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Research Article

Region of Nuclear Ribosomal DNA (*ITS2*) and Chloroplast DNA (*rbc*L and *trn*L-F) as A Suitable DNA Barcode for Identification of *Zingiber loerzingii* Valeton From North Sumatera, Indonesia

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ABSTRACT

Zingiber loerzingii Valeton is one of the species in the Zingiberaceae family found throughout Aceh and North Sumatra, Indonesia, with slimy flowers, yellowish white color, and dark orange stamens. *Z. loerzingii* is endemic in North Sumatra with a very limited distribution. The International Union for Conservation of Nature and Natural Resources classifies this plant into the vulnerable ones category. This study aims to examine the potential of DNA barcoding from nuclear DNA (*ITS2*) and DNA chloroplasts (*rbc*L and *trn*L-F) to identify *Z. loerzingii* plants. The research sample was obtained from two main distribution areas of *Z. loerzingii* in North Sumatra, Indonesia, namely Sibolangit Nature Reserve and Tangkahan Conservation Forest. The results showed that all the DNA barcode markers used were able to classify *Z. loerzingii* into the same group in the phylogenetic analysis. *ITS* marker is the most effective marker for classifying Zingiberaceae species compared to *rbc*L and *trn*L-F markers. The *ITS2* marker has the lowest level of intraspecific and intraspecific genetic distance overlap compared to the *rbc*L and *trn*L-F markers. This research is expected to provide information related to the DNA barcode of *Z. loerzingii* in an effort to conserve this rare plant.

INTRODUCTION

Zingiberaceae is a family of plants with the largest amount of members of the order Zingiberales (Kress et al. 2002; Pedersen 2004) spread throughout the Indo-Malay region and consist of about 52 genera and 1200 species (Sabu 2006). Most members of this family are used in spices, vegetables, nutraceuticals, and traditional medicine (Kala 2005; Tushar et al. 2010; Zakaria et al. 2011). Identification of the Zingiberaceae family is very difficult because of the narrow differences in morphological characteristics between species and the high degree of phenotypic plasticity (Kress et al. 2002; Vinitha et al. 2014).

The *Zingiber* genus is one of the genera in the Zingiberaceae family (Miller 1754). It is distributed throughout the tropical rainforests of Asia with the highest diversity level in the forests of Southeast Asia (Theilade 1999). Most species in the *Zingiber* genus are rare and endangered plants, and their habitat is notoriously difficult to access, complicating efforts to collect and study them (Tushar et al. 2010). *Zingiber loerzingii* is a species of the genus *Zingiber* and Zingiberaceae family (Valeton 1918) with characteristics which consist of slimy flowers, dark orange-colored stamens, and yellowish-white flowers found in Aceh and North Sumatra, Indonesia (Rugayah et al. 2017). *Z. loerzingii* is listed as vulnerable on the IUCN Red List (Nurainas & Ardiyani 2019). This plant is difficult to find in nature because of its endemic nature, its limited distribution area, and its rarity in Indonesia (Rugayah et al. 2017). Due to the decreasing population of this plant, an appropriate and fast identification method is needed for the conservation needs of this plant.

DNA barcoding is a method for species-level identification using short DNA segments (Kress et al. 2005; Hollingsworth et al. 2009). The DNA barcode used must be conserved among taxa and have sufficient polymorphism to discriminate (Chase et al. 2007; Kress et al. 2015). Commonly used plant DNA barcodes include RuBisCo (*rbc*L) and maturase K (*mat*K), nuclear-encoded ribosomal internal transcribed spacer (*ITS*), and *ITS* shorter fragment *ITS*2 (Ratnasingham & Hebert 2007; Hollingsworth et al. 2009). DNA barcodes cannot be used indiscriminately for all plants – each has a different advantage in identifying plants, even in some types of plants, but the three markers can be very useful.

*ITS*2 markers can classify genera in the Zingiberaceae family into different groups through phylogenetic analysis (Shi et al. 2011; Ren et al. 2019) . The *ITS*2 marker is also reported to be able to identify species in the genus Alpinia originating from Peninsular Malaysia with a success rate of up to 96.97% (Tan et al. 2020). The its sequence (*ITS* 1, 5.8S, *ITS* 2) was able to classify 18 species of the *Zingiber* genus from species in other genera in the Zingiberaceae family. (Theerakulpisut et al. 2012). *ITS*2 markers, trnH-psbA, *mat*K, *rbc*L, and *trn*L-F were reported to be able to group genera and species in the *Zingiberacea* family (Shi et al. 2011; Chen et al. 2014; Vinitha et al. 2014) . The markers *trn*L-F, *mat*K, and *ITS* showed greater intraspecific differences than interspecific differences in *Curcuma* (Zingiberaceae) (Záveská et al. 2012).

One of the difficulties in identifying species in the Zingiberaceae family is that there is no one effective marker for all species in this family, therefore it is necessary to test the ability of each marker in each genus or species to determine *ITS* effectiveness. The *mat*K or *rbc*L plastid loci have been reported to exhibit paraphyla results in *Hedychium coccineum* and *Hedychium flavescens* (Vinitha et al. 2014). The *mat*K, *rbc*L, *trn*H-*psb*A, and *trn*L-F markers did not show any barcode gaps in the *Curcuma* genus. Barcoding gaps are very important to show the success of using DNA barcodes in the observed samples (Hebert et al. 2003; Theodoridis et al. 2012; Pino-Bodas et al. 2013). The *rbc*L marker has no genetic variation and *mat*K is relatively difficult to amplify and is sequencing in the *Roscoea* genus (Zingiberaceae) (Zhang et al. 2014).

The use of DNA barcodes in plants does have limitations, including difficulties in DNA amplification due to degraded DNA (Särkinen et al. 2012), attachment location mismatch like *mat*K (Piredda et al. 2011; Kool et al. 2012), and low levels of discrimination such as *rbc*L (Stoeckle et al. 2011; Newmaster et al. 2013). This study aims to examine the potential of DNA barcoding *trn*L-F, *rbc*L, and *ITS*2 in identifying the *Z. loerzingii* rare plant from North Sumatra Indonesia, and is expected to provide im-

portant information related to DNA barcoding in *Z. loerzingii* rare plant from North Sumatra.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

A total of 5 samples of fresh *Z. loerzingii* were collected from Sibolangit Nature Reserve (4 samples) and Tangkahan Conservation Forest North Sumatra (1 sample), Indonesia. Fresh leaf samples were stored in a freezer at -20°C for long-term storage. DNA isolation was performed using the GeneJet Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommended protocol. The results of DNA isolation were tested qualitatively using gel electrophoresis and visualised using gel documentation (Bio-Rad Laboratories, Hercules, CA, USA).

Amplification and Sequencing

The *ITS2*, *rbc*L, and *trn*L-F DNA regions were amplified using polymerase chain reaction (PCR) in a thermocycler (LabCycler SensoQuest PCR, SensoQuest, Germany) with a total volume of 25 µL [2.5 µL of reverse primer, 2.5 µL of forward primer; 5 µL of distilled water; 2.5 µL of DNA template; 12.5 µL of PCR mix (MyTaq HS Red Mix, Bioline, USA)]. The primer used for *rbc*L sequence is *rbc*L a_f (5'- ATG TCA CCA CAA ACA GAG ACT AAA GC-3') dan *rbc*L a_rev (5'-GTA AAA TCA AGT CCA CCR CG-3') (Costion et al. 2011), whereas the primers for the *trn*L-F sequence are *trn*L-F F (5-GGT TCA AGT TCT ATC CCC CC-3') and *trn*L -F R (5'-ATT TGA GAC ACG AG ACT GGT-3') (Taberlet et al. 1991), and the primers for the *ITS2* sequence are *ITS2*-S2F (5'-ATG CGA TAC TTG GTG TGA AT-3') and *ITS2*-S3R (5'-GAC GCT TCT CCA GAC TAC AAT-3') (Yao et al. 2010). Amplification was carried out with a predenaturation program at 97°C for 4 minutes, denaturation at 94°C for 45 seconds, annealing at 52°C for 50 seconds and extension at 72°C for 1 minute. PCR products were visualized using 1% agarose gel with SYBR Safe DNA gel stain (Invitrogen, USA). PCR products with positive results (DNA bands are clearly visible) will be sent to the First Base DNA Sequencing Service in Singapore for sequencing.

Data Analysis

The results of the *ITS2*, *rbc*L, and *trn*L-F sequences were analysed using the Bioedit 7.2.5 application (Hall 1999) to determine the consensus sequence. Nucleotide composition, genetic distance, and phylogenetic tree were constructed using MEGA (Molecular Evolutionary Genetics Analysis) version 11 (Tamura et al. 2021) based on alignment of sequence data. The method used for phylogenetic tree analysis is Neighbor Joining and Maximum Parsimony with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

All samples in this study were successfully amplified using 3 main markers (*ITS2*, *rbc*L, and *trn*L-F). The results of the visualisation of the PCR product showed presence of a single band, which means the sequence was successfully amplified. The alignment analysis of the sequencing results shows that the length of the sequence consists of ± 492 bp (*ITS2*), ± 577 bp (*rbc*L), and ± 397 bp (*trn*L-F). The results of the analysis using BLAST software at the National Center for Biotechnology Information (NCBI) resulted in a homology level of *ITS2* sequences in *Z. loerzingii* of 99.73%. There is only one *ITS2* sequence data for *Z. loerzingii* in NCBI, while the *rbc*L and *trn*L-F markers have not been found in the NCBI da-

tabase so that the level of homology with the database cannot be measured. The nucleotide composition of *Z. loerzingii* using three barcode markers is presented in Table 1.

The length of the DNA barcode sequencing results varies greatly in each Zingiberaceae species (Kress et al. 2002; Shi et al. 2011; Vinitha et al. 2014; Ounjai et al. 2016; Saha et al. 2020). The average GC content that has been reported in Zingiberaceae with the *ITS2* marker is in the *Alpinia* genus (59.35%) (Tan et al. 2020), *Plagiostachys* (58,60%) (Julius & Suleiman 2008), *Myxochlamys* (Takano & Nagamasu 2007), and *Globba* (52.2%) (Williams et al. 2004). *ITS2* data on *Z. loerzingii* (59.2%) is still unpublished. The average GC content that has been reported in the *rbc*L marker is in the genus *Alpinia* (43.0%) (Davis et al. 2004), *Amomum* (42.9%) (Li et al. 2011), *Curcuma* (42.7%) (Kress & Erickson 2007), *Globba* (42.8%) (Kress et al. 2001), *Hedychium* (42.8%) (Vinitha et al. 2014), *Renealmia* (43.1%) (Givnish et al. 2010), and *Zingiber* (43.1%) (Smith et al. 1993). The GC content of the *trn*L-F barcode sequence in Zingiberaceae has not been reported. The only data reported is the *trn*L-F sequence in the Zingibereae tribe from the sample obtained from the Royal Botanic Garden of Edinburgh with GC content ranging from 31.5%-33.41% and an average of 32.78% (Ngamriabsakul et al. 2004).

The features of the three barcode markers (*ITS2*, *rbc*L, and *trn*L-F) on *Z. loerzingii* are presented in Table 2. All three show high levels of conserved sites. Variable sites are only visible on the *ITS2* marker. Due to the absence of *rbc*L and *trn*L-F *Z. loerzingii* sequence data on NCBI, intraspecific analysis of these markers did not use external comparisons, but only used study samples. The results showed that conserved sites on the *ITS2* marker were 97.97%, *rbc*L was 100%, and *trn*L-F was 100%. DNA barcode features on Zingiberaceae species in India showed that the conserved sites level on the *rbc*L marker was 88.91%, *trn*L-F was 82.93%, and *ITS2* was 48.33% (Vinitha et al. 2014).

Table 1. Nucleotide composition, AT content, and GC content using three main markers (*ITS2*, *rbc*L, and *trn*L-F) in *Z. loerzingii*.

Sample	Collection site	Barcode	Composition (%)				Content (%)		Total (bp)
			A	C	G	T	A/T	G/C	
Z. loerzingii	SNR T1	*ITS2*	20.5	24.4	32.7	22.4	42.9	57.1	492
Z. loerzingii	SNR T2	*ITS2*	20.2	24.6	32.6	22.3	42.7	57.3	484
Z. loerzingii	SNR T3	*ITS2*	20.2	24.6	32.6	22.5	42.8	57.2	484
Z. loerzingii	SNR T4	*ITS2*	20.2	24.8	32.6	22.3	42.6	57.4	484
Z. loerzingii	TCF T5	*ITS2*	20.3	24.8	32.6	22.0	42.5	57.5	472
Z. loerzingii	*NCBI (MN803334.1)	*ITS2*	17.1	25.5	33.7	22.6	40.8	59.2	368
Z. loerzingii	SNR R1	*rbc*L	27.8	19.8	23.1	29.3	57.1	42.9	576
Z. loerzingii	SNR R2	*rbc*L	27.9	19.8	23.1	29.3	57.2	42.8	577
Z. loerzingii	SNR R3	*rbc*L	27.9	19.8	23.1	29.3	57.2	42.8	577
Z. loerzingii	SNR R4	*rbc*L	27.8	19.8	23.1	29.3	57.1	42.9	576
Z. loerzingii	TCF R5	*rbc*L	27.9	19.8	23.1	29.3	57.2	42.8	577
Z. loerzingii	SNR L1	*trn*L-F	33.4	11.1	22.8	32.2	65.9	34.1	395
Z. loerzingii	SNR L2	*trn*L-F	33.2	11.6	22.9	32.2	65.5	34.5	388
Z. loerzingii	SNR L3	*trn*L-F	33.4	11.4	22.8	32.2	65.7	34.3	395
Z. loerzingii	SNR L4	*trn*L-F	33.2	11.3	22.9	32.0	65.6	34.4	397
Z. loerzingii	TCF L5	*trn*L-F	32.8	12.5	22.2	32.2	65.2	34.8	329

SNR: Sibolangit Nature Reserve; TCF: Tangkahan Conservation Forest
*Data obtained from National Center for Biotechnology Information

Table 2. Features of three barcoding markers (*ITS2*, *rbc*L, and *trn*L-F) of *Z. loerzingii*.

Locus	*ITS2*	*rbc*L	*trn*L-F
Total number of sites (bp)	6	5	5
Conserved sites (bp)	482	577	395
Variable sites (bp)	2	0	0
Parsimony Informative sites (bp)	0	0	0
Singleton sites (bp)	2	0	0

Different base substitutions in the analysis region were evaluated for all codon positions and are shown in Table 3. In general, the transitional substitution was higher in the *ITS2* region, lower in the *trn*L-F region, and the same as the transversional substitution in the *rbc*L region. The transitional and transversional substitution values tend to be the same because the *ITS2*, *rbc*L, and *trn*L-F sequences are highly conserved in *Z. loerzingii*. A small difference only occurred in the *trn*L-F marker with a difference in value of only 0.01%. Relative rates of synonymous substitution in most chloroplast DNA are more conserved (Muse 2000). The results of a comparative study of Ginger (*Zingiber officinale*) in the Zingiberaceae family using a complete chloroplast genome showed that the inverted repeat region (including *mat*K and *rbc*L) experienced slow nucleotide substitution (Cui et al. 2019). The rate of insertion and deletion of *ITS* markers is more frequent than substitution (Elbadri et al. 2002).

The relative distribution of interspecific and intraspecific variation is presented in Figure 1. The results showed that the three barcode markers used had a high intraspecific distribution at a smaller genetic distance than the interspecific distribution. Discrimination in the distribution of intraspecific and interspecific distances was clearly seen in the *ITS2* marker which was indicated by the small degree of overlap compared to the *rbc*L and *trn*L-F markers (Figure 1A). This indicates that the *ITS2* marker is more effective when used as DNA barcode to identify *Z. loerzingii*. The barcode markers *rbc*L and *trn*L-F showed a high intraspecific distribution at a smaller genetic distance, but the interspecific distribution showed high overlap with the intraspecific distribution. It also shows that there is no barcode gap between the *rbc*L and *trn*L-F barcode markers (Figure 1B, Figure 1C).

Barcoding gap is generated by plotting differences between the mean intraspecific and interspecific distances (Meyer & Paulay 2005; Bhagwat et al. 2015). Barcode gap shows no overlapping area between interspecific and intraspecific distances (Hebert et al. 2003; Pino-Bodas et al. 2013; Chen et al. 2015). In this study, there was an overlap between the intraspecific and interspecific distances in the *ITS2*, *rbc*L, and *trn*L-F markers. However, the smallest overlapping level was found in the *ITS2* marker. Analysis of *ITS2* sequences as DNA barcodes in Zingiberaceae in E'meai region, Sichuan province, China, showed a clear barcode gap (Ren et al. 2019). Several studies showed that there is no barcoding gap according to observations using the *rbc*L, *ITS*, and *ITS2* markers (Ferguson 2002; Meier et al. 2006; Shearer & Coffroth 2008; Hollingsworth et al. 2009). Research on closely related plants also did not show any barcoding gap using *rbc*L and *mat*K markers (Lahaye et al. 2008; Bhagwat et al. 2015).

Based on the phylogenetic tree analysis constructed using the Maximum Parsimony method, the three barcode markers used seemed to be able to include *Z. loerzingii* samples in the same group. However, only marker *ITS2* showed the highest level of species and genus discrimination (Figure 2A) compared to markers *rbc*L and *trn*L-F. The markers *rbc*L

Table 3. Pattern of Nucleotide Substitution of *ITS2*, *rbc*L, and *trn*L-F regions *in Z. loerzingii* (%)

DNA Barcodes	*ITS2*				*rbc*L				*trn*L-F			
Base	A	T	G	C	A	T	G	C	A	T	G	C
A	-	0.00	0.00	25,00	-	8,34	8,34	8,34	-	8,34	8,34	8,33
T	0.00	-	25,00	0.00	8,34	-	8,34	8,34	8,34	-	8,33	8,34
G	0.00	25,00	-	0.00	8,34	8,34	-	8,34	8,34	8,33	-	8,34
C	25,00	0.00	0.00	-	8,34	8,34	8,34	-	8,33	8,34	8,34	-

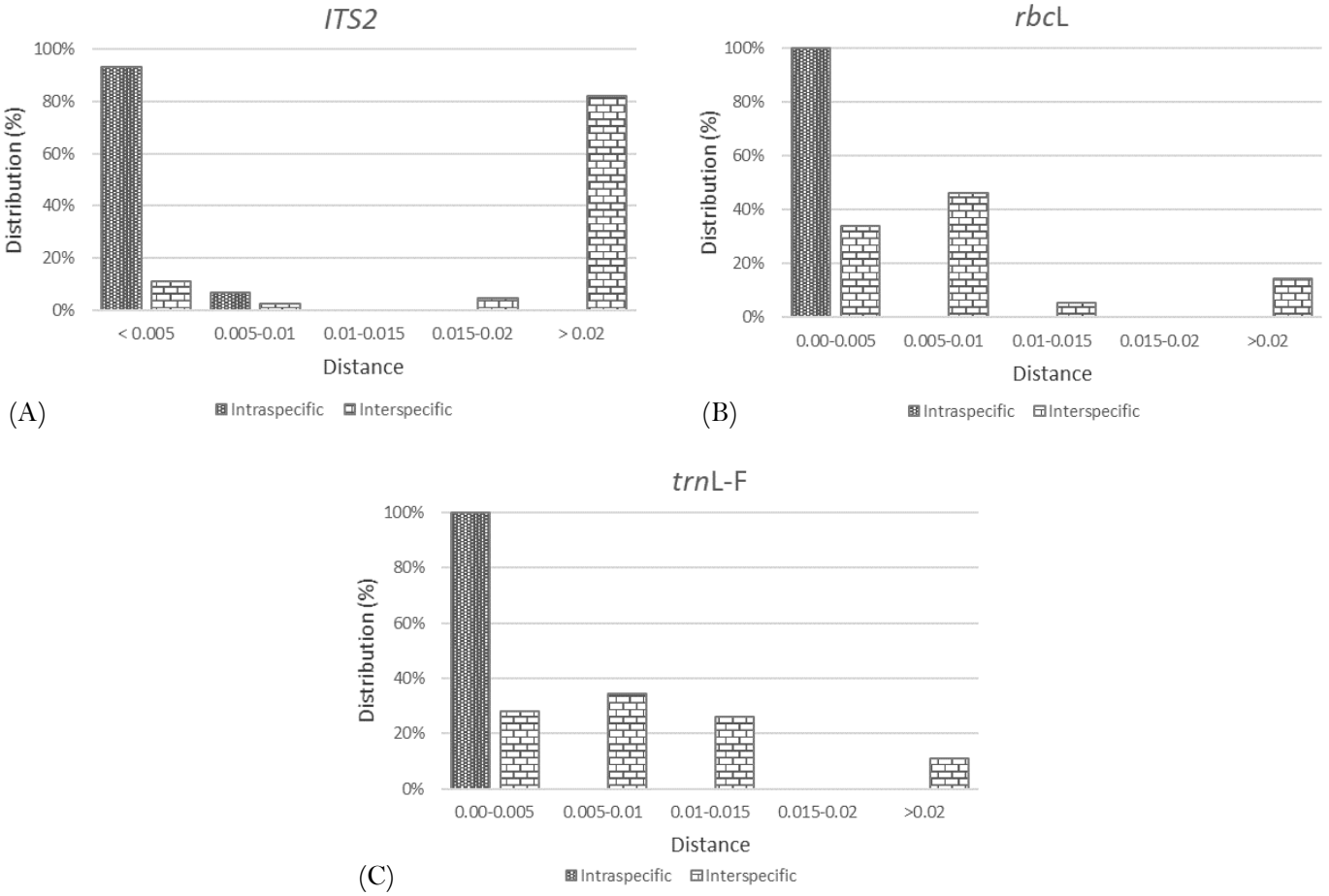

Figure 1. Barcoding gap with the intraspecific and interspecies distances

and *trn*L-F showed discrimination at family level, especially in the Zingiberaceae family (Figure 2B, Figure 2C), but were unable to classify genera or species therein.

DNA barcoding research on Zingiberaceae species in India showed that *rbc*L and *mat*K markers are more recommended as DNA barcodes for Zingiberaceae species, and highlighted the low ability of *ITS* and *ITS2* as barcoding markers for this family (Vinitha et al. 2014). This is different from the results of this study which showed that the *ITS2* marker is more effective in classifying genera and species in the Zingiberaceae family, especially *Z. loerzingii* species. In the genus *Curcuma*, it was reported that barcode markers with high success rates were *rbcL* and *trnH-psbA* (100%), *trn*L-F (95.7%), *mat*K (89,7%), and *ITS2* (82.6%) (Chen et al. 2015). Research related to DNA barcoding Zingiberaceae from North-East India shows that the *ycf1b* region is the region with the highest conserved sites, while the *ITS* region is the lowest (Saha et al. 2020). The *ycf1* gene is the most promising DNA plastid barcode for land plants (Dong et al. 2015).

The effectiveness of using DNA barcode markers on species in the Zingiberaceae family shows variations. According to our observations, species and geography factors are important in determining the effective

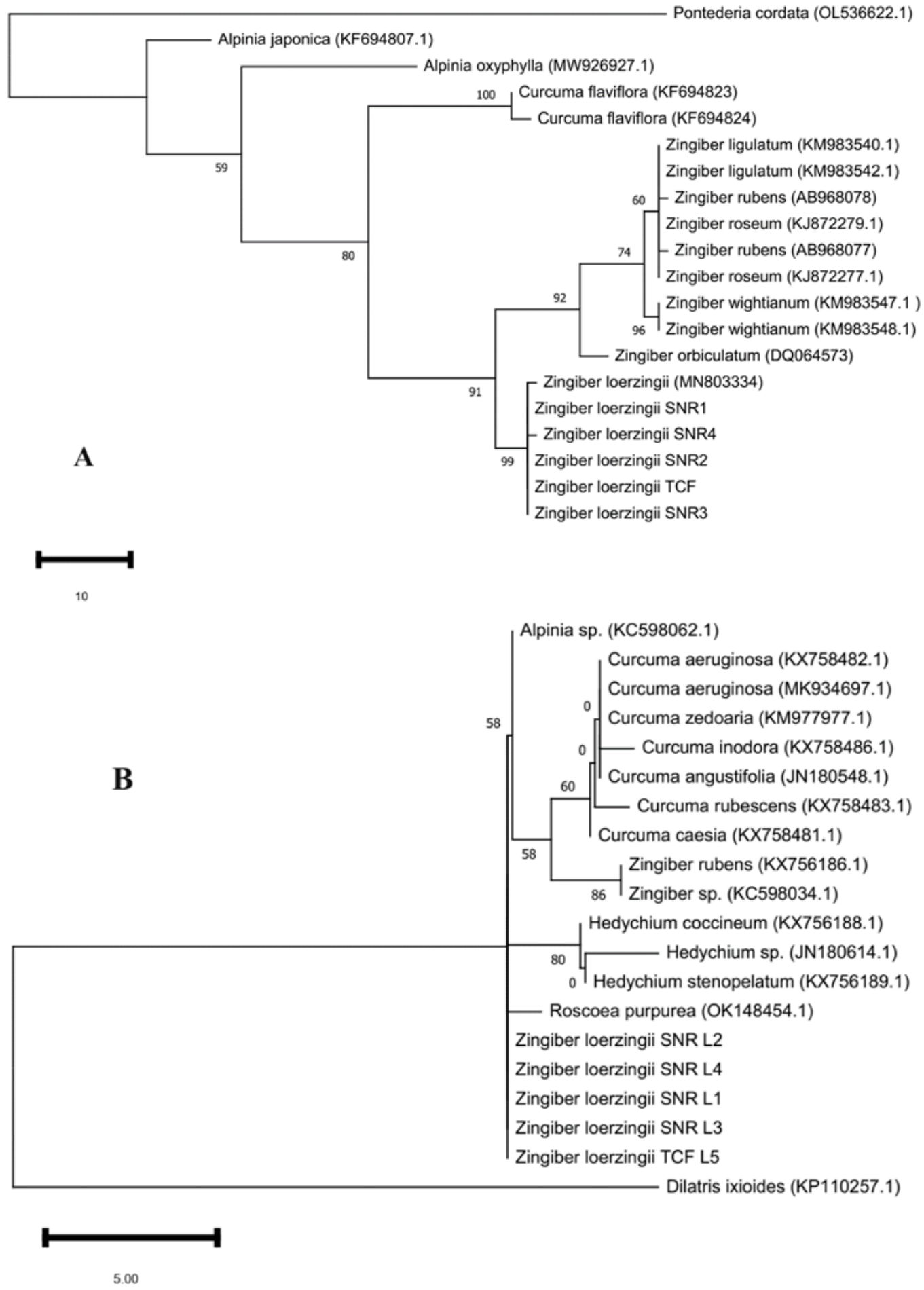

Figure 2. Maximum Parsimony tree for *Z. loerzingii*, closely related family Zingiberaceae and outgroup (*Dilatris ixioides* or *Pontederia cordata*) using *ITS2* (A), *rbc*L (B), and *trn*L-F (C) barcode.

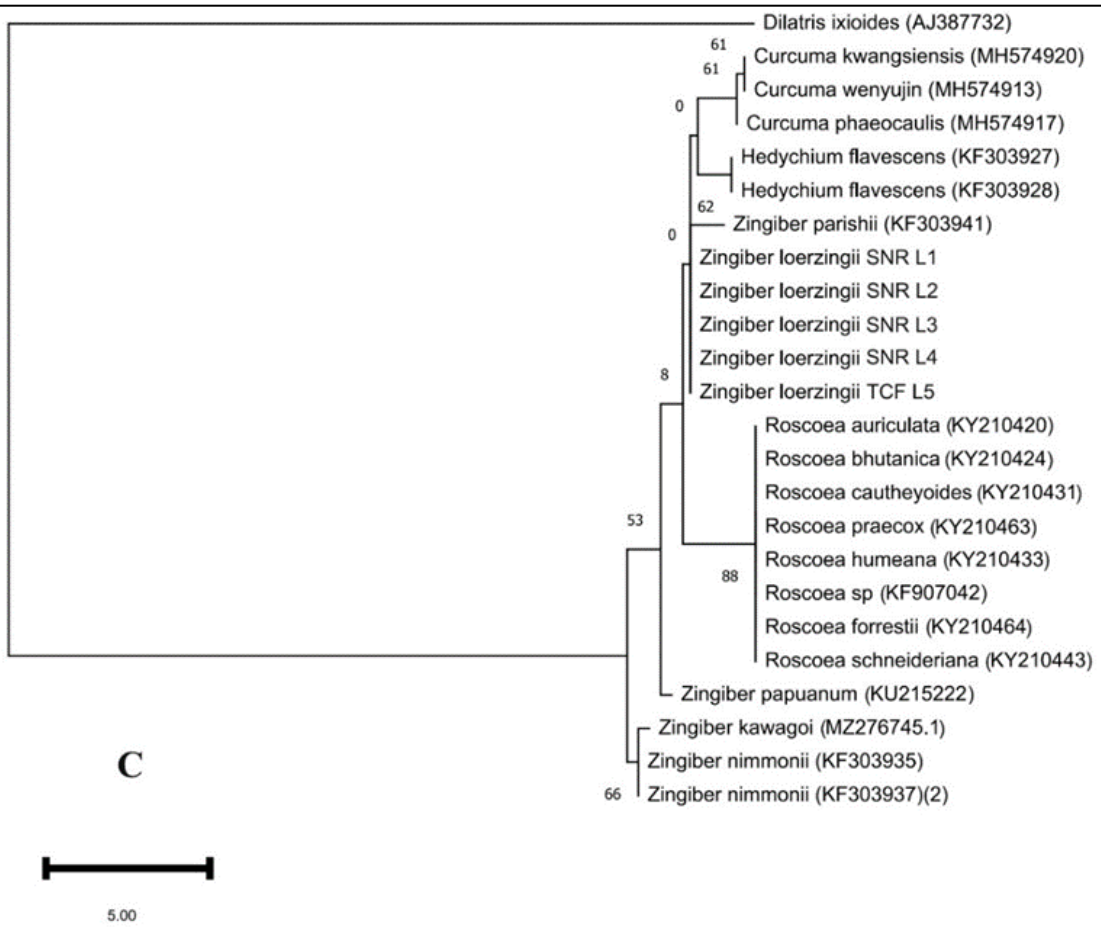

Figure 2. Contd.

DNA barcode markers to use. *ITS* markers are generally ineffective (25%) when used in Zingiberaceae species in India (Vinitha et al. 2014), while in the genus *Curcuma* (Zingiberaceae) obtained from the South China Botanical Garden and the US National herbarium, the marker showed effectiveness up to 82.6% (Chen et al. 2014). Barcode marker *ITS2* is a marker that has the highest discrimination ability at the genus (99.5%) and species (73.1%) levels in 30 Zingiberaceae genera from China (Shi et al. 2011). The very wide distribution of *Zingiberaecae*, especially in tropical Asia such as Sumatra, Borneo, and the Malayan Peninsula, gives rise to high variations due to the adaptation efforts made by the species to geo-ecological conditions (Jatoi et al. 2007). Due to the diversity of geographical areas of distribution, undoubtedly many Zingiberaceae species have not been identified (Larsen et al. 1999).

CONCLUSIONS

The conclusion that can be drawn from this study is that the markers *ITS2*, *rbc*L, and *trn*L-F were able to group *Z. loerzingii* together in the phylogenetic analysis. The *ITS2* marker is especially an effective marker for classifying the Zingiberaceae family based on genus and species compared to *rbc*L and *trn*L-F. Barcoding gap analysis showed that the *ITS2* marker has a small degree of overlap compared to the *rbc*L and *trn*L-F markers in *Z. loerzingii*, and therefore, it is more effective when used as an intraspecific species. The development of DNA barcode markers on Zingiberaceae species must take into account the geographical distribution of samples, so that the results can be more accurate.

AUTHOR CONTRIBUTION

E.P. designed the research and collected data, F.H. and L.L supervised

the entire research process, while Y.R., Y.Y. S.I., and T.H. analysed the research data. All authors were involved in writing and revising the manuscript. .

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. The authors are responsible for the content and article writing.

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