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Muhammad Haidar Amrullah, Annisa Sholikhah, Farida Aryani Dian, et al.

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Phylogenetic Study of Cantigi Ungu (Vaccinium varingifolium) Based on Universal Internal Transcribed Spacer (ITSu) DNA Barcoding

Muhammad Haidar Amrullah\textsuperscript{1,3}, Annisa Sholikhah\textsuperscript{2}, Farida Aryani Dian\textsuperscript{3}, Elhah Nailul Khasna\textsuperscript{3}, I Kade Karisma Gita Ardana\textsuperscript{1,3}, ‘Ainun Sayyidah Zakiyah\textsuperscript{1,3}, Tita Putri Milasari\textsuperscript{1}, Nur Diniyah\textsuperscript{1,3} and Dwi Listyorini\textsuperscript{1,3,a)"

\textsuperscript{1}Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang Jl Semarang, Malang 65145, Indonesia
\textsuperscript{2}Faculty of Biology, Universitas Gadjah Mada, Bulaksumur, Yogyakarta 55281, Indonesia
\textsuperscript{3}Biotechnology Division, Central Laboratory of Mineral and advanced Material, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jl Semarang, Malang 65145, Indonesia

\textsuperscript{a)Corresponding author: listyorini.aljabari@um.ac.id

Abstract. Cantigi Ungu (Vaccinium varingifolium) is a Javanese endemic plant found in mountainous area. In Java Island, it has various local names, i.e Cantigi Ungu, Mentigi, Manis Rejo, and Delima Manda. This plant is usually found at an altitude of from 1800 until 2000m above sea level. There have not been many reports of genetic data regarding this plant. Genetic conservation is needed to determine the conservation status to maintain the sustainability, availability, and existence of germplasm this plant can be known. Thus, in thesers to the conservation of this plant, in this research, using 3 individual of Vaccinium varingifolium from a different place is compared based on ITS ITS gene sequences. ITS gene amplification was performed using Forward primer ITS \textsubscript{u1} 5’-GGA AGK ARA AGT CGT AAC AAG G-3 and Reverse primer ITS \textsubscript{u4} 5’-RGF TTC TTT TCC TCT GCT TA-3. The phylogenetic analysis using MEGA X with Minimum Likelihood (ML), Minimum Evolution (ME), Neighbor Joining (NJ), and Maximum Parsimony (MP) methods shows that the samples this study is the same species with Vaccinium varingifolium.

INTRODUCTION

Cantigi Ungu or Vaccinium varingifolium is a Javanese endemic plant found in the mountainous area [1]. This plant belongs to Ericaceae family and it has various local names, Cantigi Ungu, Mentigi, Manis Rejo, and Delima Manda, people in West Java called it Cantigi Ungu [2], while in East Java, specifically in Ijen, Banyuwangi they called it “Delima Manda” [3]. Vaccinium genus mainly used as food sources and grow wild. This plant abundantly found in high altitude area (1800-3340 m.asl) [4], like in Ijen Crater, this species can be found around 2000 m.asl [5]. Only a few reports of genetic data from Cantigi in Indonesia, based on reports on genetic data are still lacking [6,7] So that there is a need for conservation to find out the existence of this plant.

Many weaknesses in terms of identifying certain types of plants through morphological characteristics that are needed experienced taxonomists because there are several types of plants that have the same morphological characteristics and identification results tend to be subjective [8]. DNA barcode could help widening knowledge about many other species quickly and efficient. The result from Genetic analysis could produce to better detail as well as it can identify a good target of species [9,10,11].

DNA barcode identification in plants using gene form primer Internal Transcribed Spacers (ITS), matK (maturaseK), psbA-trnH, rbcL, rp0Cl, rp0B, trnL. DNA barcode identification generally uses genes from the plastid
genome region (matK, rbcl, rpoB, rpoC1, trnH-psbA, and ycf1) and the genome nucleus (ITS area) the presence of variations in genes in plants such as rbcl, matK, ycf1 and ITS are used for terrestrial plants (land) [12,13,14,15,16,17]. Several studies report that the use of ITS sequences for phylogenetic studies and genomic relationships of plants at species taxonomic level [18,19,20]. It is common to use ITS for DNA barcodes, molecular phylogenetics, and biodiversity studies [21].

In plants, ITS sequences vary in length from approximately 500-700 bp in angiosperms [22]. ITS is divided into 2 types namely, Specific ITS (ITS-s) is a DNA marker intended for DNA barcode analysis in specific living things especially in certain fungi, algae or plants, while Universal ITS (ITS-u) is used in general plants especially terrestrial plants (land) [21,23]. ITS are plant DNA fragments that are often used in systematic molecular research up to the species level because of the great potential in terms of interspecific and intraspecific relationships [21,22,24,25,26,27,28]. The objective of this research is to study the phylogenetic of Cantigi Ungu (Vaccinium varingifolium) that were obtained from Ijen Crater, Bromo Mount, and Welirang Mount with another related species. This genetic conservation needs to conduct due to the lack of information about this species.

MATERIALS AND METHODS

Sampling and Isolation DNA Total

The subjects of this study is Cantigi Ungu (Vaccinium varingifolium) collected from Ijen Crater area (2769 m.asl), Banyuwangi, East Java in 2017; Bromo Mount area (2329 m.asl), Pasuruan-Lumajang-Probolinggo-Malang, East Java in 2019; and Welirang Mount area (3339 m.asl), Batu-Pasuruan-Mojokerto, East Java in 2017-2018. Total DNA obtained from 0.5 g young leaves. The samples ground with mortar and pistil in liquid nitrogen until homogenous. The isolation DNA total using High Pure DNA Plant Mini-Kit (Geneaid) and (Qiagen) with several modifications. The quantity of DNA sample determined by using Thermo Scientific NanoDrop 2000 Spectrophotometer.

Amplification DNA

The target genes using primer specification ITS_F 5’- GGA AGK ARA AGT CGT AAC AAG G -’3 as the forward primer, and reverse primer ITS_R 5’- RGT TTC TTT TCC TCC GCT TA -’3 and were amplified with PCR Mix (Intron) [21].

Electrophoresis

The 0.2 g Agarose dissolved in 20 mL TBE + 4µl EtBr to make Agarose 1%. The electrophoresis performed using Mupid-eXu in 100V for 30 minutes. The agarose then examined under UV Lightning using UV Transluminator in Biotechnology Laboratory of UIN Maulana Malik Ibrahim Malang, Biotechnology Laboratory of State University of Malang, Biology Molecular Laboratory of the State University of Malang, and Biology Molecular Laboratory of Brawijaya University.

Sequencing DNA and Phylogenetic Analysis

In First BASE Laboratories, Malaysia, the amplicons samples are then sequenced in this study. The chromatogram sequence data using FichTV software to display, the contig analysis of the forward and reverse consensus sequence using DNA Baser software. The data compares collected from the National Center for Biotechnology Information (NCBI). Finally, the methods are Maximum Likelihood (ML), Neighbor Joining (NJ), Minimum Evolution (ME), and Maximum Parsimony (MP) to analysis the samples consensus to make topology phylogenetic using MEGAX software [29].

RESULT AND DISCUSSIONS

The topological reconstruction phylogeny (Figure 1) using different kind of methods. The samples obtained from different areas, KWI in Ijen Crater, BRB in Bromo Mount, and WL4 in Welirang Mount (Table 1) are compared
sequences from NCBI with accession number AY274564.1 (Table 2). Before that, the samples to align consensus sequences with the ClustalW method using ClustalX software [29,30].

### TABLE 1. List of samples obtained from different areas.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Label</th>
<th>Sequence length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vaccinium varingifolium</em></td>
<td>Ijen crater, East Java, Indonesia</td>
<td>KWI</td>
<td>771</td>
</tr>
<tr>
<td><em>Vaccinium varingifolium</em></td>
<td>Bromo Mount, East Java, Indonesia</td>
<td>BRB</td>
<td>715</td>
</tr>
<tr>
<td><em>Vaccinium varingifolium</em></td>
<td>Welirang Mount, East Java, Indonesia</td>
<td>WL4</td>
<td>771</td>
</tr>
</tbody>
</table>

### TABLE 2. List of sequence compared from NCBI GeneBank which have validated in this research.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Species</th>
<th>Location</th>
<th>Sequence length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY274564.1</td>
<td><em>Vaccinium varingifolium</em></td>
<td>East Java, Indonesia</td>
<td>635</td>
</tr>
</tbody>
</table>

*Maximum likelihood* (ML) method shows that the samples are same species with *Vaccinium varingifolium* with bootstrap value 99 (Figure 1a), as well as *neighbor joining* (NJ) with bootstrap value 77 (Figure 1d), *Minimum Evolution* (ME) with bootstrap value 76 (Fig. 1c), while *Maximum Parsimony* (MP) with bootstrap value 65 (Figure 1d). NJ and ME analyze based on genetic distance meanwhile ML and MP analyze based on sequence character from barcode gene [31,32,33]. From the analysis, the result points out that the species happened because both of the sequences are at the same clade. It also indicates that the samples and *Vaccinium varingifolium* accession number AY274564.1 belong to monophyletic which is they have the same ancestor [29,34].

![Phylogenetic topology](image)

**FIGURE 1.** Phylogenetic topology using the (a) *Maximum Likelihood*, (b) *Neighbour Joining*, (c) *Minimum Evolution*, (d) *Maximum Parsimony*.

The Sample (Figure 2) has genetic distance 0.00 that is mean KWI, BRB, and WL4 are belongs to the same species with *Vaccinium varingifolium*. The genetic distance less than 0.03 would be considered as an intraspecific
while the genetic distance more than 0.03 would be considered as an interspecific [35,36,37,38]. The genetic
distance more than 0.05 has a mean most divergent to the others [34].

CONCLUSION

According to this study, it can be concluded that Cantigi Ungu in this study (The Sample KWI, BRB, and WL4)
were same species as exiting in the database (Vaccinium varingifolium accession number AY274564.1) and
considered as an intraspecific.

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