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Research article

Genetic diversity of 'Bangle' (*Zingiber montanum* (J.Koenig) Link ex A.Dietr.) inferred from sequence-related amplified polymorphism markers

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Abstract

Zingiber montanum (J.Koenig) Link ex A.Dietr. known in Indonesia as 'Bangle' is widely cultivated there in addition to other *Zingiber* species, such as *Z. officinale* Roscoe and *Z. zerumbet* (L.) Sm. Despite the rich properties of its rhizomes as an anti-oxidant and anti-inflammatory, knowledge of its genetic diversity is still scarce. Understanding genetic diversity is important for conservation purposes and to improve the genetic contents. Consequently, genetic diversity of *Z. montanum* was analyzed using sequence-related amplified polymorphism (SRAP) markers. In total, 54 *Z. montanum* samples collected from 12 regions in four provinces were analyzed using eight SRAP primer pairs which amplified the open reading frames region. The results showed that *Z. montanum* had high genetic diversity with mean (± SD) values for Nei's genetic diversity and the Shannon information index of 0.257 ± 0.172 and 0.400 ± 0.223. Even though *Z. montanum* is a clonal species, it was able to maintain high genetic diversity. The analysis of molecular variance showed that the genetic variation occurred mostly within the population. High genetic diversity and low differentiation among populations could suggest that rare sexual reproduction has played an important role in *Z. montanum* population history. Bayesian model-based clustering was congruent with the unweighted pair-group method of averages dendrogram at the sub-cluster level. The *Z. montanum* populations were grouped into three clusters with strong correlation within geographic locations. Based on the results, germplasm should be collected and maintained for at least one individual from each of the three clusters.

Introduction

Zingiber montanum (J.Koenig) Link ex A.Dietr. (family Zingiberaceae) known as 'Bangle' in the Javanese language is a medicinal plant widely used in Southeast Asia (Wolff et al., 1999). *Z. montanum* is probably native to India and Southeast Asia and is commonly grown in villages (Theilade, 1996). *Z. montanum* is a diploid with 2n = 2x = 22 (Raghavan and Venkatasubban, 1943; Larsen et al., 1998) presenting its inflorescence on a radical, erect peduncle and having spherical pollen (Valeton, 1918). The flowers are tubular and contain nectar and like in other Zingiberaceae species last only for 1 d (Larsen et al., 1998). The plant is the same as most other Zingiberaceae, flowering

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between July and September and fruiting from November to January (Theilade, 1996). There is no detailed information about *Z. montanum* pollinators but its relative species in Kalimantan are categorized into three groups based on their pollinator guilds, namely spiderhunters, *Amegilla* bees and halictid bees (Sakai and Kato, 1999).

In Indonesia, Bangle is widely cultivated as a minor crop planted in backyards and has been reported to be cultivated in Java, Moluccas, Sibolangit (Sumatra) and Malacca since the colonial era (Valeton, 1918). The medicinal properties of Bangle are known by the Sundanese to cure fever, postpartum drugs, dermatitis, hookworm, diarrhea and gastritis (Roosita et al., 2008). The Dayak Kenyah people have used Bangle for a wide range of treatments, including gynecological, gastrointestinal, respiratory and muscular problems (Ellen and Puri, 2016). Recent study reported that the essential oil of Bangle is a potential source of (E)-1-(3',4'-dimethoxyphenyl) buta-1,3-diene, terpinen-4-ol and sabinene for pharmaceutical products (Verma et al., 2018). In addition, Bangle is a component of Indonesian herbal medicine (*'Jamu'*), which has been industrialized.

The National Institute of Health Research and Development (NIHRD) has inventoried Indonesian medicinal plants including *Z. montanum* through the *Riset Tanaman Obat dan Jamu* (RISTOJA) or Research on Medicinal Plants and *Jamu* project and collected medicinal plants from all over Indonesia. Development of medicinal plant germplasm has been pioneered by the Medicinal Plant and Traditional Medicine Research and Development Centre, NIHRD. To gain benefit from these germplasms, assessment and characterization of genetic diversity is very important for planning meaningful breeding strategies and medicinal plant improvement.

Genetic diversity of *Z. montanum* in Indonesia is poorly known. Research on genetic diversity of *Z. montanum* has been carried out on genotypes collected from various locations in Thailand using 29 RAPD primers and showed high molecular variance (87%) within its accession (Bua-in and Paisooksantivatana, 2010). The same result was observed by Kladmook et al. (2010) who assessed genetic diversity of *Z. montanum* from Thailand using 12 amplified fragment length polymorphism (AFLP) primers and found high molecular variance (84%) within samples from the same region.

Z. montanum is a medicinal plant that will continue to be exploited. An evaluation of its genetic diversity is very important. This study analyzed the genetic diversity of *Z. montanum* from four provinces in Indonesia using sequence-related amplified polymorphism (SRAP) markers (Li and Quiros, 2001). SRAP belongs to the targeted fingerprinting marker group and is a multilocus marker that is produced semi-randomly and targets various sequence regions in the genome (Poczai et al., 2013). A SRAP marker was developed to recognize the exon, intron, spacer and promoter region (Li and Quiros, 2001). Knowledge of genetic diversity of Bangle is expected to be useful as a foundation for the breeding and development of Bangle in Indonesia.

Materials and Methods

Samples

In total, 54 samples of *Z. montanum* were used in this study to investigate the genetic variation and population structure. Samples were collected from 12 locations in four provinces in Indonesia (Table 1) during RISTOJA exploration conducted by the Medicinal Plant and Traditional Medicine Research and Development Centre, NIHRD from 2015 to 2017. The samples were obtained from limited provinces since only few ethnic communities used the plant. Medium-aged, healthy leaves of approximately 2 cm × 2 cm were cut into small pieces, then stored in a tea bag before being placed in a container filled with silica gel.

DNA extraction and SRAP analysis

Total DNA was isolated from the silica-dried leaves following the GeneJet Plant Genomic DNA Purification Mini Kit (ThermoFisher Scientific) protocol with slight modification by adding proteinase K to the sample in the second step of isolation to increase the concentration of DNA yields. The isolated DNA was diluted with nuclease-free water to a final concentration of 10 ng/µL. Then, 16 SRAP primer combinations were screened of primer pairs of four forward primers (Me1, Me2, Me3, and Me4) (Li and Quiros, 2001) and four reverse

Table 1 Genetic diversity of 12 *Zingiber montanum* populations

Population	Locality	Sample size	He	I	PPL	G_{ST}	Nm
Jawan	West Kalimantan	5	0.127 ± 0.191	0.187 ± 0.275	33.58%	-	-
Kayanath	West Kalimantan	5	0.084 ± 0.158	0.126 ± 0.234	23.36%	-	-
Kore	West Nusa Tenggara	3	0.096 ± 0.167	0.146 ± 0.243	29.20%	-	-
Pattinjo	South Sulawesi	5	0.225 ± 0.207	0.334 ± 0.292	60.58%	-	-
Bonerate	South Sulawesi	5	0.147 ± 0.198	0.218 ± 0.285	40.15%	-	-
Padoe	South Sulawesi	5	0.223 ± 0.201	0.334 ± 0.282	63.50%	-	-
Kalatoa	South Sulawesi	5	0.137 ± 0.190	0.205 ± 0.275	38.69%	-	-
Rongkong	South Sulawesi	3	0.071 ± 0.152	0.105 ± 0.223	18.98%	-	-
Tialo	Central Sulawesi	5	0.137 ± 0.200	0.201 ± 0.286	35.04%	-	-
Lauje	Central Sulawesi	5	0.083 ± 0.168	0.124 ± 0.237	23.36%	-	-
Bungku	Central Sulawesi	5	0.097 ± 0.161	0.150 ± 0.238	31.39%	-	-
Ledo	Central Sulawesi	3	0.072 ± 0.161	0.104 ± 0.231	17.52%	-	-
Species Level		54	0.257 ± 0.172	0.400 ± 0.223	98.54%	0.5112	0.4781

He = Nei's gene diversity; I = Shannon's information index; PPL = percentage of polymorphic loci; G_{ST} = population differentiation value; Nm = estimated gene flow
Values are shown as mean ± SE

primers (Em1, Em2, Em3, and Em4) (Li and Quiros, 2001). Primer pairs which produced polymorphic bands (Table 2) were used for further analysis. The polymerase chain reaction (PCR) was performed in 15 µL of reaction mixture containing 10 ng DNA template, 7.5 µL DreamTaq Green PCR Master Mix (2x) (ThermoFisher Scientific) and 2 µM of both forward primer and reverse primer. The PCR program for amplification was set following Li and Quiros (2001) with pre denaturation at 95°C for 5 min, five cycles of denaturation at 95°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, ended by final extension at 72°C for 5 min. The PCR products were electrophoresed in 1.5% agarose gel for 90 min at 100 V, and then visualized by the staining is using GelRed® Nucleic Acid Gel Stain (Biotium)

Table 2 Primer combinations and sequences

Primer pair	DNA sequence
Me1/Em2	TGAGTCCAAACCGGATA/GACTGCGTACGAATTTGC
Me1/Em4	TGAGTCCAAACCGGATA/GACTGCGTACGAATTTGA
Me2/Em1	TGAGTCCAAACCGGAGC/GACTGCGTACGAATTAAT
Me2/Em3	TGAGTCCAAACCGGAGC/GACTGCGTACGAATTGAC
Me3/Em1	TGAGTCCAAACCGGAGT/GACTGCGTACGAATTAAT
Me3/Em4	TGAGTCCAAACCGGAGT/GACTGCGTACGAATTTGA
Me4/Em3	TGAGTCCAAACCGGACC/GACTGCGTACGAATTGAC
Me4/Em4	TGAGTCCAAACCGGACC/GACTGCGTACGAATTTGA

Data analysis

Bands produced in agarose gel were scored as 1 (present) or 0 (absent) manually and using the Image Lab 6.01 (Bio-Rad) software. Data were calculated using the POPGENE version 1.32 (Yeh et al., 1999) software to calculate percentage of polymorphic loci (PPL), Nei's genetic diversity (He), Shannon information index (I) and the genetic coefficient of differentiation (GST). The composition of genetic variation was explored by analyzing the molecular variance (AMOVA) using GenAlex 6.5 (Peakall and Smouse, 2012) based on 999 permutations. Gene flow among populations was estimated indirectly using the formula Nm = 0.5 (1-GST)/GST (Slatkin and Barton, 1989). To analyze the relationship between populations, cluster analysis with the unweighted pair-group method of averages (UPGMA) algorithm using Nei and Li's distances was performed in PAUP version 4.0 (Swofford, 2003). The stability of the clusters was estimated using bootstrap analysis with 10,000 resamples. Genetic structure was calculated based on Bayesian cluster analysis implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). Initially, a simulation run of the STRUCTURE was conducted by assuming number of genetic clusters/subpopulations (K) of 1 to 10 with 20 iterations for each K and a burn-in length of 10,000 and a run length of 100,000 Markov chain Monte Carlo (MCMC) replications under the admixture model, the correlated allele frequencies model and no prior population information. The optimum number of K was calculated using the ΔK method of Evanno et al. (2005). Subsequently, each individual/sample was assigned to each subpopulation by running STRUCTURE with the optimum K value, burn-in length of 10,000 and run length of 100,000 MCMC replications. Principal component analysis (PCA) using Jaccard's similarity coefficient was performed to confirm similarity among the 54 samples. The analysis was conducted in MVSP (Kovach, 1998).

Results and Discussion

Genetic diversity

Based on the screening of the 16 SRAP primer combinations, eight primer pairs produced polymorphic bands (Table 2). The primers produced 137 bands of which 98.5% were polymorphic. The highest and the lowest polymorphic bands were produced by Me2/Em3 and Me4/Em3 primer combinations. He at the species level was 0.257 ± 0.172 and I was 0.400 ± 0.223 (Table 1). Within each population, He ranged from 0.071 ± 0.152 to 0.225 ± 0.207, I ranged from 0.104 ±0.231 to 0.334 ± 0.282 (from the lowest to the highest) and PPL ranged from 17.52% to 63.50%. The highest and the lowest genetic diversities were observed in the Pattinjo (He = 0.225 ± 0.207) and Rongkong (He = 0.071 ± 0.152) populations, respectively. The genetic diversity of *Z. montanum* was high compared to other *Zingiber* species. The expected heterozygosity of *Z. montanum* was higher than that of 13 accessions of *Z. officinale* from India (He = 0.009 ± 0.0619) according to (Thomas et al. 2016). It was also higher than the expected heterozygosity of *Z. nimmonii* from five natural populations in Kerala (He = 0.16380 ± 0.2007) according to (Thomas et al., 2016) and of *Z. neesanum* from three natural populations in Kerala (He = 0.2400 ± 0.2077) according to (Thomas et al., 2016). However, the genetic diversity of *Z. montanum* was lower than the genetic diversity of *Z. zerumbet* from 15 natural populations in Kerala, India (He = 0.2738 ± 0.1996) according to (Kavitha and Thomas, 2008).

Genetic differentiation and population structure

The value of population differentiation (G_{ST}) in *Z. montanum* was 0.5112. This indicated that 48.8% of the total genetic variability was distributed within the population. The level of gene flow (Nm) among populations was estimated to be 0.4781. The results of the AMOVA analysis (Table 3) showed significant and high variation within populations ($p < 0.001$). As much as 64% of the total variation was distributed within populations and 36% of the total variation was partitioned among populations of *Z. montanum*.

Table 3 Analysis of molecular variance of 12 populations of *Zingiber montanum*

Source of variation	df	SS	MS	Estimated variations	%	Φ-statistics	*p*
Among populations	11	483.478	43.953	7.021	36%	$\Phi_{ST} = 0.360$	0.001
Within populations	42	52.467	12.463	12.463	64%		

df = degrees of freedom; SS = sums of squares; MS = mean squares; Φ_{ST} = fixation index based on sequence data; *P* = the probability of null hypothesis

UPGMA was performed using a Nei and Li's genetic distance matrix. The analysis resulted in a dendrogram with three clusters. Cluster I consisted of two sub-clusters. Sub-cluster Ia consisted of populations from Jawan (West Kalimantan), Kayanath (West Kalimantan), Kore (West Nusa Tenggara) and Pattinjo (South Sulawesi). Sub-cluster Ib consisted of populations from Kalatoa (South Sulawesi), Rongkong (South Sulawesi) and populations from Tialo, Lauje, Bungku and Ledo in Central Sulawesi. Cluster II consisted of all populations from South Sulawesi (populations from Pattinjo, Bonerate, Kalatoa and Padoe). The last cluster, cluster III, consisted of small populations from Pattinjo and Padoe in South Sulawesi (Fig. 1). STRUCTURE analysis based on Evanno's ad hoc ΔK statistic determined K of 3 as the most likely number of genetic clusters (Fig. 2). With individual membership proportion (Q) higher than or equal to 80%, as many as 80%, 100% and 88.9% of *Z. montanum* individuals were exclusively placed in cluster A (green), cluster B (yellow) and cluster C (red) respectively (Fig. 3). Individuals with a membership proportion less than 80% were Kore5, Pattinjo1 and Pattinjo2 with dominant genetic proportions of genetic cluster A but also contained genetic parts of clusters B and C. Other admixture individuals were Bungku4 and Bungku5 being the dominant genetic proportion of cluster C but also contained a proportion of cluster A.

The UPGMA and STRUCTURE analysis showed the same number of groups but different compositions. However, the results were congruent at some level. Subcluster Ia consisted of the same individuals as cluster A (green) with the addition of an admixture individual, Kore5, Pattinjo1 and Pattinjo2. Subcluster Ib consisted of the same individual as cluster C (red) and five individuals from cluster B (yellow). Cluster II consisted of the individuals the same as in cluster C (red) except for the five individuals included in subcluster Ib.

The results of PCA analysis showed the same clustering of populations from Central Sulawesi (Tialo, Lauje, Bungku and Ledo) as that from the UPGMA result (Fig. 4). This Central Sulawesi group was close to the group that consisted of populations of Kalatoa and Rongkong. However, the rest of the populations were mixed in the plot.

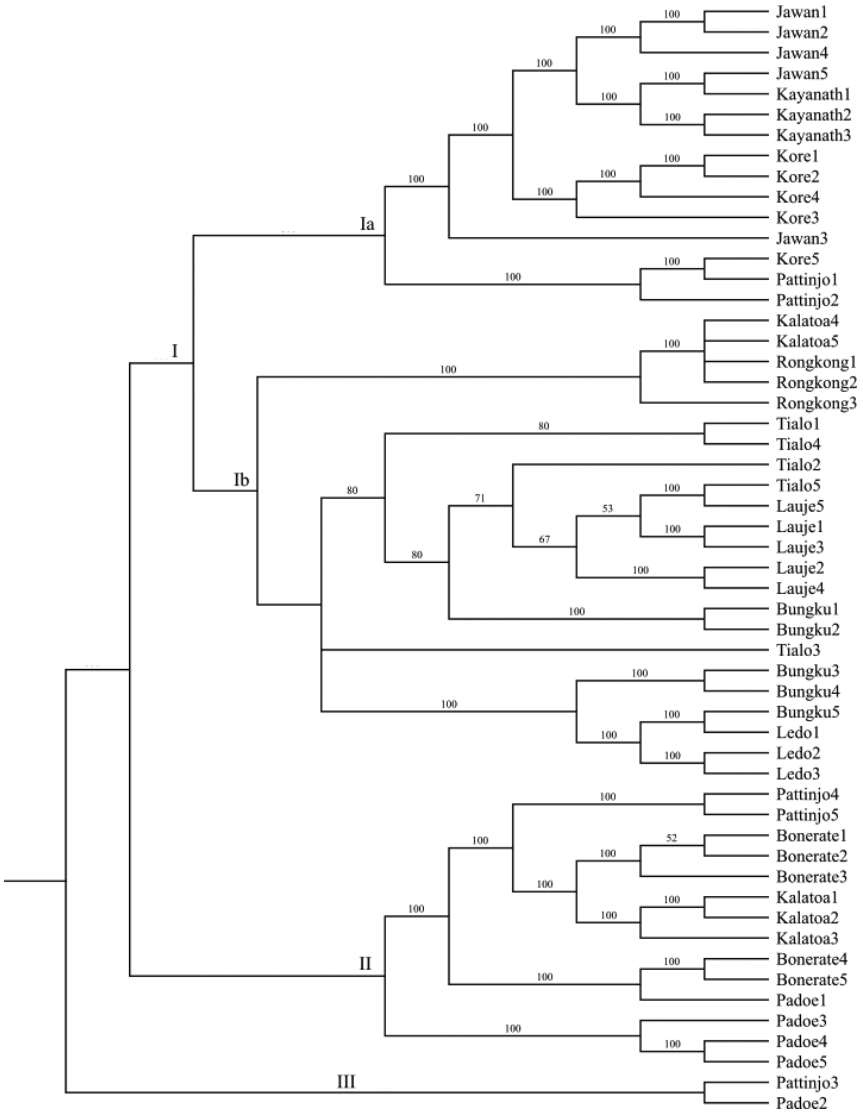

Fig. 1 Unweighted pair-group method of averages dendrogram of *Zingiber montanum* based on Nei's and Li's genetic distance

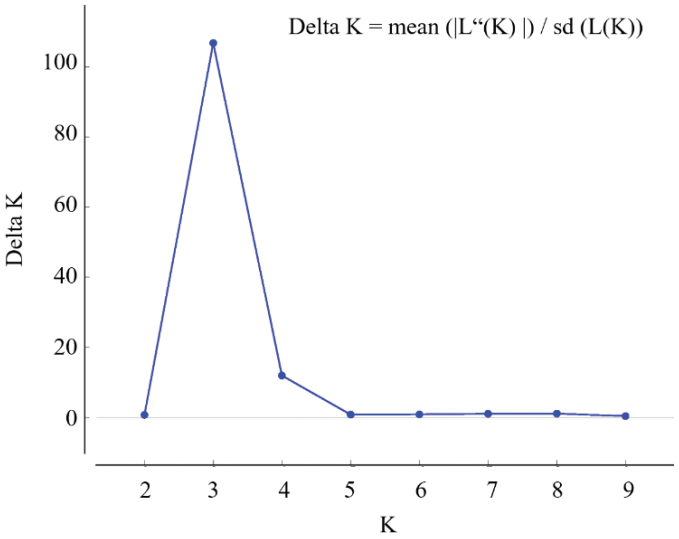

Fig. 2 Structure estimation number of clusters for K values ranging from 1 to 10, based on deltaK values

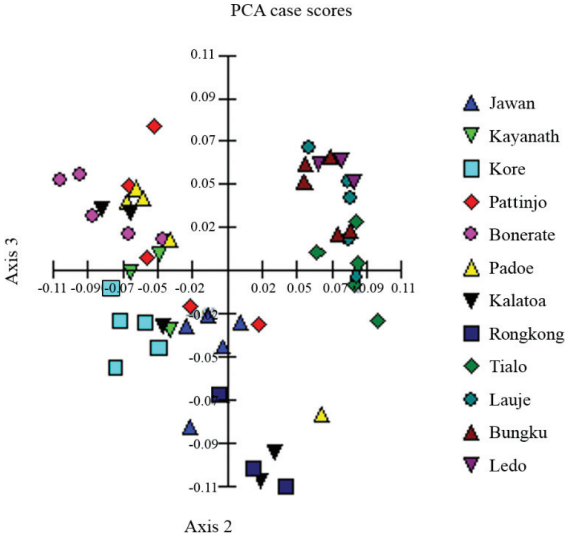

Fig. 4 Results of principal component analysis (PCA) of 12 *Zingiber montanum* populations showing some groupings relevant to results of Unweighted pair-group method of averages analysis

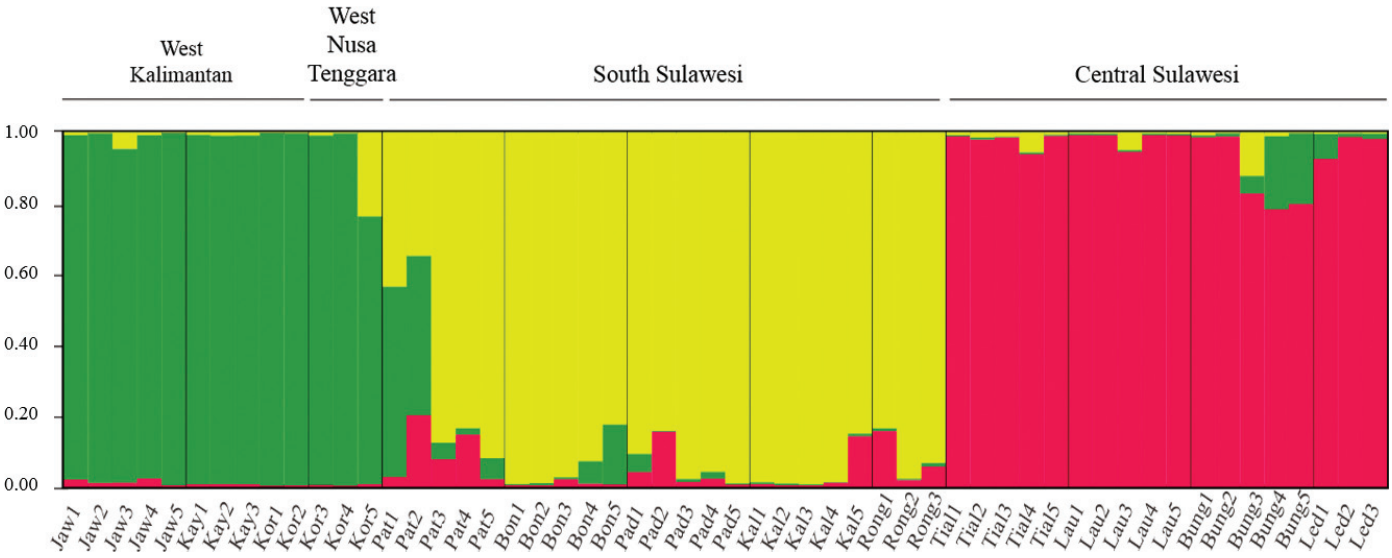

Fig. 3 Population structure of 54 *Zingiber montanum* individuals based on sequence-related amplified polymorphism markers for K = 3 with 20 iterations and a burn-in length of 10,000 and a run length of 100,000 Markov Chain Monte Carlo replications

Z. montanum has been widely used by many ethnic groups in Indonesia but its development in both breeding and genetic improvement is still very limited. The Indonesian government through the Ministry of Health initiated a project to collect *Z. montanum* from many places in Indonesia. Molecular characterization was also conducted as part of the project to identify genetic diversity and genetic structure. These data will provide basic information for the development of *Z. montanum*. Information on genetic structure will be useful for germplasm management. This will make the collecting process easier and avoid duplication of collections for accessions with the same genetic cluster. Knowledge of genetic diversity will also provide a reference for conservation management. In the current study, genetic diversity and genetic structure of *Z. montanum* was successfully analyzed using SRAP markers.

The results of the current study showed that eight combinations of SRAP primers generated 135 polymorphic loci (98.5%) for all Bangle samples. *Z. montanum* is generally cultivated with clones and clonal plant species typically show intermediate genetic diversity (Ellstrand and Roose, 1987). However, the current study showed that *Z. montanum* had high genetic diversity. This was unexpected but has also been reported from other clonal species such as *Caldesia grandis* (Chen et al., 2006), *Z. zerumbet* (Kavitha and Thomas, 2008), and *Kaempferia galanga* (Senjaya et al., 2019). Clonal propagation was expected to degrade the population variations over time. However, clonal populations have shown that they could maintain sufficient amounts of their genetic diversity (Chen et al., 2006; Han et al., 2007; Pantoja et al., 2017). Multiple origins and somatic mutations were found to be liable for maintaining high genetic diversity in clonal populations (Chen et al., 2006). *Z. montanum* in Indonesia is mostly propagated using rhizomes and planted in backyards. There is a possibility of sexual reproduction occurring at some time during the population history. Bengtsson (2003) revealed that even very low sexual recruitment was sufficient to give populations the same allelic variation patterns found in organisms that are fully sexually reproducing. Sexual reproduction also affected genetic differentiation of *Z. montanum*. The AMOVA results showed that intrapopulation variation in *Z. montanum* is higher than interpopulation variation. The pattern of genetic variation in *Z. montanum* was similar to that in species that have mixed or cross-breeding systems (Hamrick and Godt, 1996). A similar pattern was also observed in the clonal species of *Caldesia grandis* (Chen et al., 2006). A lower level of genetic differentiation among populations could also be effected by gene flow per generation (Ingvarsson and Dahlberg, 2019). Considering that sexual reproduction is rare in clonal species, pollen migration or seed dispersal might not allow gene flow. Bangle rhizomes have been traded for a long time so rhizome migration via human intermediaries might be a process that has allowed gene flow among populations. However, the Nm value of *Z. montanum* was less than one, indicating that migration per generation would not be enough to prevent genetic drift. It seemed that occasional sexual reproduction accompanied by somatic mutation was the main reason for the pattern of *Z. montanum* genetic variation.

The samples of *Z. montanum* were collected from gardens (cultivation) at several elevations, mostly at 10–400 m above mean sea level (asl). One sample was collected from almost 1,500 m asl and had the lowest genetic diversity. The highest genetic diversity was from lowland area at approximately 11 m asl. Thus, conservation management is preferably done at low elevations in the common natural habitat for the species. There was no correlation between the habitat and clustering. However, based on the Bayesian approach, the analysis showed a strong geographical correlation with genetic clusters. The Sulawesi population was divided into two clusters where the populations were restricted by the Palu-Koro fault. Populations from the western geological province were grouped in one cluster as were populations from the eastern geological provinces. Sulawesi contains three geological provinces—the Western and Eastern provinces divided by Palu-Koro fault and the Banggai-Sula province comprising the Tokala region, Banggai islands, Butung island and Sula islands (Whitten et al., 1987). The other three populations (Jawan, Kayanath and Kore) were grouped into one cluster. These three populations were separated by the sea from the Sulawesi population, so it is understandable why this population was separated from Sulawesi clusters. However, it was unclear why the Kore population from Sumbawa island had merged into one cluster with the Jawan and Kayanath populations from Kalimantan. It was assumed that rhizome trade and migration via human intermediaries might have caused gene flow between populations and somehow reduced the differentiation between these three populations.

Three genetic clusters showed that the 12 populations of *Z. montanum* originated from the introduction of three different ancestral accessions or originated from the introduction of one accession of ancestors but were cultivated in different environments, so intraspecific variations might have been formed resulting from different selection pressures. Based on the current analysis, it was suspected that the *Z. montanum* populations originated from one accession of an ancestor and experienced local adaptation. It was likely that *Z. montanum* was brought by migrants from India who came through western Indonesia and then spread out to eastern Indonesia. Population divergence in *Z. montanum* might be similar to *Z. zerumbet* that was governed by geographical spread followed by local adaptations and the expansion of genotype-adapted clones (Kavitha and Thomas, 2008). Local adaptation in a population has been reported to strengthen differentiation in selfing *Z. corallinum* which can maintain high genetic diversity (Huang et al., 2019). Unfortunately, there is no detailed information about the introduction of *Z. montanum* to Indonesia, so it is not possible to confirm the origin of *Z. montanum* in Indonesia.

The current results suggested that *Z. montanum* could have maintained high genetic diversity which resulted in population fitness. The high genetic diversity could provide variations which could be the source of genetic improvements in *Z. montanum*. This condition may have led *Z. montanum* populations to have the ability to respond to changes in the environment such as climatic conditions or biotic factors such as novel pests and diseases. Based on the results of the analysis of population structure, three *Z. montanum* clusters were formed, suggesting that germplasm collection for breeding and development as well as protection program should cover individuals representing these three clusters. The Bangle breeding program in Indonesia has been carried out by the Indonesian Medicinal and Aromatic Crops Research Institute and has produced nine accessions of superior plant varieties (personal communication). However, these varieties have not yet been released and the minimum cultivation technology means that the people harvest Bangle from the fields and from gardens on a limited scale. The breeding program is still ongoing and information about the genetic diversity produced in the current study and the germplasm that has been collected may contribute as donors to the Bangle breeding program. In addition, this research can also provide some information on the suitable environment for growing the Bangle plant. This study provided initial data of genetic diversity and genetic structure patterns for further utilization and development of *Z. montanum*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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