Jurnal Biosains Vol. 7 No. 3. Desember 2021 ISSN 2443-1230 (cetak)
DOI: https://doi.org/10.24114/jbio.v7i3.28921 ISSN 2460-6804(online)

JURNAL BIOSAINS
(Journal of Biosciences)
http://jurnal.unimed.ac.id/2012/index.php/biosains
email : jbiosains@unimed.ac.id

DNA *BARCODING Zingiber loerzingii* Valeton MENGGUNAKAN LOKUS GEN *Ribulose-1,5-biphosphate Carboxylase-Oxygenase Large subunit Gene* (*rbcL*)

Ladiez Rahmayani Sagala[1], Lazuardi[2], Fauziyah Harahap[2], Kartika Manalu[1], Zahratul Idami[1], Eko Prasetya[2]

[1]Major of Biology, Faculty of Science and Technology, State Islamic University of North Sumatra, Jl. IAIN, No. 1
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan. Jl. Willem Iskandar, Pasar V, Medan, North Sumatera, Indonesia, 20221
Email: ladiezr8@gmail.com

ABSTRAK

Barcode DNA merupakan salah satu teknik molekuler yang digunakan untuk mengidentifikasi dan mengklasifikasi makhluk hidup. *Z. loerzingii* adalah salah satu tumbuhan langka dan endemik yang hanya terdapat di Sumatera Utara. Pengkajian ilmiah mengenai *Z. loerzingii* masih sangat sedikit yang sudah dilakukan sehingga informasi mengenai tumbuhan ini terbatas sementara identitas yang jelas pada suatu tumbuhan benar-benar diperlukan untuk dapat dimanfaatkan secara maksimal. Penelitian ini bertujuan untuk mengetahui karakteristik molekuler *Z. loerzingii* dengan menggunakan *barcode* DNA serta mengkaji hubungan filogenetiknya berdasarkan lokus gen *rbcL*. DNA diisolasi dengan kit komersial. Lokus gen *rbcL* pada genom kloroplas *Z. loerzingii* diamplifikasi dengan teknik *Polymerase Chain Reaction* menghasilkan amplikon dengan panjang ± 600 bp. Penyatuan sekuens konsensus menghasilkan sekuens dengan panjang 575 bp. Rekonstruksi pohon filogenetik dilakukan dengan metode *Neighbor-Joining* dan model perhitungan Kimura-2-Parameter menunjukkan *Z. loerzingii* termasuk dalam kelompok monofiletik dengan *Zingiber mioga* dan *Zingiber officinale* sebagai *sister taxa*. Hasil analisis keragaman molekuler *Z. loerzingii* menunjukkan bahwa pada seluruh sampel *Z. loerzingii* koleksi dari Cagar Alam Sibolangit tidak ada keragaman molekuler atau keragaman genetik. Dengan demikian dapat disimpulkan bahwa *barcode* DNA dengan lokus gen *rbcL* dapat digunakan sebagai metode untuk mengidentifikasi *Z. loerzingii* secara molekuler serta efisien dalam menentukan hubungan kekerabatannya dengan spesies lain.

Kata Kunci : *Barcode* DNA, *Zingiber loerzingii*, *rbcL*

DNA *BARCODING* of *Zingiber loerzingii* Valeton USING *Ribulose-1,5-biphosphate Carboxylase-Oxygenase Large subunit Gene* (rbcL) GENE LOCUS

ABSTRACT

DNA barcode is one of the molecular techniques used to identify and classify living things. *Z. loerzingii* is currently reported as a rare and endemic plants that is only found in North Sumatra. Scientific studies for *Z. Loerzingii* is measly done so that the information about this plant is limited while a clear identity on a plant is essential to discover the potential. This study aims to determine the molecular characteristics of *Z. loerzingii* by using DNA barcodes and assessing the phylogenetic relationship based on the rbcL gene locus. DNA was isolated with a commercial kit. The *rbcL* gene locus in the chloroplast genome of *Z. loerzingii* amplified using the Polymerase Chain Reaction technique to produce amplicon with the length approximately 600 bp. Consensus sequence merging generate a sequence with 576 bp length. The phylogenetic tree reconstruction was carried out using the Neighbor-Joining method and the Kimura-2-Parameter calculation model showed *Z. loerzingii* included in the monophyletic group with *Zingiber mioga* and *Zingiber officinale* as the sister taxa. The

results for the molecular diversity analysis of *Z. Loerzingii* point out that in all samples of *Z. loerzingii* which collected from Cagar Alam Sibolangit have no molecular or genetic diversity. Therefore, it can be concluded that DNA barcoding with the *rbcL* gene locus can be used as a method to identify *Z. loerzingii* molecularly and efficient in determining their phylogenetic with other species.

Keywords: **DNA *Barcoding*, *Zingiber loerzingii*, *rbcL***

Introduction

Zingiber loerzingii Valeton is a herbaceous plant that belongs to the Zingiberaceae family with the characteristics of yellowish white flowers, slimy and flower lips and dark orange stamens. The distribution area of *Z. loerzingii* is in tropical forests from Aceh to North Sumatra (Rugayah *et al*., 2017). *Z. loerzingii* is designated as one of the plants with a vulnerable status by the IUCN Red List (IUCN, 2019). This plant is difficult to find in nature and its distribution area is very limited.

Identification of *Z. loerzingii* is very much needed as a conservation effort in preventing the extinction of this species. Conservation efforts with the aim of revealing and maintaining biodiversity are needed to ensure the sustainability of a type of living creature or rare species in the ecosystem (Rahayu & Nugroho, 2015). Molecular identification is very helpful in revealing the identity of living things quickly, easily and accurately (Hubert & Hanner, 2015).

DNA barcodes and genomic approaches have been carried out in plant identification, biodiversity studies and conservation (Kress *et al*., 2005; Vinitha *et al*., 2014; Youm *et al*., 2016; Hosein, 2017). Identification technique with DNA barcodes has been used widely and has a very important role for taxonomy, especially in the analysis of rare and endemic species (Kim *et al*., 2014; Hashim *et al*., 2020; Alaklabi *et al*., 2021). DNA barcodes consist of short DNA sequences that are unique to each species and are easily amplified. DNA barcodes performed with standardized experimental protocols are stable and can be repeated (Hu *et al*., 2019).

A number of loci have been considered and validated as ideal loci for plants (Kress *et al*., 2005; Ford *et al*., 2009). The Consortium for the Barcode of Life recommends the *matK* and *rbcL* gene loci as the main loci in plant barcodes (CBOL, 2009). The variation in *rbcL* is greater above the species level, rendering it suitable for differentiating species (Fazekas *et al*., 2008; Chen *et al*., 2010). The rbcL gene locus is also frequently used in phylogenetic analysis (Ismail *et al*, 2020).

Z. loerzingii belongs to the genus *Zingiber* which are cultivated for their rhizomes (Brickell, 2012). Studies on this species are still very limited and not much has been done. Similar studies of *rbcL* gene locus used other species from Zingiberaceae. The use of the *rbcL* gene has also never been carried out in molecular analysis of *Z. loerzingii*. Therefore, this study aims to determine the DNA barcode of *Z. loerzingii* plant based on the *rbcL* gene locus and to get its molecular characteristics.

Materials and Method

Z. loerzingii Sample

2 samples of fresh and whole leaves of *Z. loerzingii* were collected from Cagar Alam Sibolangit (Sibolangit Nature Reserve), Deli Serdang Regency, North Sumatra Province. The leaves A total of 10 in-groups and out-groups samples were taken from the alignment results on GenBank, namely *Zingiber mioga* (MW067010.1 and MW067011.1), *Zingiber officinale* (KU043478.1 and KU043479.1), *Kaempferia galanga* (KJ667620.1 and KJ667621.1), *Anigozanthos flavidus* (EF422992.1) and *Lachnanthes caroliniana* (KY626848.1).

DNA Isolation

Fresh leaf from *Z. loerzingii* was isolated with the commercial Geneaid Mini Plant DNA Isolation Kit according to the protocol provided (Geneaid, 2020). 100 mg of leaf tissue was crushed and then placed in a 1.5 ml microsentrifuge tube then added with 400 µl GP1 buffer and RNase A 5 µl. After being homogenized, it was then incubated at 60°C for 10 minutes. After incubation, the samples were added with 100 µl of GP2 buffer. The mixed

solution was then transferred to a collection tube containing a filter column and then centrifuged at 1000 x g for 1 minute. The filter column containing the supernatant was discarded and replaced with a new filter column. The solution in the collection tube was transferred to a new filter column and added buffer GP3 as much as 150% of the total volume of the solution. The GD column and collection tube were then centrifuged at 16,000 x g for 2 minutes. DNA was cleaned twice with W1 buffer and wash buffer. DNA in the GD column was then eluted with 100 µl of elution buffer which had been incubated at 60°C then centrifuged at a speed of 16,000 x g for 30 seconds. The solution that falls on the microcentrifuge tube is pure DNA. Pure DNA is stored at -20°C temperature for a longer shelf life.

DNA amplification

DNA was amplified using primers *rbcLaf* 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3' and *rbcLa-rev* 5'GTA AAA TCA AGT CCA CCR CG-3' (El-atroush *et al*., 2015; Hashim *et al*. ., 2020). DNA amplification of *Z. loerzingii* was carried out using the Myfi Mix Bioline PCR Kit with a total solution composition of 25 µl consisting of 5 µl of forward primer and 5 µl of reverse primer, 5 µl of sample DNA, 10 µl of distilled water and 12.5 µl of PCR kit. The amplification was carried out for 35 cycles with stages in the form of predenaturation at a temperature of 97°C for 4 minutes, denaturation at temperature of 94°C for 45 seconds, annealing at temperature of 52°C for 50 seconds and extension at temperature of 72°C for 1 minute. The results of DNA amplification were visualized with 1% agarose gel. The amplicon was then sent to the molecular company FirstBase in Singapore for sequencing.

DNA Analysis

The results of the sequencing in the form of a chromatogram file were edited using Bioedit 7.2.5 software to obtain a consensus sequence based on a conservative sequence of sequencing using *rbcLaf* primers and *rbcLa-rev* primers. The consensus sequences were then aligned using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Data with a high level of similarity will be included in the phylogenetic analysis. Phylogenetic tree and genetic diversity were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) X software (Kumar *et al*., 2018) and DnaSP 6th Version. The parameters will be analyzed include Genetic Distance Value, Nucleotide Composition and Haplotype and Nucleotide Diversity.

Results and Discussion

Z. loerzingii DNA Barcode

DNA isolated and sequenced from leaf tissue samples of *Z. loerzingii*. The result of *Z. loerzingii* unification sequencing is a nucleotide sequence with a length of 576 bp. The sequences showed a high degree of similarity in both samples with identical nucleotide composition. DNA barcodes consist of special regions that are sequenced and this data then becomes the "Barcode" of an organism to differentiate it from other organisms molecularly (Lebonah, *et al*., 2014). The composition of AT content in both samples is 56.51% and GC content is 43.49%. Variations in GC content in several *Zingiber* with *rbcL* gene locus ranges from 35,75%-36,27% (Li *et al*., 2020) and 40% (Osathanunkul *et al*., 2017). The low percentage of GC content indicates the primitiveness of a species (Hapsari, 2015).

Genetic Diversity of *Z. loerzingii*

Results of *Z. loerzingii* sample alignment with 8 species of Zingiberaceae taken from Genbank showing that there are 427 observable characteristics. There are 8 parsimony sites, 419 conserved sites and no SNP (Single Nucleotide polymorphism). Polymorphism occurs due to mutations that cause variations in the DNA sequence (Choudhuri & Kotewicz, 2014). Polymorphism can be in the form of bases that are exchanged, added or deleted in the DNA strand (Lesk, 2002).

The low diversity value or variation in the samples analyzed with the *rbcL* gene locus can be caused by the chloroplast genome being inherited from a single parent (maternal) so that recombination does not occur (Xu *et al*., 2021). Maternally inherited genes are much more

stable (Chase & Fay, 2009). Variations in DNA sequences generally occur due to mutations that occur and are maintained for a long time (Fitmawati & Hartana, 2010).

Table 1. Genetic Distance among *Z. loerzingii* and Genus *Zingiber* and out-group.

No	Spesies	1	2	3	4	5	6	7	8	9
1	L1(*Z. loerzingii*)									
2	L2 (*Z. loerzingii*)	0.000								
3	*Zingiber mioga*	0.000	0.000							
4	*Zingiber mioga*	0.000	0.000	0.000						
5	*Zingiber officinale*	0.007	0.007	0.007	0.007					
6	*Zingiber officinale*	0.007	0.007	0.007	0.007	0.000				
7	*Kaempferia galanga*	0.012	0.012	0.012	0.012	0.019	0.019			
8	*Kaempferia galanga*	0.012	0.012	0.012	0.012	0.019	0.019	0.000		
9	*Lachnanthes caroliniana*	0.059	0.059	0.059	0.059	0.064	0.064	0.066	0.066	
10	*Anigozanthos flavidus*	0.069	0.069	0.069	0.069	0.077	0.077	0.077	0.077	0.046

The genetic distance between *Z. loerzingii* and Zingiberaceae ranged from 0.000 to 0.019. The lowest difference is found in *Z. loerzingii* with a genetic distance value of 0.00, with the highest value is found in *Kaempferia galanga*. Genetic distance between *Z. loerzingii* and *Lachnanthes caroliniana* and *Anigozanthos flavidus*as *out-groups* is between 0.059 and 0.069.

The calculation of genetic distance relies on the presence or absence of substitution, both transversion and inversion (Hall, 2018). The calculation of genetic distance is very influential in explaining the taxon status of a species (Jarulis *et al*., 2021). The genetic distance for species that are still in the same taxon group generally ranges from <1.0% or <2% (Hebert, *et al*., 2003). Genetic distance that shows a number >3% indicates that the species being compared are at very different taxonomic levels.

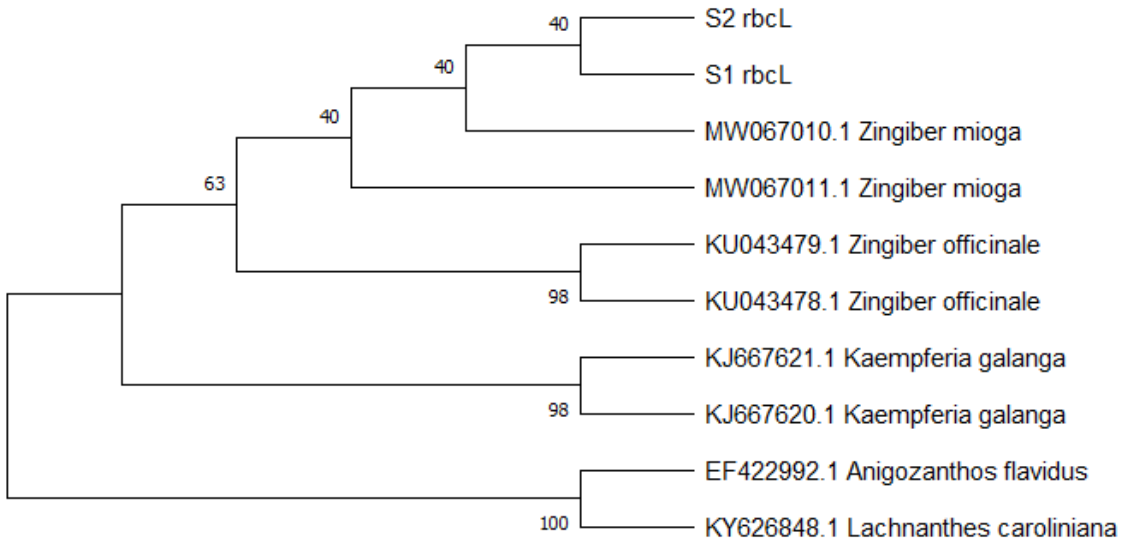
Table 2. Haplotype Diversity and Nucleotide Diversity.

Number of Haplotype	Haplotype Haplotype Variance	Hd	π
3	Hap_1: 4 [MW067011.1 *Zingibermioga* MW067010.1*Zingibermioga* S2_rbcL S1_rbcL] Hap_2: 2[KU043479.1 *Zingiber officinale* KU043478.1*Zingiber officinale*] Hap_3: 2[KJ667621.1 *Kaempferia galanga* KJ667620.1*Kaempferia galanga*]	0,7143	0,00803

Haplotype diversity analysis showed that there were 3 groups of haplotypes for all aligned species with a haplotype diversity value (Hd) of 0.7143. Nucleotide diversity value (π) is 0.00803. For both samples of *Z. loerzingii* there is 1 haplotype group with the haplotype diversity value of 0.0 and a nucleotide diversity value of 0.0. The value of diversity which shows the number 0.0 can be caused by the limited distribution area of *Z. loerzingii*, so that interbreeding or gene flow does not possible to be happened.

Phylogenetic analysis of *Z. loerzingii*

DNA barcodes have been used not only to identify living things, but also to find genetic relationships among many species (Hashim, *et al*., 2020). Previous phylogenetic analyzes of Zingiberaceae were based on morphological differences such as differences in composition of bracteole, number of flowers, and color of petals. This approach is not efficient for describing parental phylogenetic relationships. Identification technique with DNA barcodes provides a high degree of conformity in determining Zingiberaceae phylogenetic relationships (Gao *et al*., 2008).



Gambar 1. The phylogenetic tree based on the *rbcL* gene locus sequence of *Z. loerzingii* with several Zingiberaceae species and out-groups was reconstructed using the Neighbor-Joining method, Kimura-2-Parameter calculation model and bootstrap test with 1000 replications(Felsenstein, 1985).

The phylogenetic tree based on the rbcL gene locus was reconstructed in this study using the Neighbor-Joining method and resulted in 3 clades apart from the out-group. *Z. loerzingii* samples were in one clade as a monophyletic group with *Z. mioga*. In clade II there exists *Z. officinale* with bootstrap value of 98%. In Clade III there is *K. galangal* with bootstrap value of 98%. *L. caroliniana* and *A. flavidus* which is an out-group separated into its own clade with a bootstrap value of 100%.

Conclusion

Based on the results of the study, it was found that the gene locus in *Z. loerzingii* was successfully amplified with a nucleotide length of 576 bp. The composition of the GC content is lower than the AT content. Genetic distance of *Z. loerzingii* with Zingiberaceae members and the compared out-groups are in the range of 0-0.069.The alignment results show that there are 427 observable characters with 419 conserved sites, 8 parsimony sites, and no SNP regions.Haplotype analysis showed that there were 3 haplotype groups with haplotype diversity (Hd) of 0.7143 and nucleotide diversity (π) of 0.00803.Phylogenetic tree analysis using the Neighbor-Joining method separated the dendogram into 4 clades with *Z. loerzingii* separating into its own group with *Z. mioga* as a monophyletic group.

References

Alaklabi, A., Ahamed, A., Al-Qthanin, R. N., Arif, I. A., Panneerselvam, A., Al-Khulaidi, A. W. 2021. Molecular Characterization of Endangered Endemic Plant *Aloe pseudurobroviolacea* using Choloroplast *matK* and *rbcL* gene. *Saudi Journal of Biological Science*, 28: 1123-1127, https://doi.org/10.1016/j.sjbs.2020.11.042.

Arif, M.F., Aristya, G.R., Subositi, D., Sari, A.N. & Kasiamdari, R.S. 2019. Short Communication: *rbcL* and *matK* Chloroplast DNA Composition of Green Chireta (*Andrographis paniculata*) from Indonesia. *Biodiversitas Journal of Biological Diversity*, 20(12), https://doi.org/10.13057/biodiv/d201216.

Brickell, C. (ed). 2012. *Encyclopedia of Plants and Flowers*. Dorling Kindersley. New York.

CBOL Plant Working Group. 2009. A DNA Barcode for Land Plants. *Proceedings of the National Academy of Sciences of the*

United States of America, 106: 12794-12797.

Chase, M. W., and Fay, M. F. 2009. Barcoding of Plants and Fungi. *Science*, 325: 682–683, DOI: 10.1126/science.1176906.

Chen, S., Yao, H., Han, J., Liu, C, Song, J., Shi, L., Zhu, Y., Ma,X., Gao, T., Pang, X., Luo, K., Li, Y., Li, X., Jia, X., Lin, Y., and Leon C. 2010. Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS One*, 5(1): 1-8, https://doi.org/10.1371/journal.pone.0008613.

Choudhuri, S., and Kotewicz, M. 2014. *Bioinformatics for Beginners : Genes, Genoms, Molecular Evolution, Databases and Analytical Tools*. Elsevier, Inc. Maryland.

El-Atroush, H, Magdy, M., and Werner, O. 2015. DNA Barcoding of two endangered medicinal Plants from Abou Galoom protectorate. *Life Science Journal,* 12(9): 101-109. http://www.lifesciencesite.com/

Fazekas, A. J., Burgess, K. S., Kesanakurti, P. R., Graham, S. W., Newmaster, S. G. Husband, B. C., Percy, D. M., Hajibabaei, M., and Barret, S. C. H. 2008. Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well. *PLoS ONE* 3(7): e2802, https://doi.org/10.1371/journal.pone.0002802.

Felsenstein, J. 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, 39(4): 783–791, https://doi.org/10.2307/2408678.

Fitmawati, Hartana A. 2010. Phylogenetic study of Mangifera laurina and its related species using cpDNA trnL-F spacer markers. *Hayati Journal of Biosiences* 17 (1): 9-14, https://doi.org/10.4308/hjb.17.1.9

Ford, C. S., Ayres, K. L. Toomey, N., Haider, N., Staahl, J. V. a., Kelly, L. J. Wikstrom, N., Hollingsworth, P. M., Duff, R. J., Cowen, R. S., Chase, M. W., & Wilkonson, M. J. 2009. Selection of Candidate coding DNA Barcoding Regions for Use on land Plants. *Botanical Journal of the Linnean Society,* 159(1): 1-11, https://doi.org/10.1111/j.1095-8339.2008.00938.x.

Gao, L., Nian, L., Huang, B., & Hu, X. 2008. Phylogenetic analysis and genetic Mapping of Chinese *Hedychium* using SRAP Markers. *Scientia Horticulturae*, 117: 369–377.

Geneid. 2020. *Geneaid™ Genomi DNA Extraction and Purification Protocol*. Geneaid Biotech Ltd. http://www.geneaid.com

Hall, B. G. 2018. *Phylogenetic Tree Made Easy : A How-to Manual*. Oxford University Press. Oxford.

Hapsari, L. 2015. *Keragaman dan Kekerbatan Genetik Pisang (*Musa l*.) di Jawa Timur berdasarkan sekuen daerah* Internal Transcribed Spacer*.* Universitas Brawijaya. Malang.

Hashim, A. M., Alatawi, A., Al-Taf, F. M., Qari, S. H., Elhady, M. E., A., Osman, G. H., Abouseadaa, H. H. 2020. Phylogenetic Relationship and DNA barcoding of Nine Endangered Medicinal Plant Species Endemic to Saint Katherine Protectorate. *Saudi Journal of Biological Science*, 28: 1123-1127, https://doi.org/10.1016/j.sjbs.2020.12.043.

Hebert, P. D. N., Cywinska, A., Ball, S. L., and DeWaard, J. R. 2003. Biological Identifications through DNA Barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270: 313–321. https://doi.org/10.1098/rspb.2002.2218.

Hosein, F. N., Maharaj, S., Johnson, W., Rostant, L., Ramdass, A. C., & Rampersad, S. N., 2017. Utility of DNA Barcoding to Identify Rare Endemic Vascular Plant Species in Trinidad. *Ecology and Evolution*, (7): 7311-7333, https://doi.org/10.1002/ece3.3220.

Hu, S. J., Hu, H. Y., Gao, H., Xia, L., and Chen, S. L. 2019. DNA Barcoding and Rapid Identification of the Precious Herb Herba *Anoectohili*. *Chinese Journal of Natural Medicines*, 17(10): 738-745,

https://doi.org/10.1016/S1875-5364(19)30090-1.

Hubert, N.,and Hanner, R. 2015. DNA Barcoding, Species Delineation and Taxonomy: A Historical Perspective. *De Gruyter Open*, 3: 44–58, https://doi.org/ 10.1515/dna-2015-0006.

Ismail, M., Ahmad, A., Nadeem, M., Javed, M. A., Khan, S. H., Khawaish, I., Sthanadar, A. A., Qari, S. H., Alghanem, S. M., Khan, K. A., Khan, M. F., and Qamer, S. 2020. Development of DNA barcodes for selected *Acacia* species by using rbcL and matK DNA markers. Saudi Journal of Biological Sciences, 27:3735-3742. https://doi.org/10.1016/j.sjbs.2020.08.020

Jarulis, Muslim, C., Kamilah, S. N., Utama, A. F., Permana, D., Sari, M. M., Prayitno, A. H. and Jannah, I. M. 2021. DNA barcode of Enggano hill myna, *Gracula religiosa enganensis* (Aves: Sturnidae) based on mitochondrial DNA cytochrome oxidase subunit I. *Biodiversitas*, 22(3): 1635-1643. https://doi.org/10.13057/biodiv/d220407

Kim, H. M., Oh, S. H., Bhandari, G. S., Kim, C. S., and Park, C. W. 2014. DNA Barcoding of Orchidaceae in Korea. *Molecular Ecology Resources*, 14: 455-507, https://doi.org/10.1111/1755-0998.12207.

Kress, W.J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., and Janzen, D. H. 2005. Use of DNA Barcodes to Identify Flowering Plants. *Proceedings of the National Academy of Sciences of the United States of America*, 102(23): 8369-8374, https://doi.org/10.1073/pnas.0503123102.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35(6): 1547–1549, https://doi.org/10.1093/molbev/msy096.

Lebonah, D. E., Dileep, A., Chandrasekhar, K., Sreevani, S., Sreedevi, B., and Pramoda, J. 2014. DNA Barcoding on Bacteria : A Review. *Advances in Biologi*, 12:1-9 http://dx.doi.org/10.1155/2014/541787

Lesk, A. M. 2002. *Introduction to Bioinformatics*. Oxford University Press. Oxford.

Li, D. M., Ye, Y. J., Xu, Y. C., Liu, J. M., and Zhu, G. F. 2020. Complete Chloroplast Genomes of *Zingiber Montanum* and *Zingiber zerumbet*: Genome Structure, Comparative and Phylogenetic Analyses. *PLoS ONE,* 15(7):1-19, https://doi.org/10.1371/journal.pone.0236590.

Nurainas and Ardiyani, M. 2019. *Zingiber loerzingii*. *The IUCN Red List of Threatened Species* 2019:e.T117465518A124284822. https://dx.doi.org/1.0.2305/IUCN.UK.2019.2.RLTS.T117465518A124284822.en. Accessed on 08th February 2021.

Osathanunkul, M., Ounjai, S., Osathanunkul, R., and Madesis, P. 2017. Evaluation of a DNA-Based Method for Spice/herb Authentication, So You Do Not Have to Worry about What Is in Your Curry, Buon Appetito! *PLoS ONE*, 12(10):1-12, https://doi.org/10.1371/journal.pone.0186283.

Rahayu, D.A., dan Nugroho, E. D. 2015. *Biologi Molekuler dalam Perspektif Konservasi*. Plantaxia. Yogyakarta.

Rugayah, Yulita, K.S., Arifiani, D., Rustiami, H., & Girmansyah, D. (eds). 2017. *Tumbuhan Langka Indonesia : 50 Jenis Tumbuhan Terancam Punah*. LIPI PRESS. Jakarta.

Vinitha, M.R., Kumar, U.S., Aishwarya, K., Sabu, M., and Thomas, G. 2014. Prospects for Discriminating Zingiberaceae Species in India Using DNA Barcodes. *Journal of Integrative Plant Biology*, 56(8): 760-773, https://doi.org/ 10.1111/jipb.12189.

Youm, J. W., Han S. W., Seo, S. W., Lim, C. U., and Oh, S. H. 2016. DNA Barcoding of Schisandraceae in Korea. *Korean Journal Of Plant Taxonomy*, 46(3): 273-282,

https://doi.org/10.11110/kjpt.2016.46.3.273.

Xu, J., Zang, F., Wu, Q., Wang, Y., Wang, B., Huang, P., Zang, D., Ma, Y., Zheng, Y. 2021. Analysis of the Genetic Diversity and Molecular Phylogeography of the Endangered Wild Rose (*Rosa Rugosa*) in China Based on Chloroplast Genes. *Global Ecology and Conservation*, 28:1-12, https://doi.org/10.1016/j.gecco.2021.e01653