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Research Article

Assessment of Genetic Diversity Using Morphological and Molecular Characteristics of Indonesian Zoysiagrass Genotypes

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ABSTRACT

Zoysiagrass is warm-season turfgrass thriving in tropical regions. Despite the adaptive nature, the existence of Indonesian zoysiagrass as well as morphological and genetic characteristics are not available. Therefore, this study aimed to explore Indonesian zoysiagrass from western and eastern parts of the country, as well as its morphological and molecular characteristics. Morphological characteristics was conducted to measure vegetative and reproductive characters while genotyping was performed using 15 simple sequence repeat markers. Morphological characteristics cluster three major groups, namely Group 1 corresponded to short, shorter, and fine leaves. Group 2 corresponded to tall, longer, and fine leaves, fewer seeds, and short spikelet, while Group 3 corresponded to tall, long, and wider leaves, more seeds, and longer spikelet. The results showed that the expected heterozygosity (H*e* = 0.256) was lower than homozygosity (H*o* = 0.341). The high level of discriminating capacity, polymorphism, and informativeness of SSR marker was observed (Effective Multiplex Ratio = 4.20, Marker Index = 2.394, and Resolving Power = 1.574). Additionally, population structure generated two subpopulations. Group 1 corresponded to *Z. japonica* from Sumatera Island and mixed province while Group 2 corresponded to *Z. japonica* from Central Java and Bali with *Z. japonica* and *Z. matrella* from mixed province. In conclusion, the exploration of morphological and genetic diversity from Indonesian zoysiagrass provided useful insight for conservation and future breeding improvement.

1. Introduction

The zoysiagrass, *Zoysia* spp., is reported to have 11 species distributed in the western Pacific Rim and Indian Ocean. Among these species, *Z. japonica* Steudel, *Z. matrella* (L.) Merrill, as well as *Z. pacifica* (Goudswaard) Hotta and Kuroki and the commercial cultivars are interspecific hybrids used for various turfgrass applications, such as golf courses, roadsides, home lawns, and other tourist spots (Kunwanlee *et al.* 2018). Zoysiagrass is preferred among turfgrass breeders due to its easy and efficient cultivation requirements in water, as well as tolerance to shade, including resistance to biotic and abiotic factors (Culpepper *et al.* 2020, Wang *et al.* 2020, 2022, Zuo *et al.* 2020, 2021).

A survey has been conducted in Indonesia to collect zoysiagrass genotypes on Java Island. The survey covered mountain areas on the south side of Merapi Mountain in Yogyakarta and Gedong Songo in the Semarang district. The seaside areas were in Jepara, Bantul, Gunung Kidul, Pacitan, and Karimunjawa islands, while in Bali Island were Tanah Lot, and Tabanan. Other areas included the western part, namely Aceh, Bengkulu, Bangka Belitung, West

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Sumatera, and in eastern were Maluku, and Southeast Sulawesi (Rahayu *et al.* 2016). The growth response and turf quality of the collected zoysiagrass genotypes were analyzed in various growing media, different shading intensities, and under mowing conditions (Rahayu *et al.* 2018, 2020).

Morphological characteristics have been applied as the tools to characterize variations of zoysiagrass germplasm. The results showed that the characteristics were influenced by genetic composition and environmental factors (Liu *et al.* 2019). Generally, morphological characteristics show variations in the expressed region, while molecular markers indicates differences in all genomes, including expressed and non-expressed regions (Pocovi *et al.* 2020). Zaman *et al.* (2022) use foliar epidermal anatomical characteristics as micromorphological features with accurate identification information to taxonomically family species of *Lamiaceae* species. The other pollen morphological study also showed the anatomical characteristics (Bahadur *et al.* 2023).

Genetic variation of zoysiagrass was assessed using molecular markers such as SRAP (Wang *et al.* 2023), SSR (Araneda *et al.* 2013; Kimball *et al.* 2013; Kunwanlee *et al.* 2018; Wang *et al.* 2023), RADSeq and SNP-based high-density linkage map (Holloway *et al.* 2018; Yang *et al.* 2023) as well as the assembly of genome sequence of *Z. japonica, Z. matrella* and *Z. pacific* (Tanaka *et al.* 2016a). In this study, the Simple Sequence Repeat (SSR) markers, also known as microsatellites, were selected for analysis. The selection was due to the presence of short repeat units, which are distributed throughout the eukaryotic genome. Additionally, SSR are widely used due to their abundance, highly polymorphic, codominant, transferability among closely related species, easy detection and assessment compared to other markers. SSR markers are valuable for examining genetic diversity, population genetic structure, linkage mapping, and paternity testing (Hussain and Nisar 2020).

Despite the numerous benefits, there is limited study of zoysiagrass characteristics using SSR markers and no reports on genetic diversity, particularly Indonesian species. Therefore, this study aimed to assess the phenotypic and genetic diversity of zoysiagrass genotypes using morphological characters and SSR markers.

2. Materials and Methods

2.1. Plant Materials

The collected zoysiagrass genotypes were planted in a greenhouse of Sebelas Maret University (7°56'07.09" S, 110°85'65.78" E) and were maintained, as shown in Figure 1 (Rahayu *et al.* 2016). Genotypes and geographical information of sampling sites are presented in Figure 2 and Table 1.

2.2. Morphological Characteristics of Zoysiagrass

The vegetative propagated of 22 zoysiagrass and Bb genotypes from South Korea as control were planted at an experimental field. The study was arranged in a randomized complete block design with three replications. It was conducted in a large pot of 60 cm diameter, with a planting medium of mixing soil, sand, and organic material by the volume ratio of 1:10 v/v. Growth maintenance of grasses was performed by irrigation of 100% evapotranspiration with 3 days intervals. This was followed by the application of fertilizer with a par lecturer nitrogen 15 g/m^2 to ensure that turfgrass stayed healthy and developed new leaf blades (Rahayu *et al.* 2016).

Morphological characteristics of zoysiagrass genotypes was determined using 3 vegetative characters (leaf length, leaf width, and turf height) 40 Days After Seedling (DAS). Other factors included 3 reproductive characters (spikelet length, internode length, and grain per spikelet) on 60 DAS. The caliper's ruler was used for measuring turf quantity such as leaf width, leaf length, turf height, and internode length. Characteristics of turf quality was observed in the visual quality of shoot density, uniformity, texture, growth rate, and level of surface coverage. The evaluation of turf quality was carried out following the method of Visual Field Assessment (VFA) by The National Turfgrass Evaluation Program (NTEP) (NTEP 2008). A scale of 1-9 was used (1.0 = poorest possible quality and 9.0 = best possible quality) where the "6" score was the minimum value accepted for the assessment.

2.3. Molecular Analysis

Fresh leaves of all genotypes were used for genome DNA extraction. The buffer extract was added to ground leaves tissues. The suspension was incubated in a water bath temperature of 65°C for 30 minutes with gentle

Figure 1. Performance of Indonesian zoysiagrass; (A) plant character, (B) zoysiagrass plug, (C) zoysiagrass turf

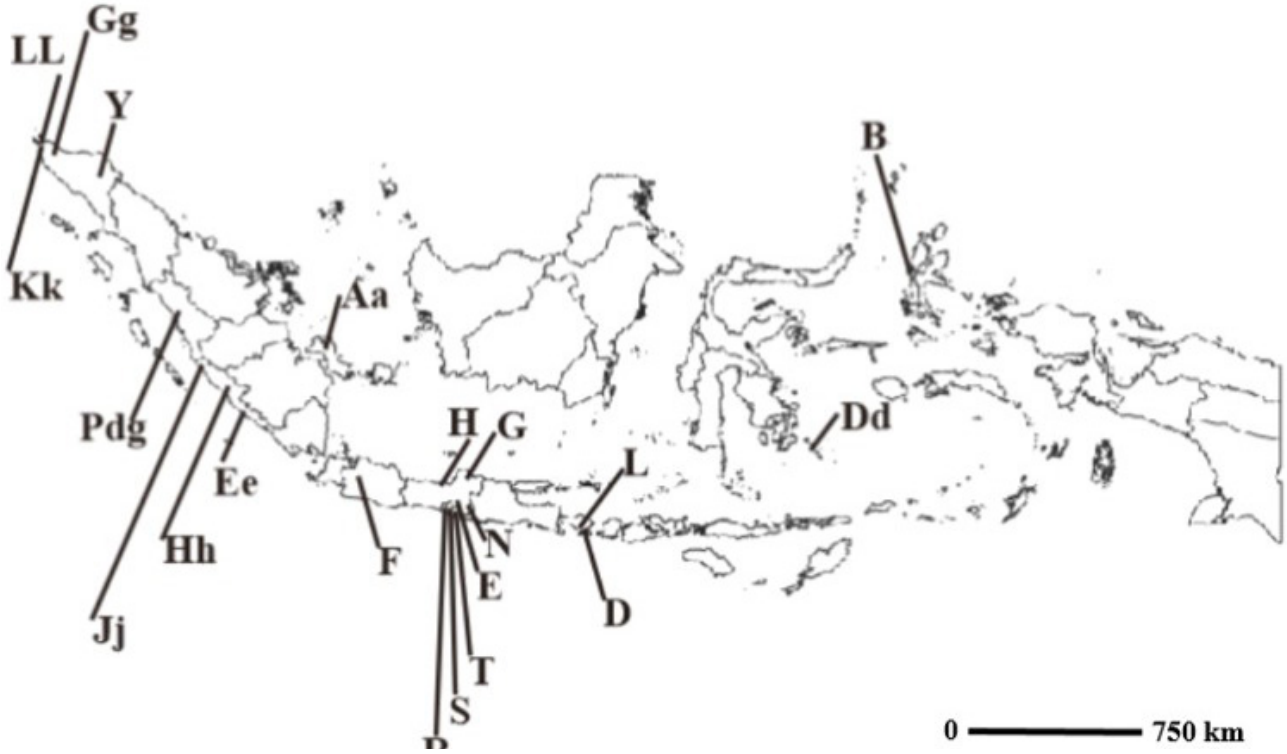

Figure 2. Map of collected zoysiagrass acession in Indonesia. B: Ternate, D: Tanah Lot, E: Klaten, F: Bogor. G: Jepara, H: Semarang, K: Kebumen, L: Tabanan. N: Solo. R: Cangkringan, S: Prambanan, T: Kepuh Harjo, Y: Romeo Aceh, Aa: Bangka, Bb: Cheonan, Dd: Wakatobi, Ee: Bengkulu, Gg: Banda Aceh, Hh: Ipuh, Jj: Muko, Kk: Banda Aceh, LL: Banda Aceh, Pdg: Padang

Table 1. Genotype and geographical information of collected zoysiagrass in this study

Geno type	Species	Specific sample collection site					
		Location	Province	Site	Altitude (m)	Latitude	Longitude
B	*japonica*	Ternate	North Maluku	Sea side	10	0° 45' 20"N	127° 20' 14"E
D	*japonica*	Tanah Lot	Bali	Sea side	7	8° 37' 19"S	115° 05' 18" E
E	*japonica*	Klaten	Central Java	Ground	197	7° 41' 45"S	110° 35' 57" E
F	*japonica*	Bogor	West Java	Ground	250	6° 34' 47" S	106° 49' 53"E
G	*japonica*	Jepara	Central Java	Sea side	3	6° 32' 56"S	110° 39' 44"E
H	*matrella*	Semarang	Central Java	Mountain	1270	7° 12' 30"S	110° 20'30"E
K	*japonica*	Kebumen	Central Java	Sea side	2	7° 47' 44" S	109° 44' 16"E
L	*japonica*	Tabanan	Bali	Road side	275	8° 32' 17"S	115° 06' 58" E
N	*japonica*	Solo	Central Java	Ground	105	7° 33' 20"S	110° 48' 22"E
R	*japonica*	Cangkringan	Yogyakarta	Road side	630	7° 47' 44"S	110° 27' 13"E
S	*japonica*	Prambanan	Yogyakarta	Park area	150	7° 45' 08" S	110° 29' 23" E
T	*japonica*	Kepuh Harjo	Yogyakarta	Mountain	855	7° 36' 16" S	110° 27' 14"E
Y	*japonica*	Romeo	Aceh	Sea side	5	5° 36' 50"N	95° 23' 50" E
Aa	*japonica*	Bangka	Bangka Belitung	Sea side	4	2° 17' 09"S	95° 13' 50" E
Bb	*japonica*	Cheonan, Korea	Control Variety	Ground	122	5° 50' 07"N	127° 09' 50" E
Dd	*japonica*	Wakatobi	Southeast Sulawesi	Sea side	6	5° 31' 48"S	123° 47' 04"E
Ee	*japonica*	Bengkulu	Bengkulu	Park area	11	3° 47' 15"S	102° 15' 08"E
Gg	*japonica*	Banda	Aceh	Sea side	2	5° 34' 27"N	95° 15' 03"E
Hh	*japonica*	Ipuh	Bengkulu	Park area	12	3° 47' 15"S	102° 15' 08"E
Jj	*japonica*	Muko	Bengkulu	Sea side	5	2° 32' 25" S	101° 05' 03" E
Kk	*japonica*	Banda	Aceh	Sea side	7	5° 32' 44" N	95° 15' 42" E
LL	*japonica*	Banda	Aceh	Sea side	4	5° 33' 25"N	95° 15' 07"E
Pdg	*japonica*	Padang	West Sumatera	Park area	20	0° 50' 46"S	100° 22' 00"E

mixing. The chloroform: isoamyl alcohol (24:1, v/v) with equal volume was added and centrifuged at 12,000 rpm for 15 minutes at room temperature. The aqueous phase was transferred to a new tube, followed by DNA precipitation with 2/3 volume of cold isopropanol and 3M ammonium acetate (1/10 volume), and stored at -20°C for 1 h. The precipitated DNA was centrifuged at 14,000 rpm for 10 minutes rinsed with cold 70% ethanol and dried for 30 minutes. DNA was dissolved in TE buffer, followed by quantity and quality confirmation by 0.8% agarose gel electrophoresis and nanodrop.

Molecular analysis was conducted using 16 SSR primers to zoysiagrass genotypes developed by Cai *et al.* (2005) from a cross between a *Z. japonica* and a *Z. matrella* clone, for polymorphism assessment, as shown in Table 2. Polymerase Chain Reaction (PCR) mixture containing 100 ng of genomic DNA, MyTaq Red Mix, was carried out following manufacturer's instructions (Bioline). The PCR conditions were initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing at a temperature of 55°C for 1 minute, and extension at 72°C for 1 minute for 35 cycles, and a final extension for 7 minutes at 72°C. Subsequently, the PCR products were electroporated on 8% acrylamide gel followed by ethidium bromide staining. Gel electrophoresis was visualized and documented using gel documentation (Bio-rad).

2.4. Data Analysis

The SSR amplification data were scored based on allele sizes of all genotypes. Genetic parameters namely the number of alleles (Na), PIC, observed heterozygosity (H*o*), and expected heterozygosity (H*e*) were calculated to measure genetic diversity of zoysiagrass genotypes using PowerMarker v3.25 (Liu and Smouse 2005) and GenAlex 6.41 (Peakall and Smouse 2006). EMR and MI were calculated to measure the usefulness of the marker system. According to Powell *et al.* (1996), EMR is the number of polymorphic fragments detected per assay. It is expressed as EMR = n × β, where β is the fraction of polymorphic markers, and is estimated after considering the polymorphic loci (np) and non-polymorphic loci (nnp) as β = np / (np + nnp). In this study, MI was calculated by multiplying the PIC with EMR. RP which showed the ability of the most informative primers to differentiate between genotypes was assessed according to Prevost and Wilkinson (1999) using: Rp = ∑Ib. In the equation, Ib is the band informativeness with Ib = 1 - [2 × (0.5-p)] and p is the proportion of clones containing the band. Moreover, RP is based on the distribution of detected bands within the sampled genotypes.

Genetic relationship between zoysiagrass genotypes was compiled from allele frequency and morphological data for similarity coefficients. Cluster analysis with

Table 2. Primer list used in this study*

SSR	Repeat motif	Forward primer	Reverse primer
ZB03J03	(TC)16	ggctttatagcgaagtttga	ctaccctgacacaagaaagc
ZB03L03	(GA)19	atagctagctgccttgagg	caagtatgcatttctgctca
ZC01P20	(GAA)11	agttctgaggagaagggaag	ggtacgtcaacatctgctg
ZB01C23	(TC)29	caagaggagtttgggctc	tcagtccctcaaggaaatta
ZB01G19	(GA)28	ttgtgatcatgtgatcgatc	tggctttgatccttcctata
ZB03B05	(GA)19	gagaggcttcttgacaagg	gtaccagaccgaaggctac
ZB10M09	(GA)12	cagcataggaaaggaagcta	agtgtaagccgttgcttg
ZA01L02	(TG)19(GA)20	ttcctaatctcgagcatagc	cgctgacaaggagatctatc
ZB03H23	(TC)18	aagaccattgtaggctcaaa	ccctggccttaaacagtt
ZA01P11	(CA)21	tccgctaccaagtaatcact	tgcgctttctagagatcttc
ZB01A21	(GA)25	ctccagataatccatggaga	cggctaagacatcatatggt
ZT-A114	(TC)18(CA)18	gatctttggaaccgcttt	ccagaacaatgctgttcac
ZB01C06	(GA)20	ataaagatacgagtggaattgg	gcacaaagaagctagaccc
ZA03F03	(TG)14	atcaaggtaacaagatcacga	gagaaggacgtaacgtaacaa
ZB01B12	(GA)18	gctagtgtttgttgatgacttg	aacttgagcgtgctatgc
ZB01C16	(GA)20	aggacaagtgaggcagtaga	aaaggtcagtctccgtcc

*Cai *et al.* (2005)

the unweighted pair-group method with arithmetic average (UPGMA) was performed by NTSYSPC software (Rohlf 2005). The reliability of the cluster was estimated by bootstrap analysis (100 replicates). Population structure of zoysiagrass genotypes was performed using Structure v2.1 (Pritchard *et al.* 2000). The Marcov Chain Monte Carlo method (MCMC) assessed the posterior probabilities in number of subpopulation (k) values between 1 and 10, with five replications for each k using the length of burning period of 10,000, followed by 100,000 of MCMC after burning. The output of LnP(D) and delta k were calculated to assess the k value. Structure Harvester was used to estimate the main population (Earl and von Holdt 2012). PCoA was performed using GenAlex 6.41 (Peakall and Smouse 2006) for further investigation.

3. Results

3.1. Existence of Indonesian Zoysiagrass

Zoysiagrass was only found on the south side of Merapi Mountain in Jogjakarta and Gedong Songo Semarang District, but not on Merbabu, Lawu, Sindoro, or the Dieng Plateau. Generally, coastal regions were more suited as habitat for zoysiagrass in Indonesia since numerous surveyed places were discovered such as Banda Aceh, Bangka Belitung, Bengkulu, Kebumen, Jepara Bandengan beach, Tanah Lot, Wakatobi, and Ternate. This showed that Zoysiagrass might be developed as a saline region turfgrass in Indonesia.

3.2. Morphological Characteristics of Zoysiagrass Genotypes

Morphological characteristics showed the performance of zoysiagrass genotypes. The coefficient variation (CV) of spikelet length, spikelet number, turfgrass height, and leaf length were high (>22%). Meanwhile, internode length, leaf width, and leaf angle were less than 16% and the CV in turfgrass quality character was 5.8%, as shown in Table 3. The results showed variation among 23 zoysiagrass genotypes in turfgrass morphological characteristics compared to quality.

Morphological characteristics of Indonesian zoysiagrass genotypes conducted in the vegetative phase were compared with genotypes from South Korea as the threshold. The plant height character was separated into short/dwarf (<10 cm) consisting of 4 genotypes of *Z. japonica*, and *Z. matrella* (H). Meanwhile, tall genotypes (>10 cm) consisted of 18 genotypes of *Z. japonica*. The leaf length characteristics were separated into short (<3.5 cm) consisting of 5 genotypes of *Z. japonica* and *Z. matrella* (H). The long leaves (>3.5 cm) comprised 17 genotypes, as shown in Table 3. The leaf width character was separated into fine leaves (<4 mm) consisting of 17 genotypes of *Z. japonica* and *Z. matrella* (H) and wider leaves (>4 mm) comprising 5 genotypes.

Morphological characteristic of local zoysiagrass genotypes in the generative phase was internode length character separated into short internode/dwarf (<2.17

Table 3. Morphological characteristic of 23 genotypes of local zoysiagrass genotypes.

Geno type	Turf quality (score)	Turf height (cm)	Leaf length (cm)	Leaf width (mm)	Internode length (cm)	Leaf angle (o)	Spikelet length (cm)	Spikelet number
B	6.92	20	18.67±0.6	3.2±0.3	2.57±0.7	40	0.0±0.0	0.0±0.0
D	7.13	24	4.83±0.6	3.8±0.2	2.78±0.6	33	2.5±0.4	14.0±0.8
E	7.10	7.5	3.43±0.4	3.7±0.0	2.69±0.4	47	0.0±0.0	0.0±0.0
F	7.25	17	5.87±0.5	3.3±0.8	2.67±0.5	45	0.0±0.0	0.0±0.0
G	7.88	10	4.00±0.8	3.1±0.8	2.96±0.4	45	1.4±0.2	18.5±3.5
H	7.63	6	3.33±0.6	2.7±0.5	2.77±0.6	42	2.5±0.2	22.0±2.1
K	7.30	21	5.00±0.8	4.1±0.8	2.20±0.8	47	1.7±0.3	14.0±2.0
L	7.63	15	5.50±0.4	4.0±0.8	2.66±0.6	50	0.0±0.0	0.0±0.0
N	7.50	22	6.17±0.6	3.5±0.0	2.75±0.6	30	2.7±0.2	15.7±0.9
R	6.50	2	1.73±0.2	3.2±0.8	2.10±0.1	45	2.9±0.3	30.0±2.2
S	7.25	11	2.43±0.4	3.3±0.0	2.98±0.4	32	2.4±0.3	14.7±1.2
T	6.38	22	5.50±0.4	4.2±0.8	2.60±0.4	42	2.3±0.2	13.3±1.7
Y	6.19	20	6.67±0.5	3.7±0.5	3.20±0.5	43	0.0±0.0	0.0±0.0
Aa	6.75	33	8.50±1.1	3.7±0.5	3.60±0.4	45	0.0±0.0	0.0±0.0
Bb	7.75	19	9.33±2.6	4.5±0.5	2.60±0.6	58	0.0±0.0	0.0±0.0
Dd	7.63	16	4.83±0.6	3.7±0.5	2.90±0.4	43	0.0±0.0	0.0±0.0
Ee	7.25	18	5.00±0.8	3.5±0.2	2.80±0.5	47	1.7±0.2	12.3±1.9
Gg	7.13	8	5.00±0.8	3.7±0.5	2.94±0.4	42	0.0±0.0	0.0±0.0
Hh	6.88	13	5.00±0.8	3.2±0.2	3.10±0.7	32	0.0±0.0	0.0±0.0
Jj	7.13	25	6.00±0.8	5.7±1.3	2.50±0.3	38	1.8±0.2	13.7±1.9
Kk	7.38	4	2.50±0.4	3.6±0.2	2.04±0.1	45	0.0±0.0	0.0±0.0
LL	7.28	12	3.33±0.2	3.2±0.5	2.20±0.5	40	1.5±0.2	13.0±1.2
Pdg	7.13	22	6.67±1.7	3.7±1.3	2.40±0.4	42	0.0±0.0	0.0±0.0
Mean		7.17	15.99	5.62	3.7	2.70	1.99	16.47
Minimum		6.19	2.00	1.73	3.1	2.10	1.35	12.33
Maximum		7.88	33.00	18.67	5.7	3.60	2.86	30.00
SD		0.42	7.45	3.30	0.6	0.36	0.46	5.05
CV (%)		5.80	46.6	58.7	16.0	13.3	22.9	30.70

List of genotypes: B: Ternate, D: Tanah Lot, E: Klaten, F: Bogor. G: Jepara, H: Semarang, K: Kebumen, L: Tabanan. N: Solo. R: Cangkringan, S: Prambanan, T: Kepuh Harjo, Y: Romeo Aceh, Aa: Bangka, Bb: Cheonan, Dd: Wakatobi, Ee: Bengkulu, Gg: Banda Aceh, Hh: Ipuh, Jj: Muko, Kk: Banda Aceh, LL: Banda Aceh, Pdg: Padang

cm) consisting of 2 genotypes and long internode (>2.1 cm) consisting of 20 genotypes of *Z. japonica* and *Z. matrella*. The spikelet length and number observed were separated into fewer seeds and short spikelet in 12 genotypes. More seeds and longer spikelet were found in 10 genotypes of *Z. japonica*, as shown in Table 3.

The turfgrass quality rated with acceptable value (score > 6) was observed in all 22 genotypes. Based on the results, the highest turf quality score was G genotype from Jepara, Central Java (score 7.78), which was higher than to Bb genotype (7.75). However, the lowest turf quality was Y genotype from Romeo (score 6.19).

UPGMA tree clustering showed three major groups based on morphological characteristics of zoysiagrass genotypes. Group 1 corresponded to *Z. japonica* and *Z. matrella*, where turfgrass height was relatively short, shorter, and fine leaves characteristics consisting of 3 genotypes of *Z. japonica* and *Z. matrellla*. Group 2 corresponded to *Z. japonica* with tall height, long and fine leaves, fewer seeds, and short spikelet characteristics consisting of 10 genotypes and clustered together with Bb genotype. Group 3 corresponded to *Z. japonica* with tall height, long and wider leaves, more seeds, and longer spikelet characteristics comprising 9 genotypes of *Z. japonica,* as shown in Figure 3.

3.3. Polymorphism Analysis and Genetic Diversity in Zoysiagrass Genotypes

A total of 15 among 16 SSR primers showed good polymorphism and were used for genotyping 23 zoysiagrass genotypes. Based on the results, only one primer (ZC01P20) monomorphic was not scored. Genetic variation showed 63 alleles with an average number of 4 per locus, ranging from 2 (ZB03J03) to approximately 8 (ZA01C06). The size variation between small and large alleles at a given SSR locus was correlated with the number of alleles per locus. Therefore, ZB03J03 presented a small allele size range (30 bp), while ZA01C06 showed a large allele size

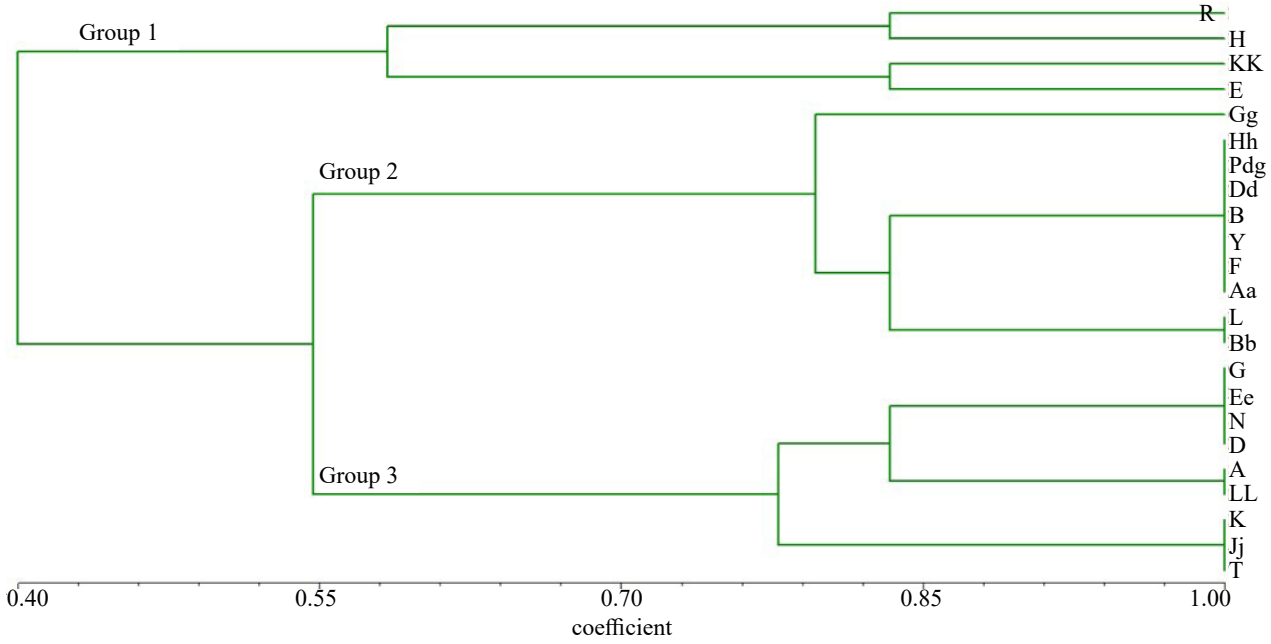

Figure 3. Genetic relationships between 23 zoysiagrass genotypes constructed by UPGMA tree showed three major groups based on seven morphological characters. B: Ternate, D: Tanah Lot, E: Klaten, F: Bogor. G: Jepara, H: Semarang, K: Kebumen, L: Tabanan. N: Solo. R: Cangkringan, S: Prambanan, T: Kepuh Harjo, Y: Romeo Aceh, Aa: Bangka, Bb: Cheonan, Dd: Wakatobi, Ee: Bengkulu, Gg: Banda Aceh, Hh: Ipuh, Jj: Muko, Kk: Banda Aceh, LL: Banda Aceh, Pdg: Padang

range (80 bp). The allele with the maximum frequency was known as major allele. ZB01G19 showed major allele frequency (0.804) and size of 180bp while ZB01C16 had major allele with minimum frequency of 0.333 and size of 120 bp. The number of alleles with a frequency ≥5% was 3.40, as shown in Table 4.

The average of PIC values was 0.53, which ranged from a low of 0.288 (ZB03J03) to a high of 0.73 (ZA01C06 and ZA01P11). Furthermore, 8 markers had a high PIC value (>0.5), including ZB03L03, ZB01C23, ZA01P11, ZTA114, ZA01C06, ZA03F03, ZB01B12, and ZB01C16). The expected heterozygosity (H*e)* varied from 0.125 (ZB03J03) to 0.377 (ZB10M09) with an average of 0.256 per locus. The expected homozygosity (H*o*) was observed from 0 (ZB01C23) to 0.614 (ZB03H23) with an average of 0.341 per locus. The average value for the expected heterozygosity was lower than homozygosity. As shown in Table 5, the inbreeding coefficient value (F*is*) was negative except for three primers (ZB03J03, ZB01C23, ZA01P11).

The performance of the examined marker systems was considered. In this study, the average of EMR was 4.20 and ZA01C06 detected the highest EMR (8). To validate the effective use of the SSR marker in zoysiagrass genotypes, the MI was calculated with average of 2.394. ZA01C06 observed the highest MI (5.870) while ZB03J03 showed the lowest MI (0.576). To determine the discriminatory capacity of the primer, RP values ranged from 0.783 (ZB01G19) to 2.00 in 7 SSR markers (ZB03L03, ZB01C23, ZA01P11, ZTA114, ZA01C06, ZB01B12, ZB01C16) with average 1.574.

Analysis of molecular variance (AMOVA) showed high genetic variation within population (98%) and low genetic differentiation among population. All zoysiagrass genotypes were collected from different regions of western and eastern Indonesia. The total number of alleles, the number of effective alleles, and the number of private alleles were positively related to the number of varieties collected from each province. Among 10 provinces, Central Java had the highest number of private alleles. The highest and the lowest number of polymorphic loci were amplified in Central Java (93.3%) and West Sumatera (13.3%), respectively. The highest (0.45) and lowest (0.06) of He values were observed in Central Java and West Sumatra population, respectively. The Central Java and West Sumatera population showed the maximum (0.76) and minimum (0.09) values of Shannon's index. Meanwhile, the highest and lowest within-population diversity was recorded in Bangka Belitung (0.60) and West Sumatera (0.133) population, respectively. The fixation index was positive in Bali, Bengkulu, Central Java, and Yogyakarta, as presented in Table 6. A

Table 4. Allelic diversity of SSR marker used in this study across 23 zoysiagrass genotypes

Marker	Allele size range (bp)	Major allele frequency		NG	NA	NAP	Polymorphic loci	PPB (%)
		Size (bp)	Freq (%)					
ZB03J03	120-150	120	0.775	3	2	2	2	100
ZB03L03	160-210	160	0.432	10	6	4	6	100
ZB01C23	120-150	120, 135	0.333	4	4	4	4	100
ZB01G19	180-210	180	0.804	3	3	2	3	100
ZB03B05	120-160	120	0.761	5	5	4	5	100
ZA01L02	150-170	150	0.652	4	3	2	3	100
ZB10M09	90-110	90	0.795	5	3	2	3	100
ZA01P11	120-140	120	0.652	3	3	3	3	100
ZB03H23	200-240	220	0.350	9	5	5	5	100
ZB01A21	250-350	280	0.636	5	3	3	3	100
ZTA114	200-300	230	0.386	10	5	5	5	100
ZA01C06	70-150	100	0.364	8	8	4	8	100
ZA03F03	130-160	130	0.522	7	5	4	5	100
ZB01B12	180-240	180, 200	0.425	7	4	3	4	100
ZB01C16	110-140	120	0.333	8	4	4	4	100

MAF: major allele frequency 2.5% l.b and 97.5% u.b; NG: number of genotypes per locus; NA: number of alleles per locus; NAP: number of alleles with a frequency >5 %, PPB: percentage of polymorphic band

Table 5. Parameters of SSR marker across 23 zoysiagrass genotypes

Marker	I	He	Ho	PIC	EMR	MI	RP	Fis	Fst	P-HWE	
										Aceh	Central Java
ZB03J03	0.177	0.125	0.091	0.288	2	0.576	0.900	0.273	0.700	m	0.046*
ZB03L03	0.566	0.373	0.455	0.669	6	4.016	2.000	-0.219	0.491	0.505	0.285
ZB01C23	0.303	0.215	0.000	0.670	4	2.679	2.000	1.000	0.692	0.157	0.025*
ZB01G19	0.248	0.163	0.291	0.295	3	0.886	0.783	-0.787	0.371	0.046*	0.804
ZB03B05	0.421	0.271	0.464	0.385	5	1.926	0.957	-0.713	0.301	0.931	0.821
ZB10M09	0.550	0.377	0.517	0.399	3	1.198	1.391	-0.370	0.190	0.775	0.655
ZA01L02	0.304	0.201	0.258	0.314	3	0.943	0.818	-0.280	0.303	0.505	0.019*
ZB03H23	0.547	0.357	0.614	0.461	3	1.383	1.391	-0.718	0.244	0.696	0.172
ZA01P11	0.303	0.190	0.127	0.724	5	3.619	2.000	0.330	0.776	0.506	0.120
ZB01A21	0.456	0.317	0.576	0.466	3	1.398	1.455	-0.819	0.451	m	0.261
ZTA114	0.479	0.318	0.545	0.699	5	3.494	2.000	-0.714	0.584	0.775	0.261
ZA01C06	0.205	0.136	0.212	0.734	8	5.870	2.000	-0.556	0.832	m	m
ZA03F03	0.454	0.318	0.427	0.603	5	3.017	1.913	-0.343	0.549	0.046*	0.804
ZB01B12	0.330	0.222	0.273	0.552	4	2.208	2.000	-0.227	0.667	0.572	0.046*
ZB01C16	0.382	0.259	0.273	0.676	4	2.704	2.000	-0.054	0.652	0.317	0.046*
Mean	0.382	0.256	0.341	0.529	4.20	2.394	1.574	-0.280	0.520		

I: Shannon Information Index, He: expected heterozygosity, Ho: expected homozygosity, PIC: Polymorphic Information Content, EMR: Effective multiplex ratios, MI: Marker Index, RP: Resolving Power, F*is*: Inbreeding coefficient, F*st*: Fixation Index, Hw: HardyWeinberg equilibrium (P<0.05) per locus (only significant province)

significant deviation from Hardy-Weinberg equilibrium (P<0.05) was observed in Aceh (ZB01G19, ZA03F03) and Central Java (ZB03J03, ZB01C23, ZA01L02, ZB01B12, ZB01C16) (Table 5).

Cluster analysis was calculated from SSR markers to construct a neighbor-joining tree (r = 0.8), which showed weak clustering into three major groups. Subsequently, Group 1 was subdivided into subgroups consisting of 11 genotypes originating from Sumatera Island and Yogyakarta (Figure 4). Based on the results, Group 1 showed weak bootstrap supports for branches separating genotypes from the same geographical distribution. Subgroup 1 consisted of genotypes Jj and Hh from Bengkulu (bootstrapping value 99), Subgroup 2 comprised genotypes S and R from Yogyakarta (bootstrapping value 87), while Subgroup 3 included genotypes Ll and Kk from Aceh (bootstrapping value 76). Similarly, Group 2 was divided into subgroups, which contained 8 genotypes originating from Central Java, Bali, and Bangka Belitung. Subgroup 1 showed weak bootstrap supports for branches separating genotypes from the same geographical distribution,

Table 6. Genetic diversity parameters of zoysiagrass genotypes analyzed by SSR marker

Population	Na	Na Freq. >= 5%	Ne	I	No. private alleles	No. LComm alleles <=25%	No. LComm alleles <=50%	H*o*	H*e*	%P	F
Aceh	2.067	2.067	1.721	0.556	0.000	0.133	1.133	0.430	0.357	80	-0.190
Bali	1.733	1.733	1.658	0.468	0.000	0.133	0.733	0.270	0.325	67	0.170
Bangka Belitung	1.533	1.533	1.533	0.416	0.067	0.067	0.600	0.600	0.300	60	-1.000
Bengkulu	2.000	2.000	1.743	0.555	0.067	0.133	1.000	0.380	0.363	73	0.010
Central Java	2.667	2.667	2.123	0.768	0.133	0.400	1.467	0.350	0.458	93	0.240
Yogyakarta	1.733	1.733	1.528	0.420	0.067	0.000	0.733	0.270	0.281	67	0.040
North Maluku	1.133	1.133	1.133	0.139	0.067	0.067	0.400	0.200	0.100	20	-1.000
South Korea	1.467	1.467	1.467	0.323	0.133	0.200	0.467	0.470	0.233	47	-1.000
Southeast Sulawesi	1.133	1.133	1.133	0.139	0.067	0.067	0.400	0.200	0.100	20	-1.000
West Java	1.467	1.467	1.467	0.323	0.067	0.200	0.600	0.470	0.233	47	-1.000
West Sumatera	1.133	1.133	1.133	0.092	0.000	0.067	0.467	0.130	0.067	13	-1.000

Na: number of different alleles; Na (Freq >= 5%): No. of different alleles with a frequency >= 5%; Ne: No. of effective alleles; I: shannon's information index; No. private alleles = No. of alleles unique to a single population; No. LComm alleles (<=25%): No. of locally common alleles (Freq. >= 5%) found in 25% or fewer populations; No. LComm alleles (<=50%): No. of locally common alleles (Freq. >= 5%) found in 50% or fewer populations; He: expected heterozygosity; P(%): number (percentage) of polymorphic loci; F: fixation index

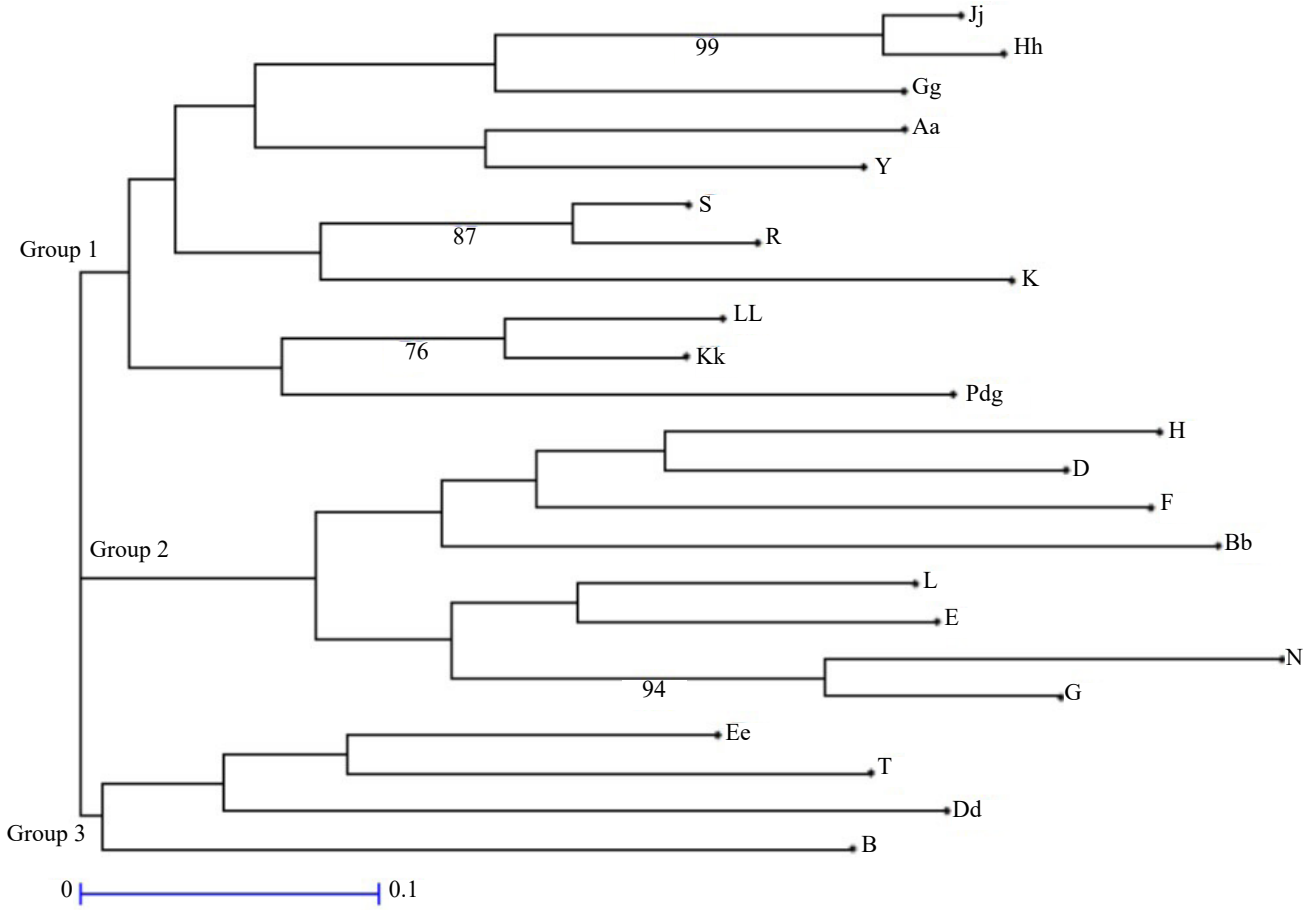

Figure 4. Neighbor Joining tree of zoysiagrass genotypes showing genetic relationships between 23 zoysiagrass genotypes based on 15 microsatellite markers constructed with 100 bootstrap replicates. B: Ternate, D: Tanah Lot, E: Klaten, F: Bogor. G: Jepara, H: Semarang, K: Kebumen, L: Tabanan. N: Solo. R: Cangkringan, S: Prambanan, T: Kepuh Harjo, Y: Romeo Aceh, Aa: Bangka, Bb: Cheonan, Dd: Wakatobi, Ee: Bengkulu, Gg: Banda Aceh, Hh: Ipuh, Jj: Muko, Kk: Banda Aceh, LL: Banda Aceh, Pdg: Padang

except in Subgroup 2, which contained genotypes N and G from Solo (bootstrapping value 94). Group 3 showed weak bootstrapping and did not share the same geographical distribution. It consisted of 4 genotypes originating from Sumatera Island, Yogyakarta, Southeast Sulawesi, and North Maluku.

Principles of coordinate analysis of zoysiagrass genotypes showed that population of Sumatera Island (Aceh, West Sumatera, Bengkulu, Bangka Belitung), Yogyakarta, and North Maluku in the right quadrant corresponded to Group 1. Meanwhile, population of Java Island (Central Java and West Java), Yogyakarta, Bali, Southeast Sulawesi, and South Korea were located in the left quadrant that corresponded to Group 2 and Group 3, as shown in Figure 5. The maximum cumulative percent variation was explained by the first three coordinates with 62.08% variance. This showed that zoysiagrass genotypes in Indonesia were highly diverse from one another.

3.4. Population Structure of Zoysiagrass Genotypes

A total of 15 SSR markers were used to determine population structure of zoysiagrass genotypes. The output results from STRUCTURE were analyzed using Structure harvester. The average estimate in probability score and lowest variance (LnP(D)), with the significant k value, were high in the evanno table (Table 7). A continuous decrease was observed in the peak of delta-*k* on $k = 2$, showing that the entire population could be divided into two subpopulations, as presented in Figure 6. Most population was homogeneous and showed little admixture apart. In $k = 2$, Group 1 corresponded to *Z. japonica* from Sumatera Island (30.4%) and mixed province (21.7%) which was colored in red. Meanwhile, Group 2 corresponded to *Z. japonica* from Central Java and Bali (26.2%), *Z. japonica* and *Z. matrella* from mixed province (21.7%) colored in green.

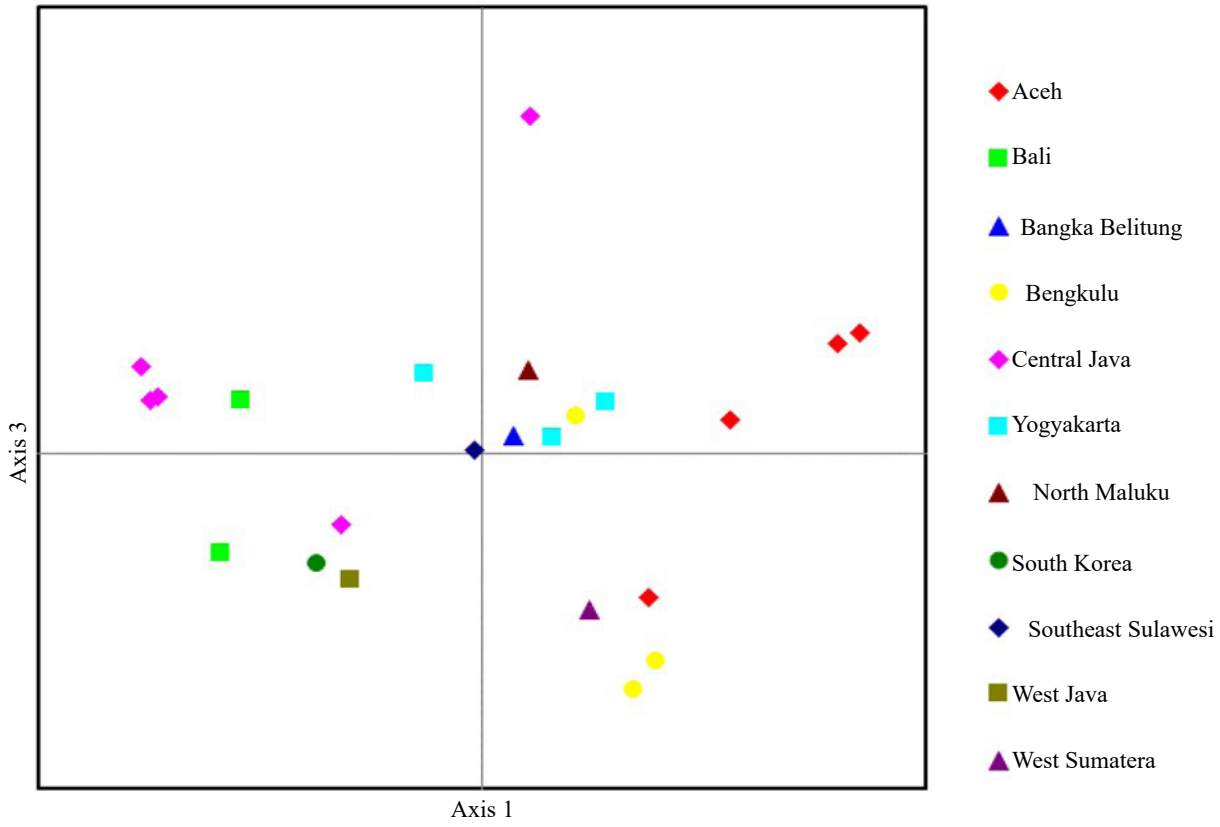

Figure 5. PCoA of zoysiagrass genotypes in Indonesia. Population of Sumatera Island (Aceh, West Sumatera, Bengkulu, Bangka Belitung), Yogyakarta, and North Maluku located in right quadrant and population of Java Island (Central Java and West Java), Yogyakarta, Bali and Southeast Sulawesi located in left quadrant

Table 7. Evanno table with mean and standard deviation of log-likelihood values along with Delta K for genetic structure analysis

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-7.331.000	0.2550	-	-	-
2	5	-6.696.800	20.632	63.420.000	31.500.000	15.267.177
3	5	-6.377.600	111.046	31.920.000	2.460.000	0.221529
4	5	-6.083.000	26.220	29.460.000	31.360.000	11.960.235
5	5	-6.102.000	144.146	-1.900.000	23.960.000	1.662.206
6	5	-6.360.600	150.913	-25.860.000	4.900.000	0.324690
7	5	-6.570.200	278.166	-20.960.000	8.040.000	0.289036
8	5	-6.699.400	166.500	-12.920.000	19.900.000	1.195.194
9	5	-6.629.600	92.891	6.980.000	29.040.000	3.126.233
10	5	-6.850.200	145.958	-22.060.000	-	-

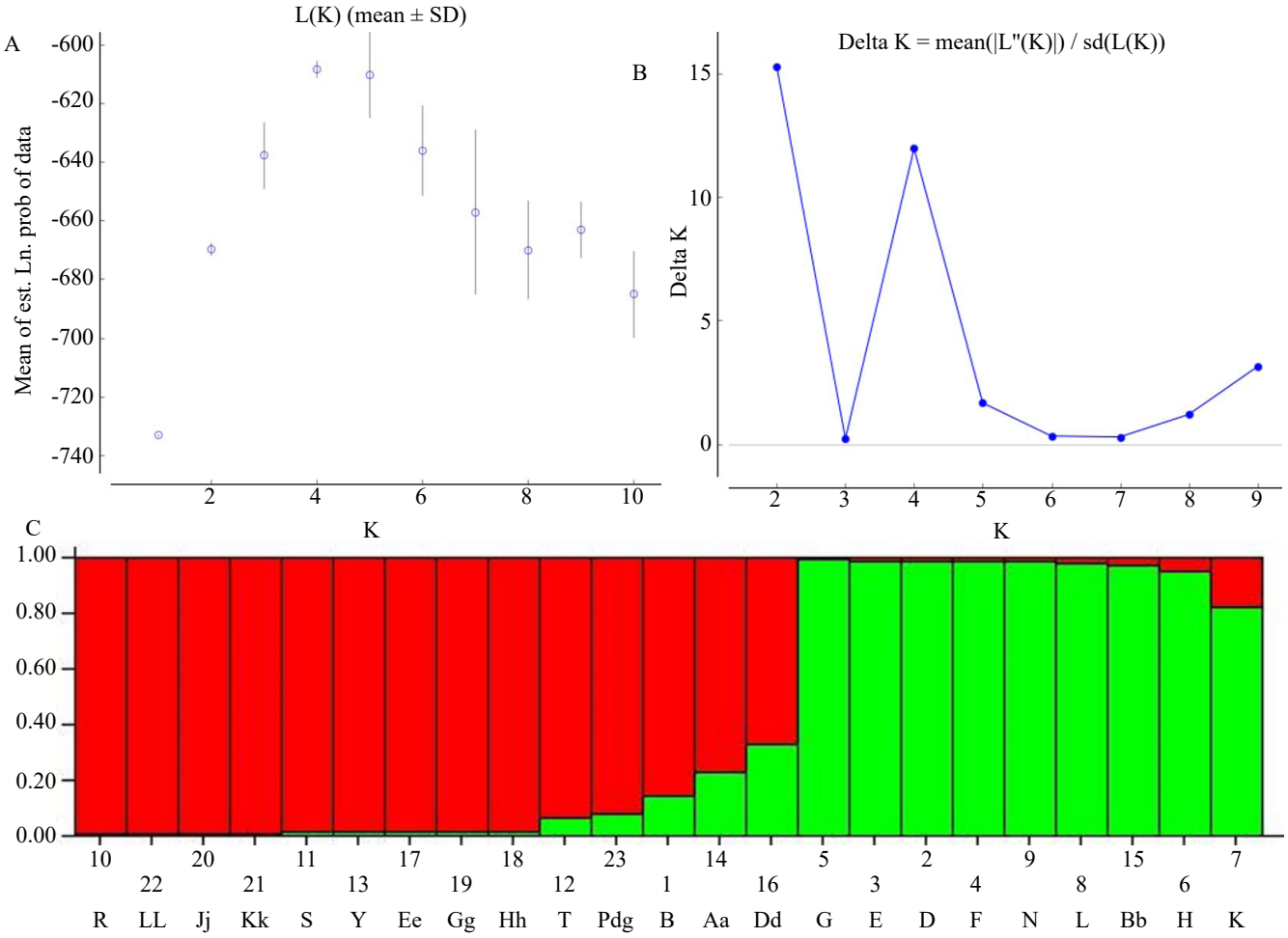

Figure 6. Population structure of 23 zoysiagrass genotypes based on 15 SSR loci. (A) The number of k clusters against the probability of the data Ln P(D) (± SD), Mean log-likelihood probabilities (k = 1-10) plotted versus Δk for determining ideal k-value (middle) and clustering based on bayesian tree plot (right), (B) population structure on k = 2. Group 1 corresponded to *Z. japonica* from Sumatera Island (30.4%) and mixed province (21.7%) which is colored in red and Group 2 corresponded to *Z. japonica* from Central Java and Bali (26.2%) and *Z. japonica* and *Z. matrella* from mixed province (21.7%) coloured in green. B: Ternate, D: Tanah Lot, E: Klaten, F: Bogor. G: Jepara, H: Semarang, K: Kebumen, L: Tabanan. N: Solo. R: Cangkringan, S: Prambanan, T: Kepuh Harjo, Y: Romeo Aceh, Aa: Bangka, Bb: Cheonan, Dd: Wakatobi, Ee: Bengkulu, Gg: Banda Aceh, Hh: Ipuh, Jj: Muko, Kk: Banda Aceh, LL: Banda Aceh, Pdg: Padang

4. Discussion

Turfgrass breeders require a wide variety of germplasm to develop improved varieties with low-cost maintenance and adaptability to environmental conditions. This study is the first major attempt to characterize Indonesian zoysiagrass collections using morphological and SSR markers to obtain information for conservation and breeding purposes. The collection of zoysiagrass genotypes in the country was classified based on morphological and habitat characteristics (Rahayu *et al.* 2016). Morphological performances have been widely used for conventional varietal identification, phenotypic, and determining its relationship (Azahar *et al.* 2019; Ullah *et al.* 2022). This leads to the investigation of morphological characteristics among Indonesian zoysiagrass.

Based on the results, the cluster analysis of morphological characteristics and turfgrass quality distinguished three major groups, as shown in Figure 3. Group 1 characteristics were classified as dwarf ecotype, showing potential applications in breeding programs. The dwarf ecotype is a good fit for a green golf course, serving as a naturally short, compact plant with fine leaf texture and short internodes. Moreover, dwarf zoysiagrass is high in lignin, hemicellulose, and fiber content, improving resistance to traffic, wear tolerance, and devotion (Wei *et al.* 2022). Dwarfism is favorable in turfgrass because it allows for high-density planting, low mowing frequency, and high photosynthetic efficiency (Lin *et al.* 2020).

Artificial mutation-induced breeding through irradiation using 30 Gy gamma ray has been applied to develop dwarf zoysiagrass, namely Halla Green 1 (Yang *et al.* 2016) and Halla Green 2 (Song *et al.* 2017). Meanwhile, Halla Green 7 (Yang *et al.* 2021a), and Halla Green 10 (Yang *et al.* 2021b) are dwarf zoysiagrass with extended greening periods during the low temperatures of winter. These species are expected to contribute to the industrialization of zoysiagrass in Korea. Lin *et al.* (2020) induced mutations of *Z. matrella* using gamma rays to identify dwarf mutants. The results showed that manipulation of auxin biosynthetic pathway genes could be an effective method for dwarfing breeding of turfgrass. Therefore, Indonesian dwarf zoysiagrass genotypes from this study could be used as raw material for phenotypic assessment, such as biotic and abiotic tolerance, which are suitable in certain water-deficient areas and tropical soil characteristics.

Molecular characteristics are generally used to obtain more features of genetic diversity within a germplasm collection. In this study, 15 out of 16 SSR primers used produced polymorphic bands. The results showed that the method was an effective and efficient method for measuring genetic variation within and among the species of Indonesian zoysiagrass genotypes. A high level of polymorphism was observed among genotypes, as shown by 63 alleles with an average of 4 alleles per marker and a PIC value of 0.5. This was similar to Araneda *et al.* (2013) who applied 24 SSR primers and 6 CISP to generate a total of 51 allele fragments among 23 zoysiagrass genotypes. However, Kimball *et al.* (2013) detected a total of 377 alleles with an average of 8 alleles and a PIC value of 0.49 from 50 SSR primer among 62 US accessions. Kunwanlee *et al.* (2018) used 24 SSR primers showing a total of 123 alleles with an average of 5.125 alleles among 26 Japanese and US cultivars.

A comparison of the discriminating capacity, level of polymorphism, and informativeness of SSR markers was carried out by estimating the EMR and MI (Powell *et al.* 1996). RP is another method used to measure the ability of primers to distinguish between genotypes (Prevost and Wilkinson 1999). In this study, RP was higher than the report by Singh *et al.* (2020) who used 18 SSR markers to genotype 20 wheats in India, resulting in 0.63. Sutkovic *et al.* (2021) investigated 505 kale genotypes with 10 SSR markers, which showed the average EMR, MI, and RP values at 1.99, 1.28, and 1.02, respectively. Therefore, the significant RP value showed the ability of SSR markers to distinguish the difference between zoysiagrass genotypes. Mir *et al.* (2022) showed the great resolving power of SSR markers for assessing variability and interrelationship among the 192 chickpea accessions using 33 SSR markers in western Himalayas.

The expected heterozygosity of 23 zoysiagrass genotypes was lower than Moore *et al.* (2017). The average value for expected homozygosity was higher than the expected heterozygosity, showing the isolate-breaking effect or the mixing of two previously isolated populations. The F value and gene diversity were high in Central Java, which showed the presence of a large genetic diversity compared to other provinces. This showed that most genetic diversity of zoysiagrass occurred within population. Meanwhile, the negative value of the inbreeding coefficient (F*is*) showed the presence of random mating in population.

Nadachowska-Brzyska *et al.* (2022) stated that heterozygote excess was caused by small reproductive population size, overdominance, negative assortative mating, or asexual reproduction. According to Tanaka *et al.* (2016b), the excess of heterozygotes in approximately all zoysiagrass population might reflect their polyploid nature and the recent hybridizations following polyploidization. However, further studies are required by increasing the number of samples with more diverse population.

The cluster analysis, PCoA, and population structure showed that Indonesian zoysiagrass genotypes from the same or adjacent regions were not classified together. Based on the study by Tanaka *et al.* (2016b), samples collected in Southern and Northern Japan correlated with latitude or longitude. The results showed that zoysiagrass genotypes grown from adjacent regions sharing a similar environment were classified together. The wide range of distribution in Indonesia showed that the species possessed adaptive characteristics to withstand different climatic conditions. These included environmental temperature and daylength in tropical regions showed by wide variations such as leaf width and plant height (Table 3), as well as geographic origins (Gouveia *et al.* 2021). Rahayu *et al.* (2018) reported that the Indonesian zoysiagrass genotypes collected at seaside had small, narrow, finer, longer, and dark green leaves than samples obtained in a mountain. In this study, HWE showed that the samples collected from seaside area were significant ($P<0.01$ and $P<0.001$) compared to mountain area (data not shown). The clustering of genotypes showed no parallelism between genetic and geographical diversity. This was because genotypes of various geographic regions were grouped in different clusters.

The results showed different clustering in Indonesian zoysiagrass genotypes when morphological and molecular data were investigated. This difference can be interpreted as a partial and insufficient genome representation. Compared to morphological characteristics, molecular markers overlay a larger proportion of genome along with coding and noncoding regions. Additionally, molecular markers detected a non-adaptive genetic variation, which was not subjected to natural or artificial selection as several morphological characteristics (Pocovi *et al.* 2020).

In conclusion, this study successfully applied morphological characteristics and SSR markers to show genetic diversity and population structure of Indonesian zoysiagrass genotypes. Morphological characteristics clustered these genotypes into three major groups, which corresponded to leaf width and plant height. The SSR markers showed abundant genetic variation within population. The cluster analysis, PCoA, and population structure showed that zoysiagrass genotypes were classified together based on geographic origins among adjacent regions. These results provided valuable information on genetic variation of Indonesian zoysiagrass genotypes for plant breeders to select parental combinations in breeding program for genetic improvement. Moreover, further studies were recommended to develop a well-characterized diverse gene pool to capture and exploit the variation.

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