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Molecular authentication of *Etlingera* spp. (Zingiberaceae) in Aceh Province, Indonesia using DNA barcoding

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Abstract. *Saudah, Zumaidar, Darusman, Firmawati, Rislim DI, Juliantari E. 2022. Molecular authentication of* Etlingera spp. *(Zingiberaceae) in Aceh Province, Indonesia using DNA barcoding. Biodiversitas 23: 3976-3983. Etlingera* spp. or locally known as *Etlingera* spp. (Acehnese) already has strong basic evidence in the treatment of traditional tribes in Aceh such as cough medicine, fever and sprains, and used to food and spices. *Etlingera* spp. used in various places varies based on morphological characteristics. On the other hand, for the development of phytopharmaceutical, the use of *Etlingera* spp. as plant herb requires authentication about the names. This study aimed to authenticate *Etlingera* spp. from Aceh using DNA barcoding. Nuclear ITS and trnL-F DNA sequences were used to authenticate among species of *Etlingera* spp. In this study different species of *Etlingera* spp. can be differentiated effectively by comparing the DNA barcoding regions. ITS sequence data can identify two different species of *Etlingera* (*E. elatior* and *E. hemisphaerica)*. The two identified *Etlingera* spp. species can be used as important information for further research related to their pharmacological studies. One marker and combined analysis of ITS and *trn*L-F data proved that the *Etlingera* spp. is a monophyletic group and provided phylogenetic information in closely related species. The intraspecific and interspecific variation of ITS markers were more discriminatory regions, while trnL-F IGS were of lower divergences. Our findings indicated that DNA barcoding is efficient and powerful to authenticate herbal medicine *Etlingera* spp..

Keywords: Chloroplast DNA, herbal medicine, internal transcribed spacer, ITS, phylogeny, *trn*L-F intergenic spacer

INTRODUCTION

Etlingera spp. or locally known as *bak kala* (Acehnese) is a plant in the ginger family (Zingiberaceae) and the tribe Alpinieae (Burtt and Smith 1986; Smith 1986). It is native to Malaya, Indonesia and Thailand and is widely distributed in Southeast Asia (Ravindran 2017). *Etlingera* spp. has involucral bracts with dark red, red, pink and white color. The white bracted form is extremely rare and occurs in the wild. There are two types of flowers, one with white-eyed labellum (cultivated) and the other with yellow-eyed labellum which is considered a wild type (Larsen 1999; Ibrahim et al. 1999). *Etlingera* spp. already has strong basic evidence in the treatment of traditional tribes in Aceh as medicinal plant (Jackie et al. 2011), vegetables, and spices. Stems and fruit are the most widely part of plant used for medicinal herbs. Processing was carried out by pounding, then squeezing and grinding either singly or in a mixture used by drinking and rubbing. The flower buds and fruit are the most widely part of plant as food ingredients (Saudah et al. 2021a; Saudah et al. 2021b). *Etlingera* has several species, both growing wild in the forest and in cultivation. The use of the *Etlingera* species in the Aceh is limited to one species, while the other *Etlingera* relatives have not been explored for their potential. The diversity of cultures and languages in Aceh Province, Indonesia causes diversity in the mention of local names for different species, thus causing local knowledge about the alleged use of *Etlingera* spp. to also differ. Inaccuracy in species identification, environmental factors and developmental stages can affect morphological characters among accessions of *Etlingera* spp..

In addition, the use of *Etlingera* spp. as traditional medicine requires authentication or clarity about the names and variations of this *Etlingera* spp. Proof of this species similarity is required for data accuracy. Identification and authentication of the plants used for the production is, therefore, an elementary and critical step at the beginning of an extensive quality assurance process. The use of a wrong herb as medicine may prove ineffective or even worsen the condition (Chaudhary et al. 2014). There are three techniques to perform proof or authentication, namely

through identification of morphology and anatomy as a whole, chemical profiles, and DNA barcoding. It is important to authenticate the name of the *Etlingera* spp. for further development. Therefore, it is necessary to identify *Etlingera* spp. plants are using efficient methods such as DNA barcoding. The research results obtained will provide new information to the public so that they can maximize their use.

DNA barcoding has upper hand in identifying medicinal plants compared to other markers. DNA marker is accepted and reliable method for species identification based on nucleotide diversity of conserved sequences (Su et al. 2010). As the DNA sequences are highly specific, they can be identified with the help of the known molecular markers which can find out a particular sequence of DNA from a group of unknown. These barcodes are used for phylogenetic analysis, genetic diversity and species discrimination in different organisms (Biswas and Biswan 2014). This system not only helps to classify the organisms but also reveals genetic information for species ancestral inheritance and flagging of new species (Wen-Liu et al. 2019; Zhu et al. 2017; Amandita et al. 2019).

The use of morphological features still has shortcomings in explaining kinship and evolution because morphological characteristics are influenced by environment. Based on morphology, white flowers are only found in highland areas, while in lowlands the flowers are pink to red. In contrast to more consistent DNA barcoding in search of evolutionary traces. In addition, the use of molecular markers will eliminate characteristic contradictions that are generally found in pattern grouping using morphological characteristics. The topology of the phylogenetic tree uses molecular markers to provide greater clarification than grouping by characteristics morphology even though it has a low bootstrap value (Zhang et al. 2015; Mishra et al. 2016).

Molecular analysis in this study, we used Internal Transcribed spacer (ITS) sequence (nuclear DNA) and *trn*L-F intergenic spacer (IGS) sequences (chloroplast DNA). These two markers were used to make comparison between them. Whether they are complementary or contradictory or even strengthening each other to shape the best phylogenetic tree. We hope combination of these two markers could show better results.

Correct identification of the plants that make up medicine is a prerequisite and fundamental to all fields of medicine and science. From the authentication results, the community especially in Aceh Province can cultivate it for *Etlingera* spp. that have the potential as medicine. Thus, authentication of the botanical sources of plants taken for research or medicinal use is a must to achieve satisfactory results and also to maintain the efficacy and therapeutic properties of the preparations in which these plants are used (Ganie et al. 2015). Therefore, the study was conducted to authenticate the name of the *Etlingera* spp. and to study the relationship among *Etlingera* spp. accessions in Aceh, Sumatra. The molecular approach also has benefit to find the best phylogenetic tree model which will be useful in conservation and cultivation strategies for developing *Etlingera* spp. in Sumatra, Indonesia as a potential source of herbal medicine.

MATERIALS AND METHODS

Plant materials and extraction DNA

Nine accessions of *Etlingera* spp. were collected from Aceh Province, Indonesia. The explored areas for the samples collected were eight districts including Gayo Lues, Aceh Tenggara, Aceh Selatan, Nagan Raya, Aceh Tamiang, Pidie, Aceh Tengah. The selection of research locations was based on the distribution center of *Etlingera* spp. plants in Aceh (Figure 1). As much as 100 mg *Etlingera* spp. leaves were extracted using Viogene DNA Mini Kit (Plant). DNA was stored in 100 µL of TE solution (1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, distilled water).

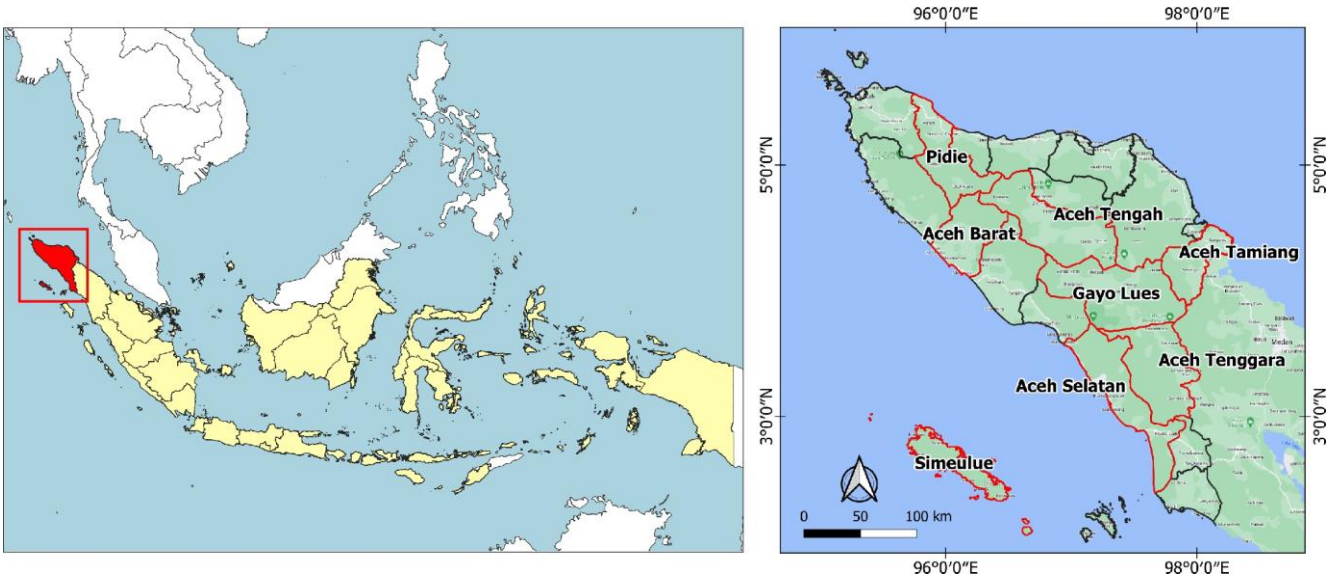

Figure 1. Map of locations for collecting *Etlingera* spp. accessions in Aceh Province, Indonesia

Amplification and sequencing

The DNA was amplified with the PCR technique. The genomic DNA was amplified using universal primer internal transcribed spacer (*ITS*) ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R(5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) (entire ITS regions, nrDNA) and primer *trn*L-F intergenic spacer E (GGTTCAAGTCCCTCTATCCC) and primer F (ATTTGAACTGGTGACACGAG) (Small et al. 2004) (entire *trn*L-F intergenic spacer region, cpDNA).

The Reaction mixture (50 µL) contained 0.5-2.0 ng genomic DNA, 10 pmol of each primer, 1 unit DreamTaq™ Hot Start Green PCR Mix (DreamTaq Hot Start DNA polymerase, 2X DreamTaq Green Buffer, 0.4 mM dNTPs and 4mM $MgCl_2$), and free nuclease water. PCR with thirty five cycles were conducted using miniPCR v1.6 thermal cycler compatible with windows under following profiles: 95ºC for 3 m, 95ºC for 30 s, 51ºC (ITS regions) and 52ºC (*trn*L-F intergenic spacer region) for 60 s, 72ºC for 1 m, 72ºC for 5 m.

The amplified PCR products were separated by electrophoresis on 1.2% agarose gel in TAE 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 were documented using Smart Doc Enclosure with the Smartphone. The sequencing was carried out at First Base Laboratories, Malaysia.

Phylogenetic analysis

To identify similar sequences used BLAST in the National Center for Biotechnology Information (NCBI). DNA sequences of *Etlingera* spp. were aligned with ClustalW Multiple Allignment in Molecular Evolutionary Genetics Analysis (MEGA) software (Small et al. 2004). Phylogenetic tree construction used the Maximum Parsimony (MP) and Neighbor joining (NJ) was carried out with the program of PAUP* version 4.0 (Swofford et al. 2002; Tamura et al. 2013). The evolutionary model in this study used NJ analysis with 1000 bootstrap replications to test the strength of each branch of phylogenetic tree obtained with a frequency of >50%. Bootstrap support category consisted of strong (>85%), moderate (70%-85%), weak (50%-69%), or poor (<50%) (Hasegawa et al. 1985; Danyan et al. 2021).

RESULTS AND DISCUSSION

Maximum parsimony (MP) analysis

The result of maximum parsimony analysis (MP) based on the sequence data of ITS region and *trn*L-F intergenic spacer are summarized in Table 1. The difference in the number of *Etlingera elatior* sequences analyzed in this study based on the ITS marker was very low, only 3% of the total sequence. Although low but it provides very accurate information in grouping In this study, the character of ITS and *trn*L-FIGS sequence give strong implications in *Etlingera* phylogenetic grouping (Figures 2, 3 and 4).

The combination analysis is presented in parsimony cladogram with the strict consensus tree (Figure 4). The combined molecular data set included 1154 characters with 32% parsimony informative characters, parsimonious trees with a length of 905 steps, CI of 0.89 and RI of 0.91. This indicates that the resulting phylogenetic tree is quite strong and has consistent branching which is indicated by the consistency index value above 50%. The index value is used to measure the consistency of a tree. The phylogenetic construct is by looking at the consistency index (CI), namely the consistency index used as a parameter of a tree topology (Farris 1989). Trees that do not have homoplasia characteristics are indicated by the value of CI=1, so the better a phylogenetic tree is characterized by a higher index value close to 1. The retention index (RI) is a stability index that is the proportion of synapomorphic characteristics that can be accepted as true synapomorphic characteristics. Therefore, the greater the value of RI (RI=1) then the character used to generate a phylogenetic tree is consistent (Klingenberg et al. 2010). The most parsimony tree can be obtained if it has a good consistency and retention index value with a bootstrap of 1000 times (Soltis and Soltis 2003).

Parsimony informative sites help reconstruct parsimony phylogenetic trees, while parsimony-uninformative characters do not provide information about variations but specific parsimony-uninformative character sequences can also be used for species identification using the DNA barcoding method (Hapsari et al. 2018; Kumar et al. 2018). The Strict consensus tree derived from the MP sequence analysis of ITS region and combination (ITS+*trn*L-F IGS) of *Etlingera* spp. separated into different clades for *E. elatior* and *Etlingera* sp 1 and 2 (Figure 2, 4). Based on the strict consensus tree of the *trn*L-F analysis IGS has also grouped the two accessions of *Etlingera* sp. become one clade but the clade is still joined to clade *E . elatior* (Figure 3).

Table 1. Summary of parsimony analysis using MP method

Variables	ITS	*trn*L-F IGS	ITS+*trn*L-F IGS
Etlingera spp. + outgroup			
Sequences length	754	400	1154
Number of characters constant (%)	35	80.3	42.5
Parsimony uninformative characters (%)	14.7	11	25.5
Parsimony informative characters (%)	50.3	8.7	32
Tree length	685	120	905
Consistency index (CI)	0.90	0.80	0.89
Retention index (RI)	0.96	0.70	0.91
Etlingera spp.			
Sequences length	754	400	1154
Number of characters constant (%)	90	85	88.4
Parsimony uninformative characters (%)	9	9	9
Parsimony informative characters (%)	3	6	2.6
Tree length	89	95	173
Consistency index (CI)	0.96	0.78	0.87
Retention index (RI)	0.57	0.61	0.52

Internal Transcribed Spacer (ITS) and trnL-F intergenic spacer profile sequences

The ITS and *trn*L-F intergenic spacer profile sequences of *Etlingera* spp. obtained were carried out by BLAST data mining to identify sequences similarity based on the NCBI database to ensure that the sequence obtained was the *Etlingera elatior* sequence or other *Etlingera* species. The analysis of BLAST parameters are important to determine species name (Madden et al. 2013). Sequence similarity identification was taken based on the highest percentage of query coverage and percentage identity (Table 2).

All *trn*L-F intergenic sequences of the *Etlingera* sp. species in this study are new data from NCBI so no specific sequence of *trn*l-F intergenic spacer was found but species similarity was found based on genomic DNA of *Etlingera elatior*. This is different from the sequence analyzed based on the ITS marker. ITS sequence data can identify two different species of *Etlingera*, namely *E. elatior* and *E. hemisphaerica*. Furthermore, the trnL-F sequence data provide phylogenetic information in closely related species. Different sequence variations in the trnL-F IGS sequence is generally caused by a single nucleotide mutation that represent mutations that occur over a very long period of time.

Phylogenetic analysis using the maximum parsimony method based on ITS markers has identified two *Etlingera* spp. accessions which are not *Etlingera elatior* species but identified as *E. hemisphaerica* from GenBank data. A strict consensus tree of 9 accessions of *Etlingera* spp. from maximum parsimony analysis based on ITS sequences is divided into two clades. The first clade is a group of the species *E. elatior* and the second clade is a group of *E. hemisphaerica* (Figure 3). In addition to using the maximum parsimony method, construction of phylogenetic tree (phylogram) was also done by neighbor joining (NJ) method (Figure 4). The resulting phylogram showed the separation of *E. elatior* and *E. hemisphaerica* very clear. NJ analysis generated phylogram that describes the evolutionary process indicated by the magnitude of the genetic distance of the branches in each type of *Etlingera*. Based on NJ phylogram, two accessions of *E. Hemisphaerica* from this study and one accession from GeneBank have greater genetic distance values than *E. elatior* (Figure 3). Phylogenetic analysis using ITS markers with two different analytical methods can authenticate *Etlingera* sp. is *E. hemisphaerica*.

Discussion

The markers used in the phylogenetic analysis in this study were internal transcribed Sequence (ITS) region of Nuclear DNA and *trn*L-F intergenic spacer (IGS) non-coding region sequences of chloroplast DNA. The molecular structure of chloroplast DNA is often used in phylogenetic analysis plant. Some of the reasons are that the chloroplast DNA structure does not undergo genetic recombination process, is more conservative and relatively stable, and is mostly inherited from the female parent (Greiner et al. 2015).

The analysis using Maximum Parsimony (MP) showed monophyletic presence in each taxa group. Monophyletic group of species means that each taxa group comes from a single common ancestor and also includes all of the descendants of the common ancestor. *Etlingera* as established by Burtt and Smith (Burtt and Smith 1986) is strongly supported as monophyletic. This specialized genus of the tribe Alpinieae is defined by eight ITS substitutions (seven unique) and characterized by an involucre of sterile bracts, a short or much elongated peduncle and tubular and elongated bracteoles.

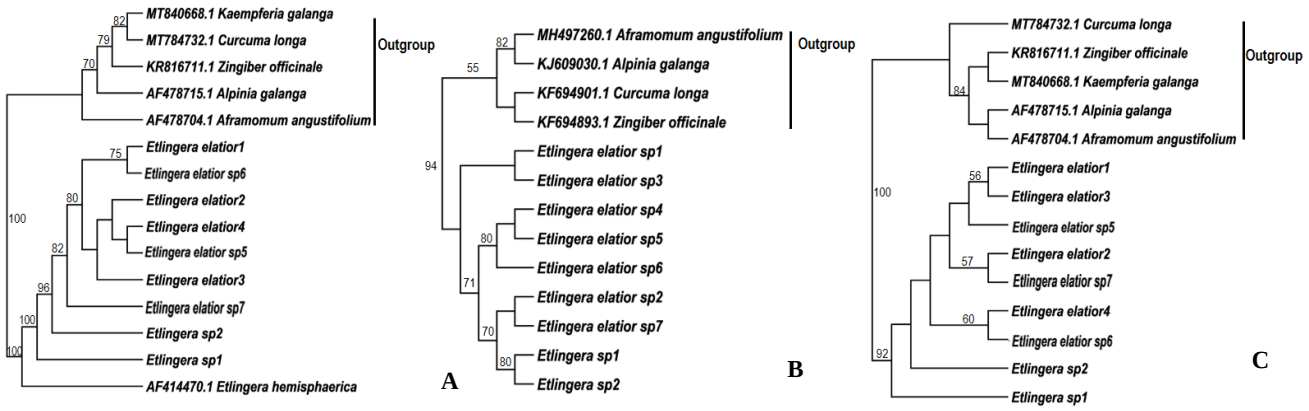

Figure 2. A. Strict consensus tree from maximum parsimony analysis based on ITS sequences of *Etlingera* spp. and outgroup taxa. CI=0.90 and RI=0.95. Numbers below branches showed bootstrap values. B. Strict consensus tree from maximum parsimony analysis based on *trn*L-F intergenic spacer sequences of *Etlingera* spp. and outgroup taxa. CI=0.80 and RI=0.70. Numbers below branches showed bootstrap values. C. Cladogram based on Maximum Parsimony analysis based on combination ITS+*trn*L-FIGS sequences of *Etlingera* spp. and outgroup taxa. CI=0.89 and RI=0.91. Numbers below branches showed bootstrap values

Table 2. Results of nucleotide base on BLAST on Genbank

GenBank acc. no.	Description	Species	Locality (District)	Coordinate	Max score	Total score	Query cover (%)	Ident (%)
	Internal Transcribed Spacer (ITS)							
AF414470.1	*Etlingera elatior* 18S ribosomal RNA gene, partial sequence; ITS1, 5.8S ribosomal RNA gene and ITS2, complete sequence; and 26S ribosomal RNA gene, partial sequence	*E. elatior* sp1	Gayo Lues	97°4'10.379"E 4°3'28.033"N	1245	1245	99	99.42
		E. elatior sp*2*	Aceh Selatan	97°14'47.629"E 3°14'4.163"N	1227	1227	99	98.84
		E. elatior sp*3*	Nagan Raya	96°30'46.393"E 3°59'27.398"N	1140	1140	98	99.52
		E. elatior sp4	Aceh Tamiang	98°4'28.700" E4°20'27.800"N	1205	1205	98	98.54
		E. elatior sp5	Pidie	96°4'59.463"E 4°51'48.526"N	1230	1230	98	99.41
		E. elatior sp6	Aceh Tengah	95°3'28.901"E 5°42'30.407"N	1234	1234	97	99.13
		E. elatior sp7	Aceh Tengah	95°3'28.901"E 5°42'30.407"N	1225	1225	98	99.26
AF414465.1	*Etlingera hemisphaerica* 18S ribosomal RNA gene, partial sequence; ITS1, 5.8S ribosomal RNA gene and ITS2, complete sequence; and 26S ribosomal RNA gene, partial sequence	*Etlingera* sp1	Aceh Tenggara	97°48'36.000"E 3°29'18.700"N	907	907	88	97.77
		Etlingera sp2	Pidie	96°4'59.463"E 4°51'48.526"N	1179	1179	99	99.49
	***trn*L-F Intergenic Spacer**							
MH603418.1	*Etlingera elatior* isolate 97-686A plastid, partial genome	*E. elatior* sp1	Gayo Lues	97°4'10.379"E 4°3'28.033"N	680	680	99	99.47
		Etlingera sp1	Aceh Tenggara	97°48'36.000"E 3°29'18.700"N	730	730	98	90.78
		E. elatior sp*2*	Aceh Selatan	97°14'47.629"E 3°14'4.163"N	601	601	90	96.44
		E. elatior sp3	Nagan Raya	96°30'46.393"E 3°59'27.398"N	752	752	99	99.76
		E. elatior sp4	Aceh Tamiang	98°4'28.700" E4°20'27.800"N	558	558	98	95.18
		E. elatior sp5	Pidie	96°4'59.463"E 4°51'48.526"N	662	662	98	98.41
		Etlingera sp1	Pidie	96°4'59.463"E 4°51'48.526"N	555	555	93	90.84
		E. elatior sp6	Aceh Tengah	95°3'28.901"E 5°42'30.407"N	719	719	99	98.30
		E. elatior sp7	Aceh Tengah	95°3'28.901"E 5°42'30.407"N	673	673	99	97.24

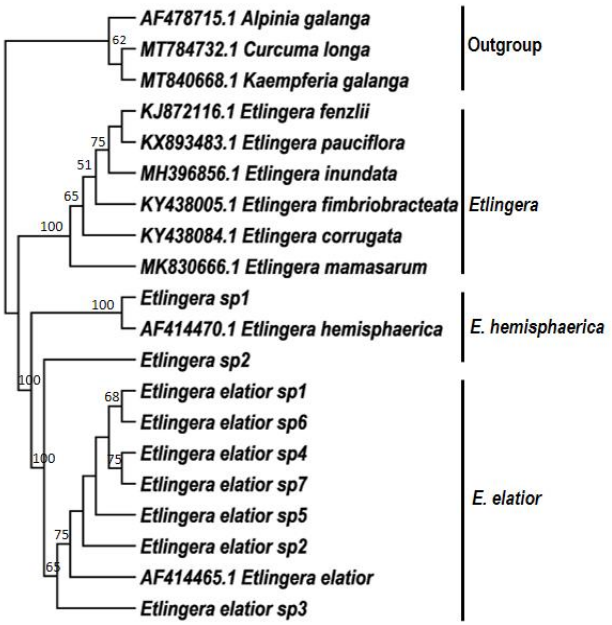

Figure 3. Cladogram based on maximum parsimony analysis of ITS sequences of *Etlingera* spp.. Numbers above branches showed bootstrap values

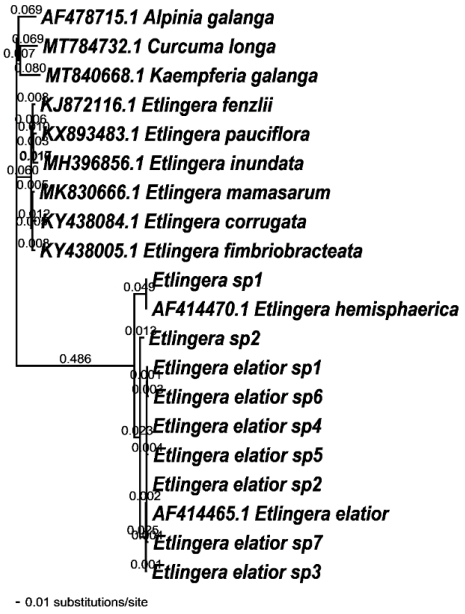

Figure 4. Phylogram from neighbor joining analysis of ITS sequences of *Etlingera* spp.. Numbers above branches correspond to the genetic distance

Monophyletic is an important parameter in phylogeny analysis, because in evolutionary analysis, the number of ancestor is more than one will cause confusion in determining the characteristic changes that occur during evolution. The confusion occurs due to the uncertainty of the ancestor which ones pass down their traits to their descendant taxa (Su et al. 2010; Soltis et al. 2019). A phylogenetic tree topology that can be accepted in biosystematics if it is monophyletic, dichotomous, has a high bootstrap value high and the clade formed is consistent and sturdy. Monophyletic group has only one ancestor and all of its descendants come from ancestors. This causes members in monophyletic groups to be considered to have a very close relationship and are assumed to carry similar genetic and biochemical patterns (Hertweck et al. 2015).

The difference in the number of *Etlingera* spp. sequences analyzed in this study based on the ITS marker only 3% of the total sequence (Table 1). This indicates that the *Etlingera* accessions studied based on the ITS markers have slight differences in nucleotide bases. Changes and variations in chloroplast DNA, although only one base, have important meaning as information to explain the process of evolution of organisms (Fitmawati et al. 2010; Daniell et al. 2016). The nucleotides of two pairs of similar sequences are derived from common ancestor, or both are of the same species.

The phylogenetic tree based on the ITS region grouped accessions of *E. elatior* originating from the lowlands of Aceh Province into one clade, namely *E. elatior* sp1, *E. elatior* sp2, *E. elatior* sp4, and *E. elatior* sp6,. Then the accessions of *E. elatior* sp7, with white involucral bracted were separated from the accession groups with red and pink white involucral bracted. This is consistent with that the white involucral bracted is extremely rare and occurs in the wild. This is also supported by the low value of the accession genetic distance.

The Strict consensus tree from the MP sequence analysis based on ITS region of *Etlingera* spp. separated into different clades for *E. elatior* and *Etlingera* sp_1 and sp_2 (Figure 2A, 2C). Based on the strict consensus tree of the *trn*L-F analysis IGS has also grouped the two accessions of *Etlingera* sp. become one clade but the clade is still joined to clade *E. elatior* (Figure 2B). Pairwise sequence divergence of ITS within *Etlingera* ranges from 0% between closely related taxa (namely *E. hemisphaerica* and *E. elatior).* Based on morphological characters, *E. elatior* and *E. hemisphaerica* with long peduncles, are deeply embedded in a well-supported clade that also includes subterranean species (Pedersen 2004). NJ analysis showed that the differences in genetic distance describe the level of evolution of each species of *Etlingera*. In this study, three *E. Hemisphaerica* accessions have greater genetic distance values than *E. elatior* (Figure 4). The greater the genetic distance, the higher the genetic adaptation developed by the species to survive in its environment. Genetic distance is the requirement for species to survive long term and adapt to environmental changes (Govindaraj et al. 2015). Evolution was usually thought to be slow or fast proses, something that happens depending on the adaptation process and environmental conditions.

Etlingera spp. is a traditional Aceh medicine generally used to relieve wind and expectorants and loss of energy. Along with the times, this plant will go to the commercial stage of the market. The similarity of morphological characters and high market demand will be feared if this plant is widely substituted and counterfeited. DNA barcodes are an approach to identifying species based on

sequences of short standard DNA regions. From the authentication results, the community especially in Aceh Province can cultivate *Etlingera* spp. that have the potential as medicine. The benefits can be used as a reference in the use of *Etlingera* spp. growth as a drug in an effort to improve public health, especially herbal medicine.

In conclusion ITS sequence data can identify two different species of *Etlingera*, namely *E. elatior* and *E. hemisphaerica*. The two identified *Etlingera* spp. species can be used as important information for further research related to their pharmacological studies. One marker and combined analysis of ITS and *trn*L-F data proved that the *Etlingera* spp. is a monophyletic group. The intraspecific and interspecific variations of ITS markers were more discriminatory regions, while trnL-F IGS were of lower divergences. In this study different species of *Etlingera* spp. can be differentiated effectively by comparing the DNA barcoding regions. Our findings indicated that DNA barcoding is efficient and powerful to authenticate herbal medicine *Etlingera* spp..

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