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Genetic variability and phylogenetic relationships of *Begonia multangula* based on *atpB-rbcL* non-coding spacer of cpDNA sequences

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Abstract. *Warseno T, Efendi M, Chasani AR, Daryono BS. 2022. Genetic variability and phylogenetic relationships of* Begonia multangula *based on atpB-rbcL non-coding spacer of cpDNA sequences. Biodiversitas 23: 5491-5501. Begonia multangula* (*Begoniaceae*) belongs to Section Platycentrum-Sphenanthera Group which has a wide distribution from Sumatra to the Lesser Sunda Islands and Sulawesi. Information on *B. multangula* genetic variability and intraspecies relationships based on molecular characters is critical for developing appropriate strategies in conservation biology, breeding activities, and many other applied fields. The genetic variability and phylogenetic relationships of *B. multangula* in Indonesia were investigated using sequence data from the atpB-rbcL intergenic spacer (IGS) cpDNA regions. The atpB-rbcL IGS fragment was amplified using atpB-1 as the forward primer and rbcL-1 as the reversed primer. Genetic variations were found in the length of the sequence and nucleotide divergences in the atpB-rbcL IGS region. The genetic distance between 822 fixed sites ranged from 0 to 0.61 %. Eighteen of the 822 sites (99.27 %) analyzed were invariable, six sites (0.73%) were variable consisting of four singleton variable sites and two parsimony informative sites, and twelve sites were gaps. The phylogenetic relationships generated by *B. multangula* based on the atpB-rbcL IGS sequence analysis indicated the genetic variation and divided it into two clades. However, the clustering pattern of *B. multangula* specimens resulting from molecular analysis based on atpB-rbcL IGS sequences did not show the geographic clustering grouping pattern.

Keywords: *Begonia*, Begoniaceae, intraspecies, molecular, phylogenetic

INTRODUCTION

Begonia (Begoniaceae) is one of the largest vascular plant genera, ranking among the top ten most speciose angiosperm genera, with approximately 2111 accepted species which have been grouped into 70 sections (Haba 2015; Hughes et al. 2015-continuously updated). *Begonia* is a pantropical genus found throughout the world's tropical and subtropical regions, with the exception of subtropical Australia. Therefore, it is an ideal model for investigating the mechanisms and patterns that underlie plant diversity (Chung et al. 2014; Moonlight et al. 2018; Tian 2018). Indonesia is one of the centers of *Begonia* distribution in Southeast Asia, with more than 200 species of *Begonia*. *Begonia multangula* Blume is one of Indonesia's natural Begonias, belongs to the Platycentrum Section Group Sphenanthera, and has a wide distribution range spread across Sumatera, Java, Bali, Lombok, Sumbawa, Flores, and Sulawesi (Thomas et al. 2018; Cahyanto et al. 2019). Generally, it grows on the forest floor, which has enough high humidity with much litter and is protected from direct sunlight (Girmansyah 2017). Due to the distinctive shape and hue of their leaf blades, most begonias have the potential to be ornamental plants with high economic value (Siregar et al. 2018). Because of its large size, attractive color, large flower, and distinctive fruit, *B. multangula* is suitable for use as a yard ornamental. It can also be used for food and medicine (Rajbhandary 2013; Suresh et al. 2016; Cao et al. 2020).

In addition, many studies have shown that this plant contains phenolic compounds, flavonoids, steroids, terpenoids, and alkaloids (Karpova et al. 2019; Bhattarai and Rana 2020). Furthermore, the compound contributes to antifungal and antibacterial activity (Siregar et al. 2018; Tamam et al. 2020; Hamzah et al. 2022). Based on its potential and utility, *B. multangula* necessitates special attention to its botany, ecology, physiology, and genetic variation for conservation and development purposes. The morphological character of *B. multangula* varies. Based on the morphological characteristics, Efendi et al. (2017) found two variations of *B. multangula* in the Remnant Forest of the Cibodas Botanical Gardens, and Girmansyah (2017) found two *B. multangula* in Sumatra that showed morphological characters variation. This morphological variability indicates the genotypic diversity among *B. multangula* accessions (intraspecific variation). Intraspecific variation trait of plants relates to their response to the changes in several important environmental factors, including temperature, light, and nutrients. The adaptation process can result in genetic differences in populations and lead to phenotypic differences (Schradin 2013).

Variability and genetic diversity are essential in the evolution and applied sciences because they will determine how an organism responds to environmental changes such as natural selection, environmental stress and susceptibility to various diseases (Nonić and Šijačić-Nikolić 2019). Therefore, information on *B. multangula* genetic diversity and intraspecies relationships based on molecular characters is important to develop the appropriate strategies in conservation biology, breeding activities, and many other applied fields. The availability of information can assist the plant breeders to determine the combination of parents to obtain the progenies that have the desired character (Mukhopaddhyay and Bhattacharjee 2016).

Genetic variability and diversity studies of Begonia using various molecular markers have been reported such as Simple Sequence Repeats (SSR) (Li et al. 2018) and nrDNA internal transcribed spacer (ITS) (Ningrum et al. 2020). The studies can be a foundation for research at the other taxonomic level of *Begonia* with more complex taxonomic issues. A non-coding region of the chloroplast genome, the *atpB-rbcL* intergenic spacer that separates the plastid genes atpB and rbcL, was one of the first non-coding markers used for phylogeny inference. It has been recently used in Angiosperms phylogenetic studies such as Rosaceae (Liu et al. 2015), *Syzygium boerlagei* (Merr.) Govaerts (Myrtaceae) (Widodo et al. 2019), *Synedrella nodiflora* (L.) Gaertn and *Eleutheranthea ruderalis* (Swartz) Sch.-Bpi (Asteraceae) (Susanto and Dwiati 2019). In Begonias, this region is used to confirm the unidirectional hybridization of *Begonia* X*taipeiensis*. from Taiwan (Peng and Chiang 2000). This paper is the first of the non-coding cpDNA sequence *atpB-rbcL* intergenic spacer of *B. multangula*. This study aimed to assess the genetic variability and phylogenetic relationships of *B. multangula* Blume by using cpDNA *atpB-rbcL* intergenic spacer sequence.

MATERIALS AND METHODS

Plant materials

A total of ten *B. multangula* samples were obtained and utilized in this research. The materials were collected from Java (Cianjur), Bali (Tabanan, Jembarana and Buleleng), and Sumatra (Table 1). The living collection plants were cultivated in Botanic Gardens Bali Botanic Gardens and Cibodas Botanic Gardens-National Research and Innovation Agency, Indonesia. A molecular character analysis was conducted at the Genetics and Breeding Laboratory, Faculty of Biology Gadjah Mada University, Indonesia. The outgroup in this study were species closely related to *B. multangula*, namely *B. longifolia* and *B. aptera* Blume. In this study, *B. longifolia* (AG 337) was isolated and sequenced with the same steps as the *B. multangula* samples, while *B. aptera* sequence data was obtained from the GenBank database (Peng and Chiang 2000).

Procedures

DNA extraction

The total genomic DNA was extracted from fresh leaves using Quick-DNA Plant/ Seed Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocols. The leaf was grounded to be powder using liquid nitrogen. A small amount of PVP (*Polyvinylpyrrolidone*) is added to aid in the removal of secondary plant compounds such as polyphenols, tannins, and quinones. The presence of total genomic DNA was determined by using the agarose electrophoresis technique. A nanodrop spectrophotometer (Simplinano spectrophotometer, Biochrom) was used to estimate the quantity and purity of genomic DNA, with the ratio on a spectrophotometer at 260 nm (optimum absorbance detection for DNA) and 280 nm from DNA samples. The extracted DNA is stored at-20^{o}C in a freezer until PCR amplification was carried out.

Polymerase Chain Reaction (PCR) amplification

A non-coding cpDNA fragment, *atpB-rbcL* intergenic spacer, was amplified using primers atpB-1 (5'-ACATCKARTACKGGACCAATAA-3) as forward and rbcL-1 (5'-AACACCAGCTTTRAATCCAA-3') as reverse (10 pM each) (Chiang et al. 1998). The PCR reaction was performed in 25 µL volume that included 12.5 µL PCR mix (Bioline MyTaq HS Red MixTM), 8 µL ddH_2O (Invitrogen UltraPure Distilled Water), 2.5 µL DNA template, 1 µL forward primer, and 1 µL reverse primer.

Table 1. List of the specimen of *Begonia multangula* used and sequenced in this study

Specimen code/ collection number	Accesion number	Origin
G1/BA 752	E201106212	Batukaru Nature Reserve, Tabanan Regency, Bali, Indonesia (BBG)
G3/RP 703	E200909104	Sengayang Mountain, Batukaru Nature Reserve, Waru, Gesing Village, Banjar Districs, Buleleng Regency, Bali, Indonesia (BBG)
G34/AG 340	E2011075	Pengelengan Hill, Buleleng Regency, Bali, Indonesia (BBG)
G35/MA 1,2	E20060634	Candikuning Village, Baturiti Districs, Tabanan Regency, Bali, Indonesia (BBG)
G40/TT 9	E20060646	Lampung, Sumatera, Indonesia (BBG)
G51/MR 349	E20091051	Batukaru Mountain Nature Reserve, Gesing Village, Banjar Distric, Buleleng Regency, Bali, Indonesia (BBG)
G39/LL 238	C2019060122	Gede Pangrango Mountain National Park, Cianjur Regency, West Java (CBG)
G41/ME 03	-	Wornojiwo Remnant forest, Cianjur Regency, West Java, Indonesia (CBG)
G47/FT 123	E2015090009	Mesehe Mountain, Jembrana Regency, Bali, Indonesia (KRE)
G50/RP 703b	E200909104	Sengayang Mountain, Batukaru Nature Reserve, Waru, Gesing Village, Banjar Districs, Buleleng Regency, Bali, Indonesia (BBG)

Note: BBG: cultivated in Bali Botanic Gardens-BRIN, CBG: cultivated in Cibodas Botanic Gardens-BRIN

The amplification settings were as follows: an initial denaturation step at 95ºC for 5 minutes followed by 35 cycles of denaturation phase at 95ºC for 1 minute, an annealing phase at 50ºC for 1 minute, extension phase at 72ºC for 1 minute, and followed by a final extension phase at 72ºC for 10 minutes (Chiang et al. 1998). The PCR products were electrophoresed in 0.8% agarose with fluorosafe staining and visualized under a UV transilluminator compared to the 50 kb DNA ladder (marker). Digital photographs of the gel profiles were captured by Gel Documentation System. The sequencing preparation process is carried out by double-checking all samples of electrophoresis visualization results. Forty µl of PCR products with a positive targeted band between 841-900 bp were sent to First BASE Malaysia for purification and sequencing bidirectionally using The Sanger Sequencing Method.

Data analysis

The *atpB-rbcL* intergenic spacer sequences were analyzed, edited, and fixed using Gene Studio Pro Program (version 2.2.0.0) and were checked manually to make sure the consensus of sequences. The sequences were compared with sequences in the NCBI GenBank database by BLAST with Megablast program selection. Alignment is done using OPAL, which is integrated with Mesquite software (version 3.31). MEGA11 software (version 11.0.11) was used to calculate the genetic distances of sequences and create the neighbor-joining (NJ) phylogenetic tree (Tamura et al. 2021). The parameter settings were as follows: 1,000 bootstrap replications were used to test the branch stability (Kumar et al. 2018). The nucleotide substitution model was constructed using the Kimura 2-parameter model (Ningrum et al. 2020). The substitution included some transitions and transversions, and the gaps/missing data treatments were set to pairwise deletion. The nucleotide diversity index (π) was used to quantify genetic diversity, which was categorized as low (0.1-0.4), medium (0.5-0.7), and high (0.8-2.00). (Nei and Li 1979; Nei 1987).

RESULTS AND DISCUSSION

Amplification of cpDNA *atpB-rbcL* spacer region

Visualization of PCR results on 0.8% agarose gel revealed a single band, indicating that the primers successfully amplified partial sequences of the *atpB-rbcL* IGS region in ten of *B. multangula* and one sample of *B. longifolia* (outgroup). The results showed that the area of the *atpB-rbcL* IGS region *B. multangula* in this study was estimated at 850 bp (Figure 1). Similar results were reported by Chiang et al. (1998), who stated that the *atpB-rbcL* IGS region band size of *B. aptera* was 841 bp. The product results were then sequenced to determine each sample's base sequence of the *atpB-rbcL* IGS region. The amplified nucleotide sequences from the atpB-1 and rbcL-1 primers were combined using GeneStudio software. The nucleotide length of the *atpB-rbcL* IGS sequences varies from 822-827 bp. Non-coding cpDNA *atpB-rbcL* IGS sequences of *B. multangula* and *B. longifolia* were submitted to NCBI GenBank on 14-15 June 2022 with accession numbers ON777989 to ON777999 (Table 2).

The BLAST analysis revealed that all samples of *B. multangula* used in this study were similar to *B. aptera* (AJ223092.1), with the identity value ranging from 98.40-98.80% (*B. multangula*), 99.88% for *Begonia longifolia* (AG 340) and the query cover value 100%. The query cover value is a number that represents how much of the query sequence is covered by the target sequence. In this study, the target sequence in the database spans the whole query sequence. Per cent identity value is a statistic that reflects how similar the query and target sequences are and how many characters in each sequence are identical. *B. aptera* belongs to Section Platycentrum Sphenanthera Group, identical to *B. multangula* and *B. longifolia* (Girmansyah et al. 2009; Thomas et al. 2011; Hughes et al. 2015-). Ghorbani et al. (2017), stated that accessions have an identity value of 95% (high), identity: 90% ≤ i <95% (medium), identity: i <90% (low). High identity represents species, medium represents genus, and low represents family.

In addition, NCBI contains no information regarding the *atpB-rbcL* IGS sequence of *B. multangula*. To date, this is the only *atpB-rbcL* non-coding spacer sequence of *B. multangula* to be submitted to NCBI by a researcher from this study. Therefore, the insufficient information available in GenBank regarding the *atpB-rbcL* IGS cpDNA region sequence of the *B. multangula*, *B. longifolia*, and *B. aptera* may have contributed to the identification inaccuracy in our work.

Genetic variability

A total of ten *B. multangula* accessions were analyzed for the *atpB-rbcL* non-coding spacer, which showed a variation in the length of nucleotide sequences, ranging from 824 to 827 bp (Table 3). The matrix's total alignment provided 834 bp. Alignment of the *atpB-rbcL* IGS sequences resulted in an 834 bp partial sequence for *B. multangula* to be used in phylogenetic analysis. Polymorphism analysis of the cladograms of the *atpB-rbcL* non-coding spacer sequences was carried out to determine the polymorphism character of the DNA molecular marker. The observed characters include conserved characters (invariable sites), singletone and parsimony informative sites (Table 5).

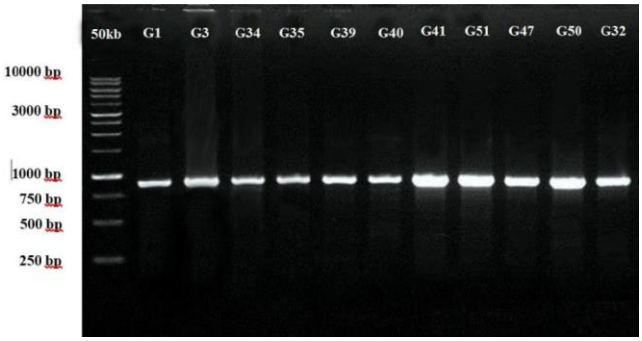

Figure 1. Electrophoregram amplicons of cpDNA *atpB-rbcL* intergenic spacer region of *Begonia multangula* and *B. longifolia* examined on agarose gel 0.8%

Table 2. Result of homology analysis of *atpB-rbcL* IGS sequence *of Begonia multangula* and *B. longifolia* with BLAST

Specimen code	Sample name/ Collection number	Accession number in NCBI	Most similar species	Accession number in NCBI	Size (bp)	Query cover (%)	Identity (%)
G1	*B. multangula* / BA 752	ON777989	*B. aptera*	AJ223092.1	829	100	98.64
G3	*B. multangula* / RP 703 a	ON777991	*B. aptera*	AJ223092.1	829	100	98.76
G34	*B. multangula* /AG 340	ON777990	*B. aptera*	AJ223092.1	829	100	98.44
G35	*B. multangula* /MA 1,2	ON777992	*B. aptera*	AJ223092.1	829	100	98.80
G39	*B.* multangula /LL238	ON777993	*B. aptera*	AJ223092.1	829	100	98.64
G40	*B.* multangula /TT 9	ON777994	*B. aptera*	AJ223092.1	829	100	98.64
G41	*B. multangula* /ME 03	ON777995	*B. aptera*	AJ223092.1	829	100	98.56
G51	*B. multangula* /MR 349	ON777998	*B. aptera*	AJ223092.1	829	100	98.40
G47	*B. multangula* /FT 123	ON777996	*B. aptera*	AJ223092.1	829	100	98.52
G50	*B. multangula* /RP 703 b	ON777997	*B. aptera*	AJ223092.1	829	100	98.64
G32	*B. longifolia* /AG 337	ON777999	*B. aptera*	AJ223092.1	829	100	99.88

A site containing the same nucleotide or amino acid in all sequences is referred to as a constant site. A singleton site contains at least two types of nucleotides (or amino acids) with, at most, one occurring multiple times. A site was identified as a singleton site if at least three sequences contain unambiguous nucleotides or amino acids. A site is parsimony-informative if it contains at least two types of nucleotides (or amino acids), and at least two of them occur with a minimum frequency of two. Among 822 sites (excluding sites with gaps / missing data sites), 816 sites were monomorphic (invariable), six sites were polymorphic (variable), and 12 sites were gaps. There were six variable sites, 4 of them were singleton variable sites, and two sites were parsimony informative sites. A single variant was discovered on nucleotides 1, 253, 270, and 832. Parsimony sites were found on nucleotides 833 and 834. Sites with gaps were on sites 8, 25, 211, 225, 247, 389, 607, 640, 648, 680, 818, and 821 (Table 5). In other words, *atpB-rbcL* non-coding spacer sequences from *B. multangula* contained 99.27% conserved sites and are therefore classified as a DNA region that is highly conserved. Variations in the nucleotide bases that occur at these locations result from nucleotide base substitution in the form of transition and transversion, while the existence of gaps results in a variable nucleotide composition due to insertion and deletion (Table 7). Based on the study, length variation on the *atpB-rbcL* non-coding spacer was prevalent, caused primarily by sequence gaps. Mechanisms such as deletions, insertions, transitions and transversions can occur naturally or through induction which causes changes in nucleotide bases in DNA sequences. Mutations can cause changes in genetic material at the genome, chromosomal, DNA or gene level, leading to genetic diversity (Nonić and Šijačić-Nikolić 2019; Stoltzfus and Norris 2015). Genetic diversity is critical for maintaining the evolutionary potential and survival ability of a species to environmental change, especially for endangered species (Gao et al. 2015).

The GC content value is 0.291. The low GC content reflects the instability of the sequence. The average frequency of nucleotide on sequences *atpB-rbcL* spacer was 36.03% (T), 14.92% (C), 34.62% (A), and 14.43% (G). The proportion of T/A in these sequences was 70.65%, whereas the G/C was 29.35% (Table 3). These results were consistent with Li's (1997) assertion that adenine and thymine constitute the majority of nucleotides in non-coding regions of chloroplast DNA. Additionally, *atpB-rbcL* has been demonstrated to be AT-rich. The majority of non-coding areas rich in these base pairs have little functions. It was suggested that GC content variety is biologically and evolutionarily significant and that plants have developed differing GC contents and codon use to adapt to diverse environments (Šmarda et al. 2014).

The genetic distance is the evolutionary divergence between homologous gene sequences from the same ancestor. Genetic distance is determined when the difference between two genes is comparable to the time since they share a common ancestor. A genetic distance analysis determines genetic differences between individuals within a species (intra-species) or between species (inter-species). It reveals the genetic phylogeny of species and individuals within a species and denotes the distinction between individuals throughout the evolutionary process. A genetic distance with a small value denotes an extremely close lineage, whereas a genetic distance with a significant value denotes a distant relationship (Ripley 2013; Tallei et al. 2016). The genetic distance analysis (Table 4) revealed that *B. multangula* RP 703 a from Sengayang Mountain Tabanan Bali has a close relationship with similar germplasm from Candikuning Village, Tabanan, Bali (MA1,2), Gede Pangrango Mountain National Park, West Java (LL 238) and Lampung, Sumatera, Indonesia (TT9). Similarly, *B. multangula* from Batukaru Nature Reserve, Tabanan, Bali (BA 752) is also closely related to *B. multangula* from Wornojiwo Remnant forest, West Java (ME 03) at a distance coefficient of 0.00%. In contrast, a far relative relationship showed by *B. multangula* from Mesehe Mountain, Jembarana, Bali (FT123) and Batukaru Mountain Nature Reserve, Buleleng, Bali (MR 349) at a distance coefficient of 0.61%. The results showed the genetic distance between specimens of *B. multangula* ranging from 0.0-0.61%, with a mean genetic distance of 0.2% (Table 4), indicating the genetic distance among specimens of *B. multangula* was low. Similarly, a low variation of *atpB-rbcL* IGS in the populations of *Synedrella nodiflora* (L.) Gaertn on Java

Island was also reported (Susanto and Dwiati 2019). Although chloroplast DNA (cpDNA) has a relatively slow evolutionary rate, moderate to high levels of genetic variation can often be detected in non-coding spacers within species, e.g. *Orostachys* (Kozyrenko et al. 2013), *Ceriops tagal* (Rhizophoraceae), and *Hygrophila pogonocalyx* (Acanthaceae) (Susanto and Dwiati 2019).

Bhandari et al. (2017) defined genetic variety as the variation in heritable traits found in a population of the same species. Genetic variation within a species can arise from a variety of sources. One source of genetic variation is mutations. The transfer of genes across various groups of organisms is another source (gene flow). Sexual reproduction can result in genetic differences, leading to novel gene combinations. Variations in cpDNA sequences were typically the result of mutations in a single nucleotide, which represent mutations that had occurred over a very long period of time (Fitmawati and Hartana 2010). In certain regions (Table 3; Table 7), the difference in length sequences of *atpB-rbcL* IGS was caused by mutations, even though the amount of change in this sequence was minimal compared to the changes in the core genome. However, it provided important information in describing the process of evolution because cpDNA was inherited maternally or uniparentally, where the changes in the nucleotide took place for a very long time. Some species can outlive others in their environment due to genetic variance among a collection of organisms. Based on the results of the alignment on 10 samples of *B. multangula*, it was found that there were point mutations consisting of 9 insertion events, 3 deletion events and 6 substitution events. The substitution mutations consisted of 3 transversion and 3 transition mutations (Table 7). In most sequences, transitions occur more frequently than transversions (Aloqalaa et al. 2019). However, in this study, the number of both was the same. According to Dharmayanti (2011), mutation mechanisms such as deletions, insertions, transitions, and transversions can occur naturally or by induction which causes changes in nucleotide bases in DNA sequences. Mutations can cause changes in genetic material at the genome, chromosomal, DNA or gene level, leading to genetic diversity. Sequence variations in the form of point mutations in *atpB-rbcL* IGS can indicate evolutionary changes that lead to species divergence. Variation within species is one of the most important sources of information for recognizing and grasping evolutionary trends. Identifying the sources of intraspecific variation is crucial for comprehending the evolutionary processes that preserve and promote variety and speciation (Alcántara-Ayala et al. 2020; Govindaraj et al. 2015).

The phylogenetic tree

A phylogenetic tree based on the *atpB-rbcL* spacer sequence in *B. multangula* (Figure 2) was generated by Neighbor-Joining (NJ). Bootstrap values are determined by the Kimura 2-parameter model method; bootstrap value > 50 indicates the reliable grouping so that the relationship between accession and their evolutionary history can be created. Relationships are indicated by the length clade of the phylogenetic tree, which is illustrated by genetic distance and similarity among accessions (Tamura et al. 2021). The results of the phylogenetic relationship analysis of *B. multangula* based on the *atpB-rbcL* spacer sequence using B. *longifolia* and *B. aptera* as outgroups displayed in the form of a phylogenetic tree (Figure 3) showed that there were two clades formed in the in-group group, namely "clade I" and "clade II". Clade I consisted of 5 sub-clades: A, B, C, D, and E. Sub-clade A consisted of BA 752, ME03, AG 340, and 703b specimens. In sub-clade A, the separation between branches was supported by a small bootstrap value of 42%. Sub-clade B consisted of FT 123 specimens. These branches were supported with a bootstrap value of 8%. Sub-clade C consisted of RP703a and MR 349 specimens. Sub-clade D consisted of TT 9 (Sumatera) specimens. Sub-clade E consisted of LL 238 (Cianjur2) specimens. Clade II consisted of 1 specimen, MA 1,2 (Tabanan 2).

Table 3. Nucleotide composition of *atpB-rbcL* IGS sequence data in *Begonia multangula*

Specimens code	Collection code	Total aligned nucleotide	Nucleotide composition			
			T (U)	C	A	G
G1	BA 752	825	298 (36.12%)	122 (14.79%)	286 (34.67%)	119 (14.42%)
G3	RP 703a	824	297 (36.04%)	123 (14.93%)	286 (34.71%)	118 (14.32%)
G34	AG 340	827	300 (36.28%)	123 (14.87%)	286 (34.58%)	118 (14.27%)
G35	MA 1,2	825	298 (36.12%)	122 (14.79%)	286 (34.67%)	119 (14.42%)
G40	TT 9	826	298 (36.08%)	122 (14.77%)	287 (34.75%)	119 (14.41%)
G51	MR 349	826	299 (36.20%)	122 (14.77%)	286 (34.62%)	119 (14.41%)
G39	LL 238	824	298 (36.17%)	122 (14.81%)	285 (34.59%)	119 (14.44%)
G41	ME 03	826	298 (36.08%)	123 (14.89%)	285 (34.50%)	120 (14.53%)
G47	FT123	825	297 (36.00%)	122 (14.79%)	287 (34.79%)	119 (14.42%)
G50	RP 703b	826	298 (36.08%)	124 (15.01%)	285 (34.50%)	119 (14.41%)
G32	AG 337 (Outgroup)	823	293 (35.60%)	126 (15.31%)	284 (34.51%)	120 (14.58%)
	B. aptera (Outgroup)	822	293 (35.64%)	126 (15.33%)	284 (34.55%)	119 (14.48%)
	Average	824.91	297.25 (36.03%)	123.08 (14.92%)	285.58 (34.62%)	119 (14.43%)

Table 4. Genetic distance (%) among *Begonia multangula* specimens. The number at the top row indicates the species as same as the first column

Specimen code	G1	G3	G34	G35	G39	G40	G41	G47	G50	G51
G1										
G3	0.12									
G34	0.37	0.49								
G35	0.12	0.00	0.49							
G39	0.12	0.00	0.49	0.00						
G40	0.12	0.00	0.49	0.00	0.00					
G41	0.00	0.12	0.37	0.12	0.12	0.12				
G47	0.24	0.12	0.61	0.12	0.12	0.12	0.24			
G50	0.24	0.12	0.37	0.12	0.12	0.12	0.24	0.24		
G51	0.24	0.12	0.61	0.12	0.12	0.12	0.24	0.24	0.24	

Table 5. Summary of polymorphic analysis among *Begonia multangula*

Phylogenetic data	*atpB-rbcL* IGS
Number of sequence used	10
Number of sites	834
Total number of sites (excluding sites with gaps / missing data)	822
Sites with alignment gaps or missing data	12
Invariable (monomorphic) sites	816
Variable (polymorphic) sites	6
Singleton variable sites	4 (site position: 1, 253, 270 and 832)
Parsimony informative sites	2 (site potition: 833 and 834)
Genetic diversity	0.000-0.0061
Nucleotide diversity (per site) (π)	0.00197
Haplotype (gene) diversity (Hd)	0.844

Table 6. Variable sites of the nucleotide sequences of the *atpB-rbcL* IGS region of the cpDNA of *Begonia multangula*

Specimens	Base nucleotide position 222223666688888 2124578044812333 185157309708081234
G1. *B. multangula* BA 752	AA---TAG-G-----AGG
G3. *B. multangula* RP 703a	.--C--..-.-----..A
G34. *B. multangula* AG 340	.-G---.AT.---TTCA.
G35. *B. multangula* MA 1,2	.-G---..-.---T-..A
G39. *B. multangula* LL 238	..G---..-.---T-..A
G40. *B. multangula* TT 9	.-G---..T.---T-..A
G41. *B. multangula* ME 03	.-G---..-----T-...
G47. *B. multangula* FT 123	C-G---..T.-G---..A
G50. *B. multangula* RP 703b	.-G---..-.G----.AA
G51. *B. multangula* MR 349	.-G-T-C.-.--C--..A

Note: The dash (-) indicates a gap or deletion at the nucleotide position. The dot (.) indicates the same base as the first row at that position

Table 7. A point mutation in *Begonia multangula*

Position	Nucleotide changes	Point mutation type	Changed accessions
1	A → C	Transvertion	FT 123
8	A → -	Deletion	RP 703a, AG 340, MA 1,2; TT 9; ME 03; FT 123; RP 703b; MR 349
25	- → G	Insertion	AG 340; MA 1,2; LL 238; TT 9; ME 03; FT 123; RP 703b; MR 349
211	- → C	Insertion	RP 703a
225	- → T	Insertion	MR 349
247	T → -	Deletion	RP 703a; AG 340; MA 1,2; LL 238; TT 9; ME 03; FT 123; RP 703b; MR 349
253	A → C	Transvertion	MR 349
270	G → A	Transition	AG 340
389	- → T	Insertion	AG 340; TT9; FT 123
607	G → -	Deletion	ME 03
640	- → G	Insertion	RP 703b
648	- → G	Insertion	FT 123
680	- → C	Insertion	MR 349
818	- → T	Insertion	AG 340; MA 1,2; LL 238; TT9; ME 03
821	- → T	Insertion	AG 340
832	A → C	Transvertion	AG 340
833	G → A	Transition	AG 340; RP 703 b
834	G → A	Transition	RP 703a; MA 1,2; LL 238; TT9; FT 123; RP 703b; MR 349

Note: A: Adenine; C: Cytosine; G: Guanine; T: Thymine; -: gap

The phylogenetic tree can be defined as a graphical representation of the evolutionary relationships among entities that share a common ancestor. The entity can be a species, gene, genome, or other Operational Taxonomic Units (OTU). A phylogenetic tree is a branching diagram or tree that depicts the estimated evolutionary relationship between a group of species based on molecular characteristics (Hall 2013; Weyenberg and Yoshida 2016). The phylogenetic analysis based on cpDNA *atpB-rbcL* intergenic spacer sequence data showed that ten specimens of *B. multangula* are monophyletic. However, the grouping of *B. multangula* based on genetic characteristics did not reveal any evidence of grouping due to regional differences. Sub-clade A consisted of BA 752 (Tabanan 1) and ME 03 (Cianjur 1) specimens that have a meagre genetic distance (0,0). The zero value of the genetic distance is due to the slight sequence variations among specimens. Based on their original habitat, both come from different districts and islands. Tabanan 1 specimen was from the Batukaru Nature Conservation area. The Batukaru Nature Reserve is located in the Baturiti Subdistrict of Tabanan and the Sukasada Subdistrict of Buleleng. The terrain varies from level to steep, with slopes ranging between 8 and 45 per cent. The reserve encompasses 8557.98 hectares and is divided into three sections: resort Pemangkuan Hutan (RPH) Pupuan, RPH Penebel, and RPH Candikuning, while ME 03 (Cianjur 1) specimen was collected from Wornojiwo Remnant forest, Cianjur Regency, West Java, Regency, Indonesia. The Wornojiwo forest represents the Cibodas sub-montane natural forest community which was previously thought to be a unit with the Gunung Gede sub-montane forest. Other members of sub-clade A are AG 340 Buleleng 3 and RP 703b (Buleleng 1) specimens from the same regency, Buleleng, but from different mountains, Pengelengan Hills and Sengayang Mount, respectively. Clade II consisted of MA 1,2 (Tabanan 2) specimens. It was collected from the Reboisation forest area (known as "*hutan kontrak*") located within the Bali Botanical Gardens-BRIN, Candikuning Village, Baturiti Sub-district, Tabanan Regency, Bali, Indonesia (08°16'00" S and 115°09'00" E). The reforestation area consists of flat, hilly terrain between 1,250 and 1,400 m asl.

Based on this study, the clustering pattern of *B. multangula* specimens resulting from molecular analysis based on *atpB-rbcL* IGS sequences did not show a geographic clustering pattern, and it differed from the grouping of *B. longifolia* conducted by Ningrum et al. (2020), which grouped based on the geographical location of the accession origin. In some clusters, the members consist of accessions from different islands. For example, *B. multangula* G41 (Cianjur 1) from West Java belongs to the same clade and subclade as *B. multangula* from Bali (BA752, Tabanan1). The results show no relationship between accession grouping with the ecotype and the distribution of the area of a particular accession. This situation may be due to *B. multangula* not having specific habitat requirements so that it can grow in various areas with various environmental conditions. The wide distribution of *B. multangula*, like that of other members of the *Sphenanthera* section, for example, *B. longifolia*, could be due to its broad ecological tolerance and the greater possibility of long-distance dispersal afforded by baccate and therefore probably dispersed in animals. Such variations of *Begonia* are dominantly caused by population isolation (Tebbitt 2003). Population-level variations are consistent with macroevolution in the genus *Begonia* (Hughes and Hollingsworth 2008). Variations in gene sequences or DNA segments can be used to calculate the rate of evolution. The length of the branches influences the close relationship between samples. Branch lengths are proportional to genetic distances calculated from multiple alignments of homologous sequences (Staton 2015; Nonić and Šijačić-Nikolić 2019).

The results of phylogenetic analysis based on the *atpB-rbcL* IGS sequence also indicated the existence of genetic diversity below the species level, as indicated by the formation of two clades in the in-group. This gives additional evidence for the function of IGS as a useful molecular marker for use in Plant Systematics research, which focuses primarily on species-level and infraspecific diversity. The phylogenetic relationships generated based on the *atpB-rbcL* spacer sequence analysis indicated the genetic variation in *B. multangula*. The genotypic variation is reflected in the presence of two clades formed in the *atpB-rbcL* spacer phylogeny tree (Figure 2). This shows an infraspecific variation in the *atpB-rbcL* spacer *B. multangula* sequence, characterized by the presence of variable sites, although it only has a low percentage of 0,37%. Although the chloroplast genome is conservative in its evolution, reports of intraspecific variation are frequent. This research reveals that *atpB-rbcL* IGS sequence variation in the Java, Bali, and Sumatera population of *B. multangula*, similar to *atpB-rbcL* IGS sequence variation of *Synedrella nodiflora* (L.) Gaertn. populations in Java Island (Susanto et al. 2018), *Pistachia vera* L. (Talebi et al. 2016). However, in several plant species populations, *atpB-rbcL* intergenic spacer sequences were very variable. For example, in various Alismataceae species in China, such as *Sagittaria trifolia*, *S. potamogetifolia*, and *S. lichuanensis*, it was shown to be varied (Susanto and Dwiati 2019), *Quercus acutissima* Carruth (Zhang et al. 2015), *Prunus armeniaca* L. (Batnini et al. 2019). This result was similarly accurate for Southeast Asia's *C. tagal* (Rhizophoraceae) population (Mori and Kajita 2016).

As with other species of the Platycentrum section, *B. multangula* has the characteristics of an erect and robust habitus and rhizomatous. It belongs to the Sphenanthera Group since it bears fleshy fruit, flowers with often quite fleshy tepals, and male flowers with large androecia consisting of stamens with extended connectives (Tebbitt et al. 2006). *Begonia longifolia* and *B. aptera* Blume, as outgroups in this study, were successfully separated between specimens of *B. multangula* specimens. *B. aptera* is part of the *B. longifolia* Blume species complex. Thomas et al. (2012) stated that *B. longifolia* and *B. aptera* were shown to be sister taxa in cpDNA-based molecular phylogenies. In addition, the two species are similar in morphological character and have been distinguished primarily by leaf shape and leaf margin characteristics. The

comparison of several morphological characteristics of the two species of begonias with the *B. multangula* used in this study is shown in Table 8. *Begonia multangula* can be distinguished from the other Sphenanthera Group because it has sub-orbicular to broadly ovate leaves, consisting of 5-7 wide pointed lobes (Hughes and Girmansyah 2011). Based on morphological observations, *B. multangula* used in this study showed morphological variations, including the primary color of the upper surface of the leaves (green to dark green), the color of the underside of the leaves (pale green to silvery), stems (pale green to brownish red), hair density (rare to thick), stem color (green to brownish red). Some samples in this study also showed red spots at the base of the leaves that were not found in other samples. These morphological differences indicate that there is genetic variation among the samples used. Morphological and molecular characteristics can be used to determine genetic information and relationship (Arif et al. 2020; Uslan and Pharmawati 2020). Because morphological characteristics are significantly impacted by environmental conditions and phases of plant growth (Zhang et al. 2015), the study of variability and genetic diversity, as well as the identification of taxa in plant groups and their arrangement, necessitates a combination of morphological characters and other characters, such as molecular characters. Because they are more diverse and comprehensive in studying evolution, molecular characters can reveal genetic variation and their relationship to taxa. The cpDNA regions are extensively utilized as markers in phylogenetic and phylogeographic research. The *atpB-rbcL* spacer, which is located between the large subunit of the ribulose-1-5-bisphosphate-carboxylase (rbcL) and the beta subunit of the chloroplast ATP-synthase (atpB) genes, has been beneficial in evolutionary studies of angiosperms (Susanto et al. 2018).

Based on the study, the results of the phylogenetic analysis using the Neighbor-Joining method confirmed the status of atpB-rbcL spacer as a conservative region in *B. multangula*. Therefore, the low bootstrap values are due to a low number of characters supporting the clades. Because of the low bootstrap values and the small number of informative sites, it appears that the atpB-rbcL IGS region does not represent a sufficiently variable region for the study. The lack of obvious alternative regions to sequence made it necessary to explore other sources of molecular data. The atpB-rbcL IGS sequence has a limited level of determining the difference in infraspecific levels, so for other plants with a low level of phenotypic variation, it is not recommended to use the atpB-rbcL IGS sequence as a molecular marker.

Table 8. The morphological difference between *Begonia multangula*, *B. longifolia*, and *B. aptera*

Morphological character	*Begonia multangula*	*Begonia longifolia*	*Begonia aptera*
Leaf shape	Sub-orbicular to broadly ovate with 5-7 broadly triangular lobes	Lanceolate or occasionally the larger leaves broadly elliptic	Broadly elliptic
Leaf margin	Dentate-denticulate	Single toothed to almost entire	Bidentate to-serrate
Female flower	Five tepals	Six tepals	Six tepals
Fruit	Berry-like fruit, with thick and unequal wings	Berry-like fruit, with thick and equal wings	Berry-like fruit, wingless

Note: Tebbitt (2003); Ardi et al. (2018)

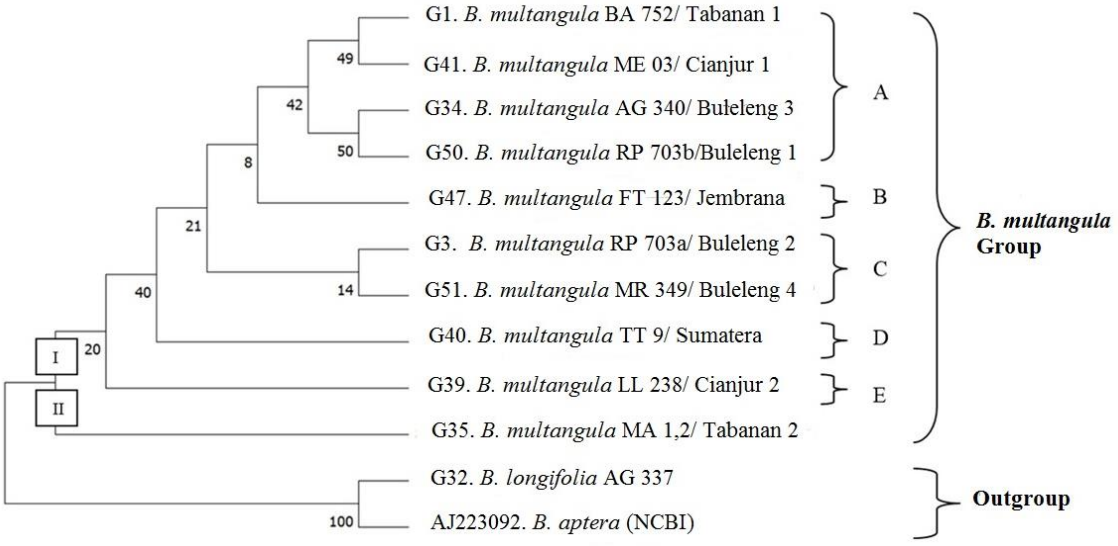

Figure 2. Phylogenetic tree of *Begonia multangul*a derived from Neighbor Joining method based on cpDNA *atpB-rbcL* spacer sequence data

For further research, it is recommended to use other cpDNA, nrDNA molecular markers such as the nrITS region or a combination of both, or EST-SSR marker so that the phylogenetic tree can better describe the relationship among *Begonia* accession. Sequencing data analysis from the nrDNA ITS region has been used for species identification (Forrest and Hollingsworth 2003; Forrest et al. 2005; Jiao and Shui 2013), genetic variability and infraspecific relationship in *B. longifolia* (Ningrum et al., 2020). Thomas et al. (2011) also revealed that a sequence of non-coding plastid DNA (ndhA intron, ndhF-rpl32, and rpl32-trnL spacers) supported the polyphyly of section *Sphenanthera*. The combination of nrDNA and trnL intron sequences has also been utilized to construct phylogenetic analyses within the Begoniaceae (Plana et al. 2004). An examination of the *B. luzhaiensis* population in China utilizing the EST-SSR marker and the chloroplast trnC-ycf6 intergenic spacer revealed that historical fragmentation and isolation by distance impacted the phylogeographic analysis (Tseng et al. 2019). According to Azman et al. (2020), populations with low genetic diversity values are threatened, fragmented, and degraded by human activity. In contrast, the high genetic diversity in the plant population could be due to some factors, including i) the genetic diversity has been high since the beginning of the population's existence, ii) the population has not been significantly affected by human activities, so its condition is better preserved, and iii) the occurrence of random mating between individuals, resulting in genetic recombination and increasing genetic diversity within the population.

Overall, the results of this study support the use of the noncoding cpDNA intergenic spacer marker atpB-rbcL to analyze the diversity and genotype relationships in *B. multangula*. This study was the first use of the atpB-rbcL spacer sequence to study the genetic diversity of *B. multangula* in Indonesia. The results show that the genetic diversity of *B. multangula* in Indonesia is low. Due to the low polymorphism, the atpB-rbcL intergenic spacer has a limited capacity to reveal the relationship among *B. multangula* accessions. The high level of atpB-rbcL IGS sequence similarity (98%) in ten accessions showed that the atpB rbcL IGS was a conserved DNA region, making it suitable for studying the relationship between species. This study supports the use of highly variable molecular markers in future research to get detailed genetic information that facilitates the conservation and management of *B. multangula* genetic resources. The information obtained in this study can be used as a resource to support the conservation and breeding efforts of *B. multangula* both locally and globally. Nonetheless, information on genetic diversity and its relationships are helpful for future breeding and conservation programs, notably for parental selection and the generation of novel better cultivars. Furthermore, the results of this study are expected to encourage the further development and protection of *B. multangula* as a potential ornamental and medicinal plant.

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