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GENETIC DIVERSITY OF BAK-KALA (*ETLINGERA ELATIOR* (JACK) R.M. SM.) IN ACEH PROVINCE, INDONESIA

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SUMMARY

Torch ginger – *bak-kala* (*Etlingera elatior* (Jack) R.M.SM.) already has been proven an effective traditional medicine by tribes in Aceh Province, Indonesia. Solid primary evidence for the torch ginger, *bak-kala's* medicinal properties include the healing effects experienced by the traditional tribes in Aceh, when treated for illnesses, such as, cough, fever, and sprains. Likewise, it has been proven to be a use for food ingredients, i.e., vegetables and spices to enhance the taste of food. Much of the available documented information focused on its biochemical and pharmacological aspects. Even though the utilization of *bak-kala* resources is mainly at the level of diversity, there is no information on its genetic diversity in Aceh. The high intensity of *bak-kala* utilization is not simultaneous with information on its genetic diversity. On the other hand, many uses of *bak-kala* are specific for certain accessions with particular benefits. The study aimed to create a classification system for *bak-kala* variations based on inter-simple sequence repeats (ISSR) markers and to provide information on the genetic diversity of *bak-kala* in Aceh Province. The 35 accessions were divided into eight populations based on the geographical locations from which they were collected. Data were recorded and analyzed using 10 ISSR primers with 77 total bands. The molecular characters divided the accessions into four major groups. As revealed by expected heterozygosity (He), genetic variability among the population showed that the Simeulu population possessed a greater level of variability than other populations. The results of the analysis of molecular variation (AMOVA) showed that the genetic variation within the population was higher (60%) than the genetic variation among populations (40%). The studies can be used to plan conservation strategies, optimal utilization of the species, and crop improvement programs in the future.

Keywords: *Bak-kala* (*Etlingera elatior*), diversity, genetic variability, germplasm, ISSR markers, population structure

Key findings: The genetic diversity of *bak-kala* accessions collected from eight populations in Aceh Province, Indonesia was studied. The molecular characters divided the *bak-kala* accessions into four major groups. This study provides pioneering data for the future use and conservation of *bak-kala* (*E. elatior*) in Sumatra, especially in the Aceh Province.

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INTRODUCTION

Bak-kala (*Etlingera elatior* Jack. R.M.Sm.), a member of the Zingiberaceae family, has the potential to be developed as an essential herb with various benefits and is rich in phytochemical content, such as, flavonoids, terpenoids, and phenols (Saudah *et al*., 2021); has the ability as antimicrobial, antioxidant, and antitumor (Juwita *et al*., 2019); and can be categorized as an essential ingredient of pharmacological agents. *E. elatior* is a medicinal plant endemic to the Indo-Pacific region (Ud-daula *et al*., 2019). *Bak-kala* already has solid primary evidence in treating illnesses of traditional tribes in Aceh, such as, cough, fever, and sprains, and is used as food ingredients. i.e., vegetables and spices to enhance the taste of food (Saudah *et al*., 2021).

The high intensity of *bak-kala* utilization is not simultaneous with the information on its genetic diversity. On the other hand, many uses of *bak-kala* are specific for certain accessions with particular benefits, such as, green or yellow fruit used for the post-delivery concoction "*makjun sejuk*", while the red fruit is used for anti-hypertensive. This is related to its phytochemical compounds and is closely correlated with these morphological variations. For flower color in *bak-kala*, variations were found, namely, light pink, pink, red, and white. The fruit was found with only two color variations, red and yellow-green. All parts of the plant are considered medicinal regardless of the flower color variations.

To develop *bak-kala* as a pharmacological agent, the data need to be accurate regarding its genetic diversity. The genetic diversity of *bak-kala* is important because it gives species a better chance of survival. However, genetic diversity can be lost when the population gets smaller and isolated, which decreases a species' ability to adapt and survive.

It is feared that the uncontrolled use of *bak-kala* germplasm will cause its availability in nature to decrease. Moreover, indigenous knowledge on treatment is getting scarce. Based on the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List, *E. elatior* is in the Data Deficient (DD) status whose extinction risk depends on the distribution and level of the population (Encyclopedia of Life, 2018; Abduova *et al*., 2022). Studies to evaluate genetic diversity are urgently needed before *bak-kala* germplasm disappears.

The combined differences in the DNA of all individuals in a species make up the genetic diversity—the overall diversity in the DNA between the individuals of a species (Minter *et al*., 2021). Genetic diversity causes individuals to have different characteristics. Genetic diversity can be evaluated within and between populations at the molecular level by DNA analysis (Guliyef *et al*., 2018). Various marker-based techniques can be used to characterize the genetic variation, i.e., the Inter Simple Sequence Repeats (ISSR) marker. ISSR markers produce highly polymorphic band fragments and have high efficiency and accuracy for studying genetic diversity between taxa at the intraspecies level (Lu *et al*., 2011; Jalil *et al*., 2021). ISSR markers can determine a genetic similarity between 57 accessions of *E. elatior* in the Malaysian Region (Ismail *et al*., 2019). Currently, there is no documented information regarding the genetic diversity of *bak-kala* in Aceh Province, Indonesia.

Information on the genetic diversity of future generations is very important for breeding and conservation purposes, to determine the relationship between individuals and their population structure. In addition, genetic diversity data can be used as a factor to select accessions with high genetic variation to produce superior accessions for the standardization of medicinal plants. Studying genetic diversity in *bak-kala* will be beneficial in completing the identity of accessions collected from different locations. The study aimed to create a classification system for *bak-kala* variations based on inter-simple sequence repeats (ISSR) markers and to provide information on the genetic diversity of *bak-kala* in Aceh Province.

MATERIALS AND METHODS

Plant material and procedure

Thirty-five *bak-kala* (*E. elatior)* were collected from Aceh Province, Indonesia. The explored areas for the samples collected were eight regency, namely, Gayo Lues, Aceh Tenggara, Aceh Selatan, Nagan Raya, Aceh Tamiang, Simeulu, Pidie, and Aceh Tengah (Figure 1 and Table 1). The selection of research locations was based on the distribution center of *bak-kala* plants in Aceh. Each area was considered one population because it has a different habitat type from other populations, so there were eight populations studied.

Molecular analysis

The DNA was extracted by using Viogene DNA Mini Kit (Plant). The DNA was amplified using 10 ISSR primers (Table 2). Polymerase chain reaction (PCR) was carried out in 15 µl volume, which consisted of 1 µl DNA (0.5-2.0 ng), 1 µl primer ISSR, 1.25 µl DreamTaq Buffer 10x, 1.25 µl dNTP Mix, 2 mM, DreamTaq DNA Polymerase 0.05 µl, and 7.45 µl free nuclease water. The PCR program consisted of an initial denaturation at 94 °C for 2 min; followed by 35 cycles of denaturation at 93 °C for 30 s; annealing at 48 °C–54 °C for 30 s, with an extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min, followed by cooling at 15 °C.

The amplified PCR products were separated by electrophoresis on 1% agarose gel in TBE IX buffer and stained with ViSafe Green Gel Stain (10000x in water). The results were recorded under the blue light Accuris Smart Blue Transilluminator, and documented using Smart Doc Enclosure with the Smartphone.

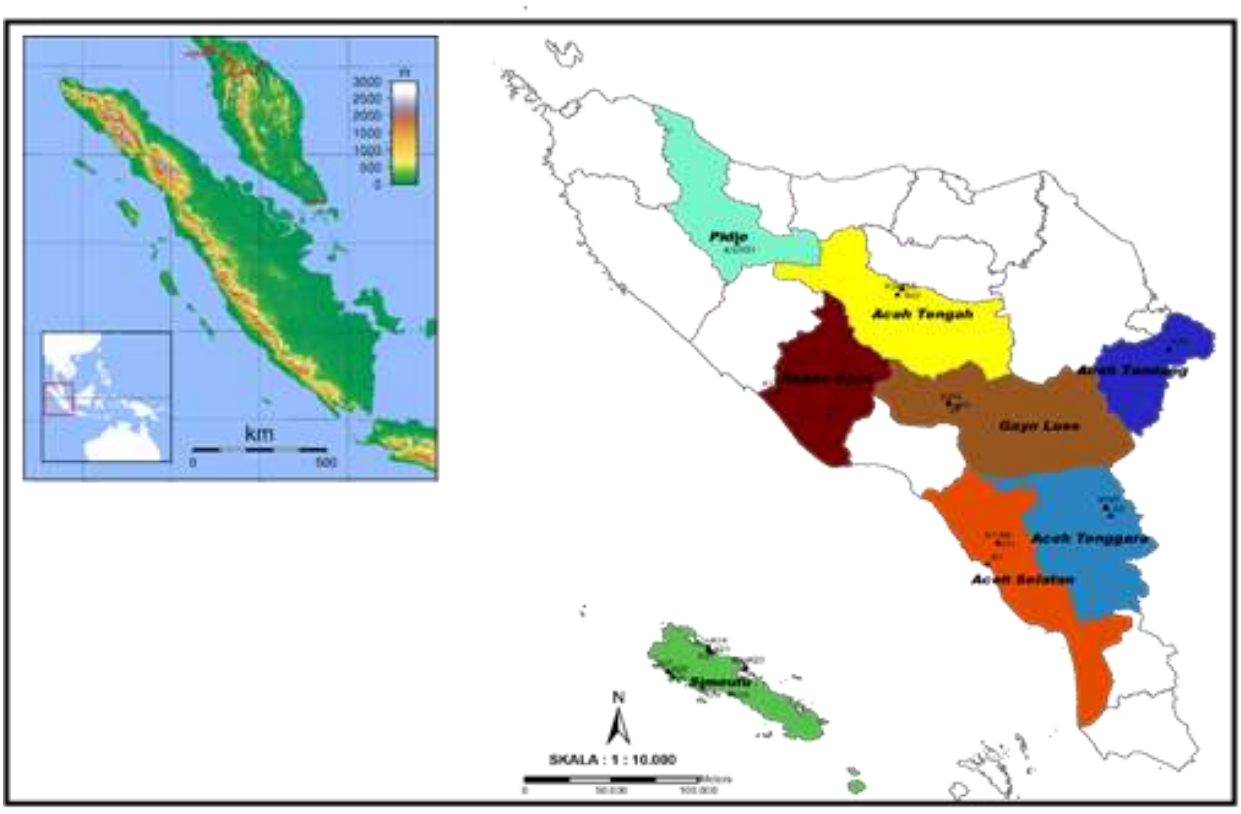

Figure 1. Map of locations for collecting *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.) accessions in Aceh Province, Indonesia.

Table 1. Different accessions of *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.) in Aceh Province, Indonesia.

Populations/coordinate	Location	Total	Code sample
Gayo Lues	Padang, Trangun Districts	4	K1,K2
(97°4'10.379"E	Makmur Jaya, Trangun Districts		K3
4°3'28.033"N)	Rime Jaya, Trangun Districts		K4
Aceh Tenggara	Perjuangan IV, Babussalam Districts	3	K5
(97°48'36.000"E	Kumbang Jaya, Bandar Districts		K6
3°29'18.700"N)	Kampung Baru, Bandar Districts		K7
Aceh Selatan	Air Pinang, Tapak Tuan Districts	4	K8
(97°14'47.629"E	Alue Keujurun, Kluet Tengah Districts		K9, K10, K11
3°14'4.163"N)			
Nagan Raya	Alue Billie, Darul Makmur Districts	3	K12
(96°30'46.393"E	Simpang Peut, Kuala Districts		K13
3°59'27.398"N)	Nigan, Seunagan Districts		K14
Aceh Tamiang	Medang Ara, Karang Batu Districts	2	K15, K16
(98°4'28.700"E	Titolor, Teluk Dalam Districts		
4°20'27.800"N)			
Simeulu	Sinar Bahagia, Simeulu Barat Districts	11	K17, K18, K19, K20
(95°59'117"E	Karya Bakti, Salang Districts		K21, K22, K23, K24
2°47'187"N)	Panton Laweh, Salang Districts		K25, K26, K27
Pidie	Leutung, Mane Districts	4	K28, K29
(96°4'59.463"E	Mane, Mane Districts		K30, K31
4°51'48.526"N)			
Aceh Tengah	Jagok Meluem, Kebanyakan Districts	4	K32
(95°3'28.901"E	Aceh Kemili, Bebesen Districts		K33, K34, K35
5°42'30.407"N)			

Table 2. The primers used in this study.

Primers	Sequences	Annealing temperature (°C)
UBC-811	$(GA)_8C$	54
UBC-891	$HVH(TG)_7$	54
UBC-808	$(AG)_8C$	52
UBC-809	$(AG)_8G$	52
UBC-859	$(TG)_8RC$	48
UBC-888	$(AC)_8G$	50
UBC-847	$(CA)_8RC$	50
UBC-841	$(GA)_8YC$	52
UBC-834	$(AG)_8YT$	48
UBC-868	$(AC)_8YT$	48

Data analysis

The banding pattern obtained by each ISSR primer was scored using the Gel Pro Analyzer program 3.1 version. The polymorphic band is scored zero (0) if there is no band and one (1) if there is a band in the same position. The binary data were used to calculate the genetic similarity matrix using the SIMQUAL (Similarity for Quality Data) procedure. Based on the genetic similarity index, cluster analysis was constructed using SAHN (Sequential Agglomerative Hierarchical and Nested Clustering) procedure. The similarity coefficient with the SM (Simple Matching) method and the clustering with the Unweighted Pair Group Method Arithmetic Average (UPGMA) method were performed using the NTSYS pc version 2.01 (Numerical Taxonomy and Multivariate System) (Rohlf, 2000). Analysis of molecular variance (AMOVA) and genetic diversity was analyzed by using GenAlex 6.5 (Peakall and Smouse, 2012).

Table 3. ISSR primer sequences and DNA amplification product band profile *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.)

Primers	Fragment size (bp)	Band total	Polymorphic band total	Percentage polymorphic band (%)
UBC-811	200–1000	12	11	91.66
UBC-891	200–1200	7	6	85.71
UBC-808	250–600	8	7	87.5
UBC-809	250–500	6	4	66.66
UBC-859	250–750	4	2	50
UBC-888	250–1500	9	6	66.6
UBC-847	100–600	7	6	85.71
UBC-841	100–750	6	4	66.66
UBC-834	200–750	7	6	42.85
UBC-868	300–2000	11	10	90.90
Total Average		77	62	73.425

RESULTS

ISSR polymorphism

A total of 10 primers have been obtained, which exhibit a clear and polymorphic banding pattern. Ten ISSR primers were used to identify the genetic diversity of 35 *bak-kala* accessions. The pattern, number, and band size varied between accessions and primers. The amplification results obtained 77 bands, and 62 bands are polymorphic, with the percentage of each primer ranging from 50%–91.66%, having an average of 73.425%. This indicates the existence of genetic diversity among *bak-kala* accessions from Aceh. The number of bands amplified in each primer ranged from 4–12 with lengths ranging from 100–2000 base pairs (bp) (Table 3).

The amplification results of the UBC-868 primer obtained the band with the largest size of 2000 bp, while the UBC-841 and UBC-847 primers obtained the band with the smallest size of 100 bp. The bands that always appear from all the primers used are 250 bp and 500 bp. This shows that the ribbon that always occurs is the ribbon that is generally found in *bak-kala*. The number of bands produced by the UBC-859 primer is the least (four bands), while the number of bands produced by the UBC-811 primer is the most (12 bands).

Phenetic analysis of *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.)

The grouping, based on the 77 bands of primary amplification of ISSR using the UPGMA method and simple matching coefficient. grouped 35 *bak-kala* accessions into four groups (A, B, C, and D) at a similarity coefficient of 58%–100% (Figure 2). Group A consisted of four accessions collected from the Gayo Lues population and five accessions from Simeulu. Group B consisted of three accessions from the Aceh Tenggara population, four accessions from the Simeulu, four accessions from the Pidie, and four from Aceh Tengah. Group C consisted of two accessions from the Aceh Tamiang population and two accessions from the Simeulu. Lastly, group D consisted of four accessions from the population of South Aceh and three from Nagan Raya.

In the dendrogram, *bak-kala* tends to group based on the origin of the accessions taken, except for the Simeulu populations in three accession groups (A, B, and C). This showed that the genetic variation within the Simeulu population was very high. It does not cluster only in one group but also clusters with accessions from other genetically similar populations. Simeulu Regency is an archipelago consisting of large islands that are also surrounded by small islands separated from the mainland of Sumatra Island, Indonesia. The high similarity value within the population or between populations from the exact location can be due to habitats and environmental conditions (Wang, 2020). Ismail *et al*. (2019) reported no correlation between clustering based on molecular characteristics of ISSR markers and the geographic location of origin for collecting torch ginger (*E. elatior*) in Malaysia.

The populations of Aceh Tenggara and Aceh Selatan have the highest genetic distance value (0.426), while between Pidie and Simeulu populations have the lowest genetic distance value (0.141) (Table 4). The relatively small genetic distance value indicates the closeness of accessions from the Pidie and Simeulu populations. This can occur due to

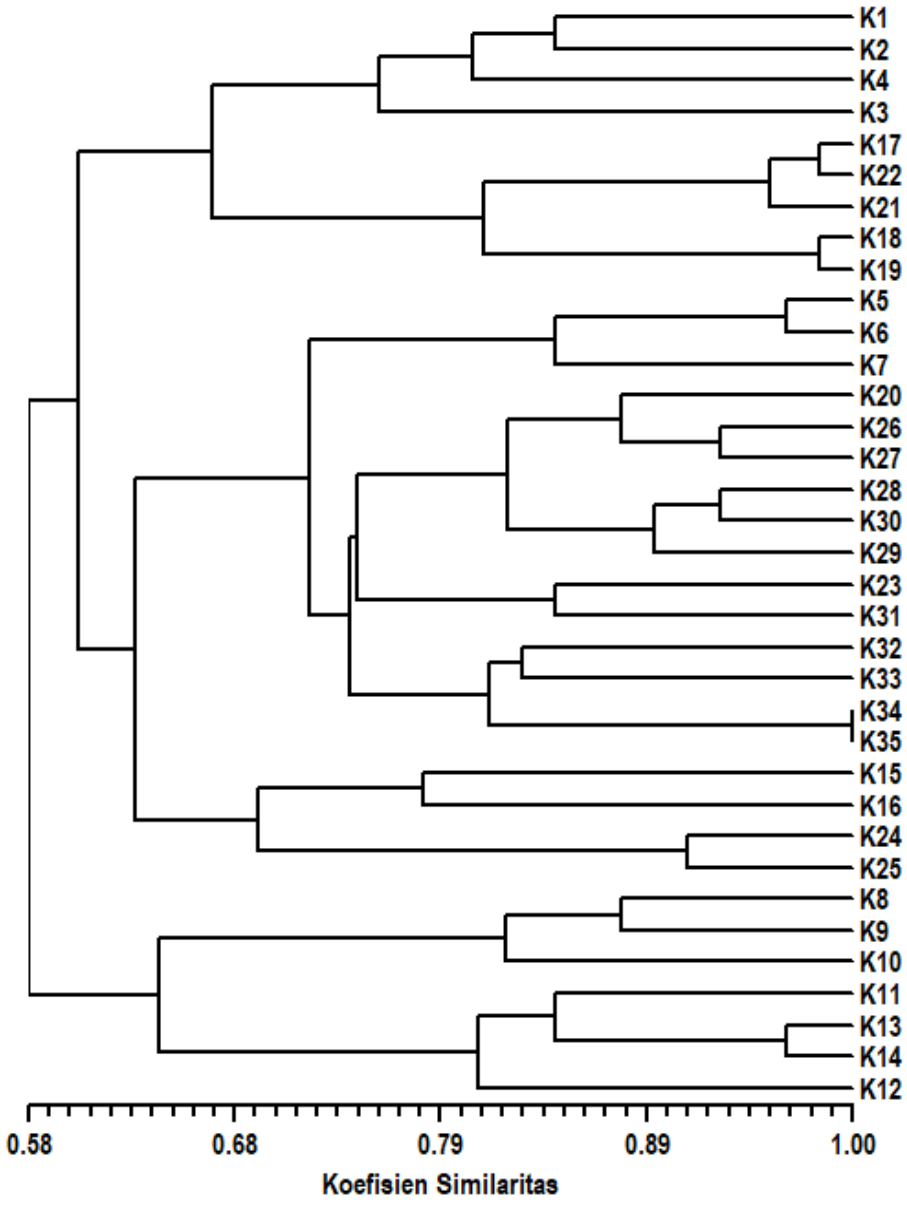

Figure 2. Dendrogram of 35 *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.) accessions with unweighted pair group method with arithmetic mean (UPGMA) method based on 77 ISSR bands.

Table 4. Pair-wise population matrix of Nei's genetic distance.

Populations	Gayo Lues	Aceh Tenggara	Aceh Selatan	Nagan Raya	Aceh Tamiang	Simeulu	Pidie
Aceh Tenggara	0.190						
Aceh Selatan	0.218	0.286					
Nagan Raya	0.353	0.426	0.182				
Aceh Tamiang	0.373	0.322	0.268	0.179			
Simeulu	0.206	0.244	0.165	0.224	0.175		
Pidie	0.275	0.142	0.311	0.333	0.287	0.141	
Aceh Tengah	0.275	0.153	0.267	0.328	0.287	0.144	0.881

seed dispersal and domestication by humans so that the genetic diversity of the two populations is similar. Humans essentially influence the distribution directly by transporting individuals and indirectly by altering natural landscapes and vectors (Bullock *et al*., 2018).

Genetic diversity and population structure of *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.)

The results of the AMOVA showed that the genetic variation within the population was higher (60%) than the genetic variation among populations (40%). Genetic diversity in the population indicates that the interpretation of the *bak-kala* is more influenced by the variation within the population (Figure 3). This suggests that sufficient donors provide the emergence of new alleles in the population to give rise to high diversity (Ozawa *et al*., 2013).

Genetic diversity is the basic information for accession selection and breeding. In this study, data on the genetic diversity of *bak-kala* in Aceh Province was obtained by analyzing variations in the number of alleles (Na), the number of effective alleles (Ne), Shannon information index (I), and diversity (H). Parameters of genetic diversity of *bak-kala* varied in eight populations (Table 5).

The observed Na values ranged from 1.093 to 1.324, while the Ne values ranged from 0.844 to 1.377. Shannon information index varied from 0.088 to 0.272, and the genetic diversity varied from 0.081 to 0.185. These results indicate that the genetic diversity of *bak-kala* is relatively low. Genetic diversity is declared high if it has a Shannon index value and diversity close to 1 (Morris *et al*., 2014; Silva *et al*., 2015; Yun *et al*., 2020). The percentage of polymorphic loci (% P) varied between 16.68%–49.35%, with a mean of 27.92%. The value of the Shannon information index (I) and diversity (h) are two important things that can indicate the level of genetic diversity (Liu *et al*., 2019).

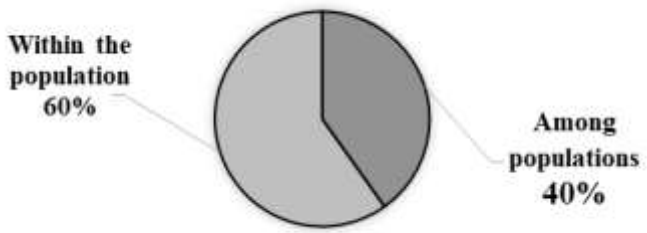

Figure 3. Percentage of molecular variation within and among *bak-kala* (*Etlingera elatior* Jack. R.M.Sm.) populations in Aceh Province, Indonesia.

Table 5. Genetic diversity parameters in *Etlingera elatior* Jack. R.M.Sm. accessions as detected by ISSR markers.

Populations	N	Na	Ne	I	H	P (%)
Gayo Lues	4	1.161	0.935	0.149	0.098	28.57
Aceh Tenggara	3	1.153	0.844	0.116	0.081	18.18
Aceh Selatan	4	1.244	1.078	0.206	0.139	37.66
Nagan Raya	3	1.093	0.909	0.088	0.058	16.68
Aceh Tamiang	2	1.165	1.013	0.141	0.097	23.38
Simeulu	11	1.324	1.377	0.272	0.185	49.35
Pidie	4	1.163	1.013	0.133	0.090	23.38
Aceh Tengah	4	1.228	0.974	0.169	0.120	25.97
Rata-rata		1.191	1.018	0.159	0.108	27.92

Na: Number of observed alleles, Ne: Effective number of alleles, I: Shannon's information index, H: Diversity index, P: Percentage of polymorphic loci

DISCUSSION

Thirty-five *bak-kala* (*E. elatior)* were collected from Aceh Province, Indonesia. The explored areas for the samples collected were eight distribution centers of *bak-kala* plants in Aceh. Human activities influence diversity in the archipelago through plant dispersal. *Bak-kala* plants found in Simeulu Regency are probably the result of plant dispersal and domestication by humans. The genetic diversity of the location is the same and has similarities with other populations. Plant dispersal is critical for gene flow among plant populations. It affects genetic diversity in plants of the family Bignoniaceae (*Adenocalymma schomburgkii* (DC.) LG Lohmann, *Anemopaegma paraense* Bureau & K. Schum and *Bignonia aequinoctialis* L.) through plant dispersal (Nazareno *et al*., 2021).

Identifying plant genotypes using molecular markers is more effective than morphological markers because it allows direct access to inherited genetic substances. On the other hand, molecular markers sometimes cannot distinguish phenotypically different plants so specific accessions cannot be easily detected by their phenotype (Ismail *et al*., 2019).

The highest similarity coefficient values were in accessions K34 and K35 (100%) (Figure 2). This indicates that the two accessions are genetically very similar. The similarity index can identify genetically identical accessions. Based on the morphological characteristics, these two accessions have flowers with white bracts and are also different from other accessions, namely, red or pink. A high similarity value indicates a lower level of similarity of genetic characteristics. Genetic distance is a measure of genetic variation between populations or at a certain taxon level, which is calculated based on the accumulation of differences in alleles per locus (Sere *et al*., 2017). The smaller the genetic distance between individuals in a population indicates that the population is more uniform (Nei and Kumar, 2000).

The similarity in climate can lead to genetic similarity (Ni *et al*., 2018). Similarities in habitat and environmental conditions can cause the low value of the genetic distance between two different locations. The similarity of environmental conditions or geo-climatic causes plants to not adapt, so they do not cause significant genetic changes. As a result, they are genetically similar even though the accessions studied are from different locations (Ni *et al*., 2018; Gunawan *et al*., 2019). Based on Ratnam (2009), the occurrence of natural selection and sexual mating processes originating from the same habitat in an area will cause individuals in the population to tend to have the same or similar genes and phenotypes.

The higher variation in genetic diversity between populations occurs in self-pollinating plants (Wright, 1965). The type of cross-pollination in *bak-kala* plants causes gene flow between different individuals, resulting in diversity. Information on diversity among and within populations is useful in the selection of accessions and determination of conservation measures. Population crossbreeding in a large population causes a larger gene pool. A larger gene pool provides genetic variation and adaptability to survive being selected by the environment (Ratnam, 2009). Information on genetic diversity is essential in the management, utilization, and conservation of available germplasm by assessing the distribution of its diversity in various populations (Tabin *et al*., 2016; Ismail *et al*., 2019).

The population of Nagan Raya has a low diversity value and Shannon index due to two things, namely, the environmental conditions of the place of origin of the accession and the number of individuals collected (Yang *et al*., 2016). The accessions found in the Nagan Raya population have habitat types that tend to be the same, which is the west coast of Sumatra. They may come from elders with similar morphological or genetic characteristics, causing genetic variations based on the ISSR band pattern of the two accessions from Nagan Raya. In addition, the number of accessions obtained in Nagan Raya is also small (three accessions).

Suppose accession is obtained in a small population. In that case, the number of alleles observed is also small so that the diversity value will also be low and have fewer allele differences in the gene pool (Ratnam, 2009; Yang *et al*., 2016). Thirty-five *bak-kala* accessions collected from eight districts in Aceh Province had low Shannon index values (I) and diversity values (h). This research studied diversity under a species taxon (intraspecies), so it has low genetic variation. The genetic diversity of 57 accessions of *E. elatior* in Malaysia, based on the ISSR marker. was also in the low category (0.14) (Ismail *et al*., 2019). Genetic diversity was low in *bak-kala* from Aceh, making it necessary that *bak-kala* germplasm needs to be conserved to maintain its existence in Aceh.

The ISSR markers were successfully implemented to define the genetic diversity and population structure in *bak-kala* populations. The recent results indicated that ISSR markers can be used and helpful in recognizing the genetic diversity in *bak-kala* (*E. elatior*), and the study also recorded the highest genetic diversity and polymorphisms in *bak-kala* populations. Information on genetic diversity patterns is essential for designing conservation, management, and sustainable use strategies for plant species (Chung *et al*., 2013; Liu *et al*., 2019; Yun *et al*., 2020). Efforts that need to be made, so that the genetic resources of the future generations continue to grow, are by using an ex-situ conservation approach.

Germplasm resources can be propagated by artificial cross pollination from genetically different accessions to produce a heterozygous seedling. The importance of the conservation of *bak-kala* in Sumatra is due to its enormous potential for pharmacology. This plant is a medicinal plant inherited from the ancestors in the tribes of Aceh. Before the knowledge on its use and existence is lost in nature, it is necessary to study its potential as a drug base material. This study provided pioneering data for the future use and conservation of *bak-kala* (*E. elatior*) in Sumatra, especially in the Aceh Province.

CONCLUSIONS

The molecular characters divided the accessions of *bak-kala* (*E. elatior*) into four major groups. As revealed by expected heterozygosity (He), genetic variability among the populations showed that the Simeulu population possessed a greater level of variability than other populations. The results of the analysis of molecular variation (AMOVA) showed that the genetic variation within the population was higher (60%) than the genetic variation among populations (40%). This study can be used to plan conservation strategies, optimal utilization of the species, and crop improvement programs in the future.

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