplasm collections constructed using simple sequence repeats (SSR) and sequence-related amplified polymorphism (SRAP) markers.

Table 6. Mean value of main morphological characteristics of the six clusters of 45 Chinese *Zoysia* germplasm collections.

Cluster/subgroup		Leaf width (mm)	Leaf length (cm)	Internode length (cm)	Stem diameter (mm)	Turf height (cm)	Turf density (tiller number cm^{-2})
I	A	4.66	10.24	5.13	1.58	11.44	4.66
	B	3.42	7.83	3.59	1.35	10.55	3.42
	C	3.62	6.75	4.62	1.34	14.00	3.62
II	–	3.70	7.29	3.36	1.23	10.08	2.81
III	–	3.74	6.80	3.82	1.37	11.03	3.03
IV	–	3.66	6.66	3.72	1.41	22.96	2.17
V	–	3.47	4.15	3.11	1.38	9.07	2.81
VI	–	3.27	6.33	4.63	1.37	8.14	2.80
Mean		3.72	7.51	4.22	1.38	10.83	3.19

varieties, including radish and cucumber[48,49]. Accurate identification of cultivars is essential for cultivation and breeding[50]. This study demonstrates that both SSR and SRAP are effective methods for constructing fingerprints of *Zoysia* germplasm. The 21 polymorphic bands amplified by one pair of SSR primer Xgwm234-5B were able to distinguish all 45 germplasm collections, while the SRAP molecular marker required a total of 43 bands amplified by two pairs of primers Me3-Em1 and Me3-Em2 to generate the fingerprints of the 45 germplasm collections. As *Zoysia* breeding advances, the number of varieties will continue to increase. To ensure the accuracy and reliability of the fingerprint of increasing number of new varieties, additional primers need to be developed, and it is essential to expand the fingerprint database of *Zoysia*. In this study, 45 *Zoysia* lines were divided into six clusters based on both SSR and SRAP markers and fingerprints of those plants were constructed. The findings of this study provided a theoretical basis for the subsequent evaluation and identification of *Zoysia* germplasm as well as new variety development.

Table 7. Fingerprints of 45 Chinese *Zoysia* germplasm collections generated by simple sequence repeats (SSR) and sequence-related amplified polymorphism (SRAP) markers.

No.	Germplasm ID	Digital DNA fingerprint	
		SSR (Xgwm234-5B)	SRAP (Me3-Em1+Me3-Em2)
1	ZG003	111000100010100110000	000001000000000001000000011011-0001101010010
2	ZG004	000001000010000100000	000000100001000001000001010010-0001101010010
3	ZG007	100000100010000100100	000000100010001000010001001110-0000001100001
4	ZG008	001000000010000100111	100000000010000000001000001100-0000001110011
5	ZG009	101010000010100101100	000001000000010100010001001110-0000001110001
6	ZG011	100000000010000100010	000000000000000001000000000000-1001101010010
7	ZG013	000000100010000100000	010001000000010001010010010000-0001101010010
8	ZG015	001000000010000100001	100010001100000000001000000100-0000001000011
9	ZG017	000100100010000100101	000010001000000000010010001100-0000101100010
10	ZG018	000000000000000100001	000001000010000000110010110010-1001101010010
11	ZG021	101000100010000100000	010001000000010001000010110001-0001101010010
12	ZG022	100000110010001100000	000001000000000000000010100000-0001101010010
13	ZG023	000000000010000000000	000010000100010001010010100100-1001111010010
14	ZG025	000000000010000101100	010010001000010000000010001110-0000101100010
15	ZG026	001001100011100100110	010001101100000001010000100100-1001111010010
16	ZG028	000001100010000110000	010001000000000010000010101010-0001101010010
17	ZG029	001000000010001100100	000010000000010000000000100100-1001101010010
18	ZG030	001001000111000100011	100010100100000001010010100000-1000111010011
19	ZG032	000001000010001110000	100010000010010001010110100001-0000001010001
20	ZG035	001010000010000100010	000010001010010000000000100010-1001101010011
21	ZG037	000001100010001100000	000010001000010000000010100000-1001101010011
22	ZG038	000000000010000100000	010010000100010000000000010000-1001101010011
23	ZG040	000000000010000100100	000010010100000000001010010000-0000101010011
24	ZG041	010010100010000100000	010010000100000001000100100000-0001101010011
25	ZG043	000000100011000100000	000000000000000000001010101000011-0000101010011
26	ZG044	011100010010000100000	010001001010001000000010100000-1001101011011
27	ZG046	000000001000000110010	010000100100010000110010001000-0000111110011
28	ZG047	000000000000000100010	000000000001010000000010101000-0000101110011
29	ZG048	001001110010001101010	010010000001010001001000001000-0001111110011
30	ZG049	100000000010000111110	100010000100010100001010001000-0000101110011
31	ZG050	000001000010000100011	010010000000110001010000000000-1001111010011
32	ZG053	001000000011000101010	010010000001010001001000001000-0001111110010
33	ZG056	000001000010010100010	010000001100000000010010001010-0000111110011
34	ZG057	001000100010101100010	010001000000010010010010001010-0000001110011
35	ZG058	001001100010000100110	010001000010010000010010001000-1000111010011
36	ZG059	010000000010000100010	010001000000010000000001001010-0000101110001
37	ZG060	000000000010000100110	001001000100110000010000001000-0000101110001
38	ZG061	000001100010000100000	000000100001000000100000000001-1001101010001
39	ZG062	100001100010000100101	000100100001001000100001010000-0001101010001
40	ZG063	000000000011000100100	000000100001010000100001010110-0001101010101
41	ZG081	010001000010010100110	000100000000100100010010101100-0011101010101
42	ZG082	001000010010000000110	000100010000100100010001100101-0011101010100
43	ZG083	000001000010100100110	000100000000100100010000101100-0011101010101
44	Lanyin No. 3	000000000000100000000	000000000000100100000001100001-0001011001001
45	Qingdao	110001000010000101000	000000000000000010001000101100-1001001010001

DNA from 45 Chinese *Zoysia* germplasm collections were amplified with primers Xgwm234-5B, Me3-Em1, and Me3-Em2. The presence or absence of bands in the same location was transformed into the corresponding digital information of 1 or 0, respectively, to form the digital fingerprints.

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Conflict of interest

The authors declare that they have no conflict of interest.

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