International Conference on Biology Education, Natural Science, and Technology
Vol. 2 No. 1 (2024)
E-ISSN : 3026-5428

In Cilico Study and Genetic Variation of Genus Rhododendron using DNA Barcode trnL–trnF Intergenic Spacer

Syifara Chika*
[1]Master of Biology Study Program, Faculty of Science and Mathematics, Universitas Diponegoro, Semarang 50275, Jawa Tengah, Indonesia
*Corresponding Author. E-mail address: syifarachika7@gmail.com

KEYWORDS:
DNA barcoding, in cilico, Rhododendron, trnL-trnF

ABSTRACT

Rhododendron is a flowering plant which is the largest genus in the Ericaceae family. Around 1,157 *Rhododendron* species have been assessed using the IUCN Red List criteria, and 316 are categorized as endangered species. Apart from maintaining species population numbers, conservation activities also need to pay attention to genetic aspects through DNA barcoding techniques. This research uses molecular in-silico methods using sequence data from NCBI's GenBank. This research aims to carry out an inventory of *Rhododendron* types based on genetic aspects, analyzing genetic variations and kinship relationships in the *Rhododendron* Genus. The resulting phylogenetic tree is divided into three clades in the *Rhododendron* group and one outgroup. The phylogenetic tree results show that the *trnL-trnF* DNA barcode can differentiate and identify up to species level in the genus *Rhododendron*. The *trnL-trnF* DNA barcode region of the *Rhododendron* genus is mostly conserved because it only has eight genetic variations, namely T71C, G225C, T245C, T229A, T485C, A491G, T705C, and A783G. The *trnL-F* gene is an informative chloroplast and can show the relationship between types

1. INTRODUCTION

Rhododendron is a flowering plant, the largest genus in the Ericaceae family. The word *Rhododendron* comes from two syllables originating from Greek, namely Rhodos (rose) and dendron (tree), which means rose tree (Mellieve, 2002; Masnawati et al., 2017). The *Rhododendron* genus is a flowering plant of which more than 1,000 species have been recorded with an average height of 1.5 m, a habitus ranging from shrubs to small trees, terrestrial or epiphytic (Sleumer, 1966). The *Rhododendron* genus is often found in mountain forests at altitudes up to 4,000 m above sea level, but some are also found in lowland and mangrove environments. These plants can grow on grassy ground and rocks. The *Rhododendron* genus can grow well in acidic soil with a pH range of 4.5-5.5 (Bowers, 1960; Sekar & Srivastava 2010; Putri, 2011). Based on its economic and ecological aspects, the *Rhododendron* genus has crucial aesthetic value, namely its flower morphology, which is unique, beautiful, and attractive (Goetsch et al., 2005). In the Papua region, the presence of *Rhododendron* species is utilized in ecotourism activities, which can increase the use value of this genus for the economy of the surrounding community (Beljai et al., 2016; Putri & Warseno, 2020). Around 1,157 *Rhododendron* species have been assessed using the IUCN Red List criteria, and 316 are categorized as endangered species (Gibbs et al., 2011; Putri & Warseno, 2020). In the Malesia region, several *Rhododendron* species are potentially threatened with extinction due to habitat destruction. Differences in pollinators and flowering time are also contributing factors causing *Rhododendron* to become threatened (Singh & Gurung, 2009). The availability of very few seeds/saplings in nature has also caused many *Rhododendron*s to be threatened (Semwal & Purohit 1980).

There is a tendency for the population of the genus *Rhododendron* to be threatened globally, making conservation efforts for this plant important. The initial steps that can be taken to preserve *Rhododendron*s are exploration, characterization based on morphology and molecular, and grouping according to character (Hamid et al., 2017). Besides maintaining species population numbers, conservation activities must consider genetic aspects (Chika et al., 2024). Conservation activities provide various information, such as bioecology and taxonomy, including genetic information (Nuryanto & Solihin, 2006). This information is necessary to determine appropriate conservation methods (Saleky & Dailami, 2021). Molecular identification is a step in plant breeding efforts, conservation biology, and other aspects of plant science (Hanifa et al., 2021). DNA barcoding is a technique that uses one or several DNA regions with short sequences to identify a species (Hebert et al., 2003). DNA barcodes that are very often used for DNA barcoding analysis in plants are *trnL-F* (de Groot et al., 2011), ITS (Chen et al., 2011), *rbc*L (de Groot et al., 2011), matK (Techen et al., 2014), and trnH-psbA (Zhang et al., 2014). The *trnL-trnF* DNA barcode is an informative chloroplast gene and can show relationships between types. The *trnL-trnF* genes are very suitable for identification up to the species level, uniparental, or detecting up to the hybrid level (Hoggard et al., 2004; Khaira et al., 2024). Research on the genus *Rhododendron* using the DNA barcoding approach has not been carried out much. Therefore, initial studies on the analysis of kinship relationships in the genus *Rhododendron* using the in silico-based DNA Barcode *trnL–trnF* Intergenic Spacer need to be carried out. This research aims to carry out an inventory of *Rhododendron* types based on genetic aspects, analyzing genetic variations and kinship relationships in the *Rhododendron* Genus.

2. MATERIALS AND METHODS

This research uses molecular in-silico methods using sequence data originating from GenBank NCBI (National Center for Biotechnology Information). The research steps are as follows:

2.1. Rhododendron Sequence Selection on the NCBI website

Sequences were searched and selected on the NCBI website (https://www.ncbi.nlm.nih.gov/) by choosing the nucleotide category and writing the keywords "*Rhododendron*" *trnL-trnF*. Next, the sequences that have been obtained are subjected to a BLAST (Basic Local Alignment Search Tool) process, and several target sequences are selected by considering the appropriate sequence length, the highest query cover value, and the highest percent identity value. The outgroup sequence used is from the *Andromeda polifolia* species. All selected nucleotide sequences are then downloaded and saved in FASTA format.

2.1.1. Analysis of Genetic Data of the Genus Rhododendron

Several sequences that have been downloaded in FASTA format are then analyzed in the MEGA 11 application (Tamura et al., 2021). The sequence was aligned using the ClustalW method. The alignment process is based on sequence homology to identify sequences with similarities. Sequence alignment is a way of arranging DNA sequences to identify the same or different regions in the nitrogen base sequence, which is a consequence of the evolutionary relationship between sequences from several related species (Wiltgen, 2019). Genetic variation analysis was performed using the MULTALIN website-based application (Multiple Sequence Alignment by Florence Corpet) via link http://multalin.toulouse.inra.fr/multalin/.

2.1.1.1. Phylogenetic Tree Reconstruction and Genetic Distances Analysis

Phylogenetic tree reconstruction was carried out using the MEGA 11 application (Tamura et al., 2021). The phylogenetic tree was reconstructed using the Maximum Likelihood method and the Tamura 3-parameter model with a bootstrap value of 1000x (Tamura et al., 1992). Next, genetic

distance analysis was carried out using the pairwise deletion method with the maximum composite likelihood model in the MEGA 11 application (Tamura et al., 2021).

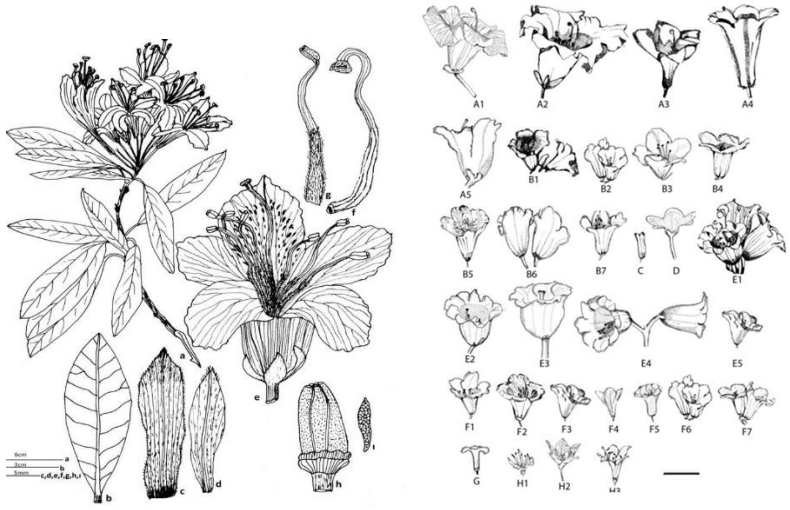
3. RESULTS AND DISCUSSION

3.1. Description and Morphology of the Genus Rhododendron

Rhododendron is better known in Indonesia by the common name Azalea, and its growing location has different regional names. The distribution of *Rhododendron*s in Indonesia is as follows: Sumatra has 22 types, Java has seven kinds, Nusa Tenggara has three types, Kalimantan has 55 types, Sulawesi has 29 kinds, Maluku has nine types, and Papua has 113 types (Hiller & Pollard, 2010). This plant has aesthetic value because its flowers are colorful, have various shapes according to their type, and also have a distinctive aroma, so they have the potential to be used as ornamental plants. In mountainous areas with cold temperatures, the color of *Rhododendron* flowers becomes sharper and more attractive to the eye (Wawo et al., 2021). According to GBIF (2023), the classification of the genus *Rhododendron* is as follows:

Kingdom : Plantae
Phylum : Tracheophyta
Class : Magnoliopsida
Order : Ericales
Family : Ericaceae
Genus : *Rhododendron* L.

Rhododendron is a flowering plant that appears to be a shrub or small tree, terrestrial or epiphytic. It has the general character of brown scales under the leaves or on other organs. The stem often swells at the base. The leaves have stalks, long and spirally arranged, facing each other, sometimes alternately. Compound interest. Pansy flower; curved petals, tubular or bell-shaped corolla, asymmetrical; stamens consist of 5-10, stuck at the base of the flower corolla, and are often unbalanced; The anther stalk is long, and the anther head is upright, generally facing inward. The ovary is attached to the base of the flower. The fruit is a capsule that rises upwards and has several seeds. Seeds are numerous, small, and thin (Sleumer, 1966). *Rhododendron*s usually form in groups, with the flowers at the branches' ends. The flowers are usually single and colorful. Some have contrasting spots (flares) and have a scent (*R. dalhousiae*, *R. edgeworthii*, *R. johnstoneanum*, etc.) (Goetsch et al., 2005; Beljai et al., 2016). The morphology of several *Rhododendron* species is presented in Figure 1.



A **B**

Figure 1. (A) *Rhododendron ponticum* subsp. ponticum. a : plant; b : leaf; c : bracts; d : bracteols; e : flower; f : pistil; g : stamen; h : capsule; i : nutlets. (Küçük et al., 2018).

(B) Floral morphology of Rhododendron. Type A: A1, R. griffithianum; A2, R. lindleyi; A3, R. maddenii; A4 R. dalhousiae var. rhabdotum; A5, R. edgeworthii. Type B: B1, R. lanatum; B2, R. ciliatum; B3, R. campylocarpum; B4, R. wallichii; B5, R. tsariense; B6, R. aeruginosum; B7, R. wightii. Type C, R. keysii. Type D, R. baileyi. Type E: E1, R. kesangiae; E2, R. hogsonii; E3, R. falconeri; E4, R. grande; E5, R. niveum. Type F: F1, R. argipeplum; F2, R. thomsonii; F3, R. barbatum; F4, R. cinnabarinum; F5, R. neriiflorum; F6, R. arboreum; F7, R. succothii. Type G, R. anthopogon. Type H: H1, R. setosum; H2, R. triflorum; H3, R. virgatum (Namgay & Sridith, 2021).

*Rhododendron*s are often found in cool weather areas (temperate climate), especially on mountain peaks 800-6000 m above sea level. However, it grows well at 3001-3500 m above sea level (Sekar & Srivastava, 2010). It can grow and adapt to lower altitudes and hot weather, but its development could be more optimal. Environmental factors such as temperature and humidity greatly influence the number of types of *Rhododendron*s that flower and bear fruit. *Rhododendron*s will grow well with good water and air circulation, sufficient water conditions, and no excess or lack of water (Kelley & Drain, 1994; Masnawati et al., 2017). The community has widely used several types of *Rhododendron* as medicinal and ornamental plants. In the Malesiana area, *Rhododendron* has been widely used as an ornamental plant and is an essential horticultural commodity, be it the original type or the hybridized one. Apart from that, some Southeast Asians use several types of *Rhododendron* as traditional medicines. According to several studies conducted in Indonesia, another potential of *Rhododendron* is as an antibacterial (*R. kanori* and *R. macgregoriae*) and producer of flavonoid compounds (*R. javanicum* and *R. macgregoriae*). *Rhododendron* mucronatum varieties are also helpful for relieving fever, gland stimulants, rheumatism, and coughs (Putri & Sudiatna, 2009; Beljai et al., 2016).

3.1.1. In cilico analysis of Rhododendron based on DNA Barcode trnL-trnF

A total of 13 *Rhododendron* nucleotide sequences and one outgroup based on the DNA barcode *trnL-trnF* underwent phylogenetic tree reconstruction. The outgroup used in this research is the *Andromeda polifolia* species, which is still in the same family as *Rhododendron*, namely the Ericaceae family. The outgroup aims to determine the primitive characters (plesiomorphs) and derivative characters (apomorphies) of the in-group group and to determine the starting point for the formation of a phylogenetic tree (Subari et al., 2021). A reconstruction of the phylogenetic tree of the genus *Rhododendron* is presented in Figure 2.

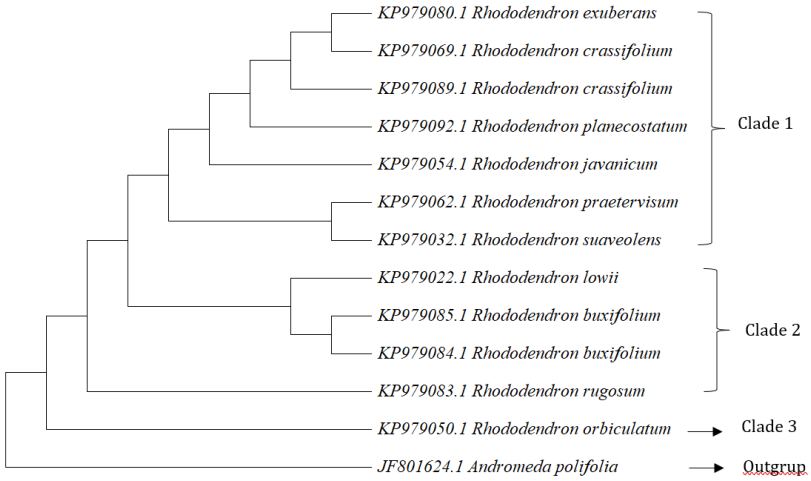


Figure 2. Reconstruction of the phylogenetic tree of the genus *Rhododendron* based on the *trnL-trnF* DNA barcode using the Maximum Likelihood method and the Tamura 3-parameter model with a bootstrap value of 1000x

Phylogenetic tree reconstruction was carried out using Maximum Likelihood and Tamura 3-parameter models with a bootstrap value 1000x. This method and model are used based on the model program in the MEGA11 application, which recommends methods and models for reconstructing phylogenetic trees of all sequences (Tamura et al., 1992). The resulting phylogenetic tree is divided into three clades in the *Rhododendron* group and one outgroup. The in-group in clade 1 consists of *R. exuberans*, *R. crassifolium*, *R. planecostatum*, *R. javanicum*, *R. praetervisum*, and *R. suaveolens*. The group in clade 2 consists of *R. rugosum*, *R. lowii*, and *R. buxifolium*. The in group in clade 3 only consists of one species, *R. orbiculatum*. The outgroup is the species *Andromeda polifolia*. The phylogenetic tree also shows that *R. crassifolium* accession KP979069.1 groups with *R. crassifolium* accession KP979089.1 and *R. buxifolium* accession KP979085.1 and *R. buxifolium* accession KP979084.1 are also in the same clade and branch. This shows that the *trnL-trnF* DNA barcode can differentiate and identify up to species level in the genus *Rhododendron*. The figure's cladogram indicates that the *Rhododendron* species in the in-group are grouped within each clade based on similarities in nucleotide sequence. The similarities and differences in characters between these species are used to determine their kinship relationships (Anafarida & Badruzsaufari, 2020).

Phylogenetic analysis aims to compile phylogenetic relationships which is generally depicted in a line that branches like a tree, called a phylogenetic tree (Irawan, 2013). A phylogenetic tree is a tree that shows the evolutionary line of different species, organisms, or genes from a common ancestor. Phylogeny helps know biological diversity, compile classifications, and explain phenomena during the evolutionary process (Baum, 2008). Maximum Likelihood is a character-based statistical method that compares all sequences in an alignment to calculate the probability value for each tree (Yang & Rannala, 2012). This method considers all possible numbers of changes/mutations in the sequence for each tree. Therefore, it is suitable for reconstructing phylogenetic trees with few sequences. A phylogeny test was used as a bootstrap method that carried out repeated resampling to see the tree arrangement's validity level, with several replications of 1000x.

Analysis of genetic variation in the genus *Rhododendron* is presented in Figure 3. The length of the entire *Rhododendron trnL-trnF* sequence is 894 bp. Based on this image, it shows that the *trnL-trnF* DNA barcode region of the *Rhododendron* genus is mostly conserved because it only has eight genetic variations, namely T71C, G225C, T245C, T229A, T485C, A491G, T705C, and A783G. The *trnL-F* gene is an informative chloroplast and can show the relationship between types. This region is located in the large single-copy region of the chloroplast genome. This region consists of the *trnL* gene, an intron group, and the *trnL-trnF* intergenic spacer (Hao et al., 2009). The *trnL-F* gene is a sequence located in the *trnL* (UAA) 5'exon to *trnF* (GAA), which is then called *trnL-F* (Adjie et al., 2008; Khaira et al., 2024). Next, all *Rhododendron* sequences were characterized to determine the nucleotide composition of each sequence. The nucleotide composition of the genus *Rhododendron* using the *trnL-trnF* DNA barcode is presented in Figure 4.

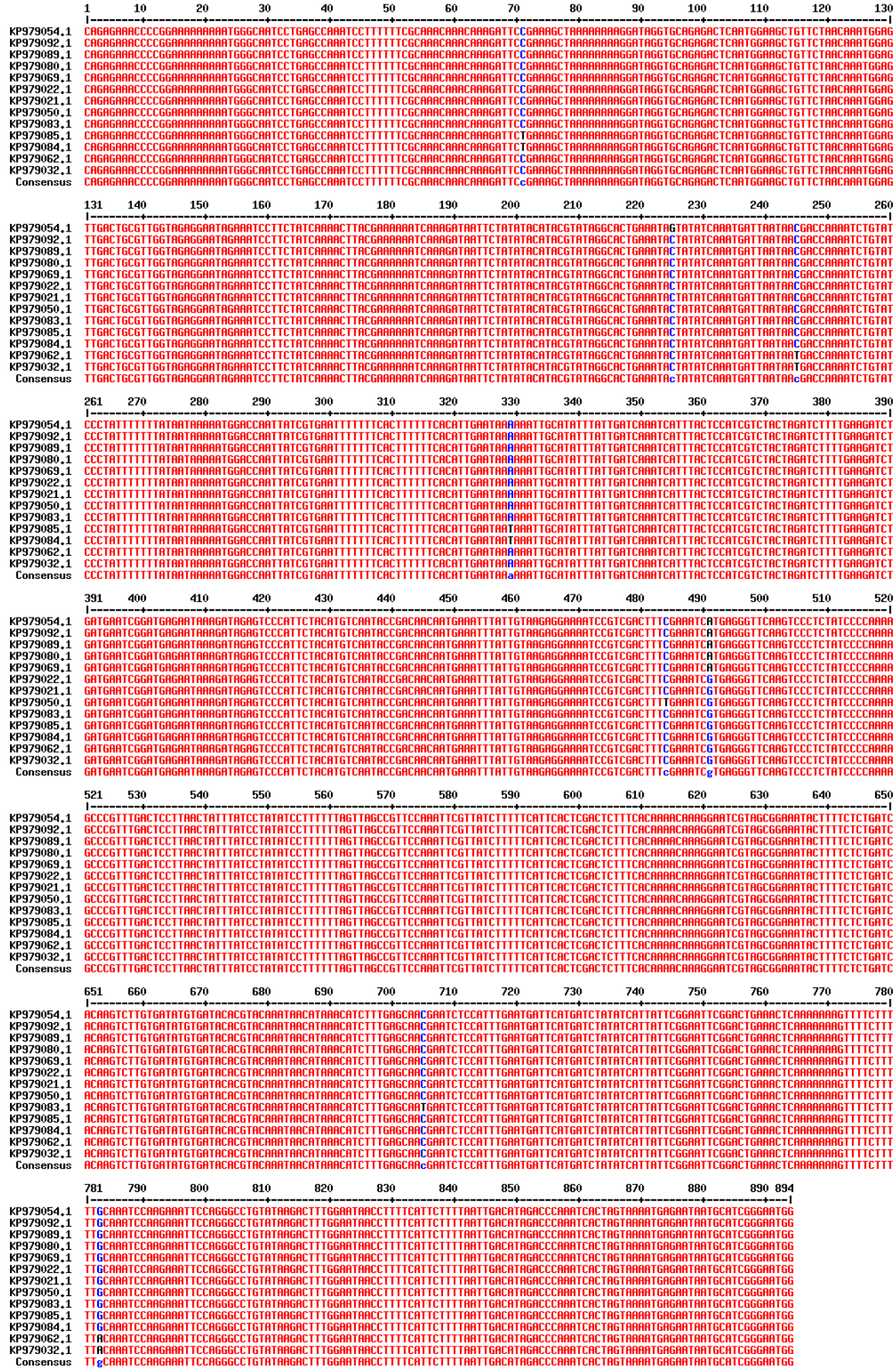


Figure 3. Genetic variation of the *Rhododendron* genus based on the *trnL-trnF* DNA barcode analyzed using the MULTALIN (Multiple Sequence Alignment by Florence Corpet)

		T(U)	C	A	G	Total
KP979054.1	Rhododendron javanicum	30.6	18.5	35.9	15.0	894
KP979092.1	Rhododendron planecostatum	30.6	18.6	35.9	14.9	894
KP979089.1	Rhododendron crassifolium	30.6	18.6	35.9	14.9	894
KP979080.1	Rhododendron exuberans	30.6	18.6	35.9	14.9	894
KP979069.1	Rhododendron crassifolium	30.6	18.6	35.9	14.9	894
KP979022.1	Rhododendron lowii	30.6	18.6	35.8	15.0	894
KP979083.1	Rhododendron rugosum	30.8	18.5	35.8	15.0	894
KP979050.1	Rhododendron orbiculatum	30.8	18.5	35.8	15.0	894
KP979085.1	Rhododendron buxifolium	30.9	18.5	35.7	15.0	894
KP979062.1	Rhododendron praetervisum	30.8	18.5	35.9	14.9	894
JF801624.1	Andromeda polifolia	31.5	17.1	35.5	15.9	889
KP979084.1	Rhododendron buxifolium	30.9	18.5	35.7	15.0	894
KP979032.1	Rhododendron suaveolens	30.8	18.5	35.9	14.9	894
Avg.		30.8	18.4	35.8	15.0	893.6

Figure 4. Nucleotide composition of the genus *Rhododendron* using DNA barcode *trnL-trnF*

Based on Figure 4, the *Rhododendron* genus has a nucleotide composition with the highest percentage of thymine (T) and adenine (A), 30.8% thymine and 35.8% adenine. The higher the content of the A and T base pairs, the higher the melting point of DNA. This is because the A and T pairs are more stable and require more heat energy to decompose than the G and C pairs. The high percentage of AT content compared to GC content is influenced by the location of the amplified *trnl-F* gene, which has many nucleotide substitutions. The low percentage of CG content indicates that this species is more primitive, as seen from the average calculation results (Hapsari et al., 2015; Khaira et al., 2024).

4. CONCLUSIONS

Based on this research, the phylogenetic results show that the *trnL-trnF* DNA barcode can differentiate and identify up to species level in the genus *Rhododendron*. The *trnL-trnF* DNA barcode region of the *Rhododendron* genus is mostly conserved because it only has eight genetic variations, namely T71C, G225C, T245C, T229A, T485C, A491G, T705C, and A783G. *Rhododendron* has a nucleotide composition with the highest percentage of thymine (T) and adenine (A), namely 30.8% thymine and 35.8% adenine.

5. REFERENCES

Adjie, B., Takamiya, M., Ohta, M., Ohsawa, T. A., & Watano, Y. (2008). Molecular phylogeny of the lady fern genus *Athyrium* in Japan based on chloroplast *rbc*L and *trnL-trnF* sequences. *Acta phytotaxonomica et geobotanica*, 59(2), 79-95.

Anafarida, O., & Badruzsaufari, B. 2020. Analisis Filogenetik Mangga (*Mangifera* Spp. Berdasarkan Gen 5,8S rRNA. *Ziraa'ah*, 45(2), 120–126.

Baum, D. (2008). Reading a phylogenetic tree: the meaning of monophyletic groups. *Nature Education*, 1(1), 190.

Beljai, M., Runtuboi, Y. Y., Manuhua, D., Worabai, M. S., & Renwarin, D. M. (2016). Aspek ekowisata *Rhododendron*: Tinjauan potensi dan strategi pengembangannya di daerah Intan Jaya. *Jurnal Kehutanan Papuasia*, 2(1), 24-33.

Bowers, C.G. 1960. *Rhododendron and Azaleas (Their Origins, Cultivation, and Development). The Macmillan Company*. New York. 525 pp.

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., & Leon, C. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *Journal PLoS ONE*, 5(1).

Chika, S., Meilina, T. D., Arfan, M. R., Febriana, A., & Mukaromah, A. S. (2024). Genetic Variation of Wax Apple (*Syzygium samarangense* (Blume) Merr. & LM Perry) Cultivar using Internal Transcribed Spacer 2 (ITS2). *Floribunda*, 7(4), 153-165.

de Groot, G. A., During, H. J., Maas, J. W., Schneider, H., Vogel, J. C., & Erkens, R. H. (2011). Use of *rbc*L and *trnL-F* as a two-locus DNA barcode for identification of NW-European ferns: an ecological perspective. *PLoS one*, 6(1), e16371.

GBIF Secretariat: GBIF Backbone Taxonomy. https://doi.org/10.15468/39omei Accessed via https://www.gbif.org/species/5284517 [30 May 2024]

Gibbs, D., Chamberlin, D., & Argent, G. (2011). *The Red List of Rhododendrons*. Richmond, UK: Botanic Gardens Conservation.

Goetsch, L., Eckert, A. J., & Hall, B. D. (2005). The molecular systematics of *Rhododendron* (Ericaceae): a phylogeny based upon RPB2 gene sequences. *Systematic Botany*, 30(3), 616-626.

Hanifa, Y. R., Pujiyanto, S., Ferniah, R.S., & Kusumaningrum, H.P. 2021. Identifikasi Molekuler Jeruk Nipis Tegal Berdasarkan Fragmen Gen 18s Ribosomal RNA. Jurnal Bioteknologi & Biosains Indonesia, 8(2), 244–254.

Hao, D. C., Huang, B. L., Chen, S. L., & Mu, J. (2009). Evolution of the Chloroplast *trnLtrnF* region in the gymnosperm lineages taxaceae and cephalotaxaceae. *Biochemical Genetics*, 47(5–6): 351–369

Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270 (1512): 313–321.

Hoggard, G. D., Kores, P. J., Molvray, M., & Hoggard, R. K. (2004). The phylogeny of *Gaura* (Onagraceae) based on ITS, ETS, and *trnL-F* sequence data. *American Journal of Botany*, 91(1), 139-148.

Kelley, J. D. and O. Drain (1994). Top Causes of Death in *Rhododendron*s. *Journal of the American Rhododendron Society*. 48 (2): 85.

Khaira, N., Tambunan, E. P. S., & Idami, Z. (2024). DNA Barcoding of Harimonting (*Rhodomyrtus tomentosa*) using the *trnL-F* Gene. *BEST Journal (Biology Education, Sains and Technology)*, 7(1), 373-379.

Küçük, S., Kürkçüoğlu, M., & Başer, K. (2018). Morphological, indumentum and chemical characteristics and analysis of the volatile components of the flowers of *Rhododendron ponticum* L. subsp. ponticum (Ericaceae) of Turkish origin. *Rec. Nat. Prod*. 12:5. 498-507

Masnawati, M., Pitopang, R., & Suleman, S. (2017). Inventarisasi Jenis-jenis *Rhododendron* (*Ericaceae*) pada Hutan Pegunungan Taman Nasional Lore Lindu (TNLL) Sulawesi Tengah. *Biocelebes*, 11(2).

Melleville, R. D. (2002). *The Rhododendron of Nepal*. Himal Books. Lalitpur. Nepal.

Namgay, S., & Sridith, K. (2021). The morphological variation of the genus *Rhododendron* (Ericaceae) in Himalayan Ranges of Bhutan. *Tropical Natural History*, 21(2), 299-320.

Nuryanto, A., & Solihin, D.D., 2006. Variasi Sekuens Gen Mitokondrial Sitokrom C Oksidase I dari Siput Lola (*Trochus niloticus*). *Biosfera*, 23(1):31–37. doi: 10.20884/1.mib.2006.23.1.143.

Putri, D.M.S. 2009. Masa berbunga Beberapa Jenis *Rhododendron* spp. (Sub.Sect.Vireya) Koleksi Kebun Raya Bali. *Prosiding Seminar Peranan Konservasi Flora Indonesia Dalam Mengatasi Dampak Pemanasan Global*. Kebun Raya Bali, 14 Juli 2009. Hal 196-205.

Putri, D. M. (2011). Fenologi *Rhododendron* spp.(subgenus Vireya) Koleksi Kebun Raya Eka Karya Bali. *Jurnal Hortikultura*, 21(3).

Putri, D. M., & Warseno, T. (2020). Variasi Status Air pada *Rhododendron javanicum* Benn. *Jurnal Penelitian Hutan dan Konservasi Alam*, 17(2), 143-153.

Saleky, D., & Dailami, M. (2021). Konservasi genetik ikan kakap putih (*Lates calcarifer*, Bloch, 1790) melalui pendekatan DNA barcoding dan analisis filogenetik di sungai Kumbe Merauke Papua. *Jurnal Kelautan Tropis*, 24(2), 141-150.

Sekar, C.K. and S.K. Srivastava. 2010. *Rhododendron*s in Indian Himalayan Region: Diversity and Conservation. *Am J. Plant Sci*. 1:131-137.

Semwal, J. K. and Purohit, A. N. Germination of Himalayan Alpine and Temperate Potentilla. *Proc. Indian Acad.Sci.* 1980;89 61–65.

Singh, K.K., Kumar, S. and Pandey, A. 2008a. Soil Treatments for Improving Seed Germination of Rare and Endangered Sikkim Himalayan *Rhododendron*s. *World Journal of Agricultural Sciences*; 4 (2):288-296.

Sleumer, H. (1966). *Flora Malesiana, Seri 1, Seed plants: Rhododendron*. Vol. 6(4). Groningen: Noordhoff.

Subari, A., Razak, A., & Sumarmin, R. 2021. Phylogenetic Analysis of *Rasbora* spp. Based on the

Mitochondrial DNA COI gene in Harapan Forest. *Jurnal Biologi Tropis*, 21(1), 89–94. https://doi.org/http://dx.doi.org/10.29303/jbt.v21i1.2351

Tamura K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution* 9:678-687.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027.

Techen, N., Parveen, I., Pan, Z., & Khan, I. A. (2014). *DNA barcoding of medicinal plant material for identification. In Current Opinion in Biotechnology*, 25 (1): 103–110. Elsevier Ltd.

Wawo, A. H., Hidayat, R. A., Setyowati, N., & Lestari, P. (2021). Keragaman Koleksi *Rhododendron* dan Tanggapan Bunganya Terhadap Suhu Udara di Sekitarnya Serta Kendala dalam Pengayaan Jenisnya di Kebun Raya Biologi Wamena. Biota: *Jurnal Ilmiah Ilmu-Ilmu Hayati*, 203-215.

Wiltgen, B. J., & Goel, A. K. (2019). A computational theory of evaluation in creative design. IBM *Journal of Research and Development*, 63(1), 1-1.

Yang, Z., & Rannala, B. (2012). Molecular phylogenetics: principles and practice. *Nature reviews genetics*, 13(5), 303-314.

Zhang, D., Duan, L., & Zhou, N. (2014). Application of DNA barcoding in Roscoea (Zingiberaceae) and a primary discussion on taxonomic status of *Roscoea cautleoides* var. pubescens. *Journal Biochemical Systematics and Ecology*, 52: 14–19.