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Phylogenetic analysis of Indonesian gandaria (*Bouea*) using molecular markers of cpDNA *trnL-F* intergenic spacer

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Abstract. *Harsono T, Pasaribu N, Sobir, Fitmawati, Prasetya E. 2017. Phylogenetic analysis of Indonesian gandaria (Bouea) using molecular markers of cpDNA trnL-F intergenic spacer. Biodiversitas 18: 51-57.* Genus *Bouea* is a member of the Family Anacardiaceae which is widespread in Malesia region. Genus *Bouea* consists of two types, namely, *B. oppositifolia* (Roxb.) Adelb. and *B. macrophylla* Griffit. This study aims to uncover the genetic diversity of *Bouea* in Indonesia based on molecular markers of cpDNA *trnL-F* intergenic spacer. The samples of genetic material analyzed are 7 accessions of *B. oppositifolia* and 8 accessions of *B. macrophylla* coming from the collection of the Bogor Botanical Gardens and the field exploration activity in Sumatra, Java, Kalimantan and Ambon, and *Anacardium occidentale* and *Mangifera indica* are also used as an out-group comparator. Based on phylogenetic analysis using the maximum parsimony and neighbor-joining method, genus *Bouea* is a monophyletic group or is derived from the same ancestor. *Bouea* derived from Gunung Tua, North Sumatra, has the longest branch and showed up earlier than the other sample so it can be considered that the ancestor of genus *Bouea*. *B. oppositifolia* has branches longer and show up early so it can be considered the ancestor of *B. macrophylla*. Both types of genera *Bouea* grouped separately in the phylogenetic tree and supports grouping types in genera *Bouea*. The results of phylogenetic tree construction do not indicate a geographical grouping of accession. Based on the phylogenetic tree, genus *Bouea* has a closer relationship with *Anacardium occidentale* compared with *Mangifera indica*. The results showed that the markers cpDNA *trnL-F* intergenic spacer effectively used to determine relationship in genus *Bouea*.

Keywords: *Bouea*, cpDNA, gandaria, phylogenetic, *trnL-F* intergenic spacer

INTRODUCTION

Bouea Meisner (Fam.: Anacardiaceae) is an Indonesian native plant and is spread in Sumatra, Java, Kalimantan and Maluku. This plant is also found in Malay and Thailand peninsula. *Bouea* grows in the wet tropic region. By nature, this flora, which is an identity of West Java province, is grown in the lowlands to highlands with a height of 300 meters above sea level, but cultivated *Bouea* is able to grow well in highlands with an altitude of 850 meters above sea level (Rifai 1992). There are other reports stating that *Bouea* is an endemic typical plant of Maluku (Rehatta 2005; Papilaya 2007).

Hou (1978) reports that genus *Bouea* includes two species namely *Bouea oppositifolia* (Roxb.) Adelb. and *Bouea macrophylla* Griffith. Based on observations of herbarium specimens and fresh specimens, Harsono (2013) reports that the variation in the leaves and fruit morphology of *Bouea oppositifolia* (Roxb.) Adelb. and *Bouea macrophylla* Griffith is still quite high, thus it requires another approach beyond morphology to reveal the diversity in the genus *Bouea*.

Morphological markers are common to be used to reveal a diversity of plant species, but since it has high plasticity, other more accurate and stable marker such as molecular marker is required. The stability of these molecular markers is used to support the clarity of morphology diversity and to reveal the genetic diversity and their suspect relationship among species. A more stable genetic diversity data can be used for various activities such as breeding, management, and conservation of germplasm.

There are differences of opinion about the origin of genus *Bouea*. Rudini (1990) determines *Bouea* as the mascot of West Java because it is a native plant with cultural value to the Sundanese. Rifai (1992) stated that genus *Bouea* comes from North Sumatra, while Rehatta (2005) and Papilaya (2007) stated that genus *Bouea* is endemic in Maluku. Harsono (2012) reported that Dayak tribe in Borneo hinterland also uses this plant for various activities of life. To address this problem, accurate and stable phylogenetic analysis is required to explain the origin, elders and evolution process of genus *Bouea* of Indonesia. Information on *Bouea* relationship is important for predicting the common elders of genus *Bouea* of Indonesia.

Phylogenetic analysis requires morphological markers as well as molecular markers which are more conservative

(nonvolatile) for example marker of cpDNA *trnL-F* intergenic spacer. These markers are often used by the experts as they are easily isolated and purified, characterized and cloned, and very conservative with the low rate of evolution, so they can be used in the reconstruction of inter-taxa phylogeny at the level of the family of flowering plants (Clegg and Dulbin 1990; Kajita et al. 1998).

The use of molecular markers chloroplasts (cpDNA) to reveal diversity, to discover the relationship with the basis of evolution and to clarify the status of Indonesian genus *Bouea* has never been done. These markers are useful to support the existing molecular data of genus *Bouea*, as well as to understand the evolution of *Bouea* on the basis of chloroplast DNA sequences. Evolution information on genus *Bouea* is used to predict the common elders of genus *Bouea* in Indonesia today. CpDNA markers have been widely used to study other plant phylogenies, for example, Morus by Weiguo et al. (2005), Cucumis spp. by Chung et al. (2006). cpDNA markers are often used as markers for they are easily isolated and purified, characterized and cloned, and very conservative with the low rate of evolution, so they can be used in the reconstruction of inter-taxa phylogeny at the level of the family of flowering plants (Clegg and Dulbin 1990; Kajita et al. 1998).

The aim of this study was to obtain data on the genetic diversity of the Indonesian genus based on markers of cpDNA *trnL-F* intergenic spacer, resulting phylogeny tree of the Indonesian genus *Bouea* in order to obtain the direction of evolution and the spread of elders of Indonesian *Bouea*, as well as to determine *Bouea* relationship with its close relatives.

MATERIALS AND METHODS

Sample plant

Samples of *B. macrophylla* and *B. oppositifolia* were obtained from various regions in Indonesia representing 7 analyzed regions, namely Ambon, Banten, Aceh, West Sumatra, West Kalimantan, Bangka-Belitung, North Sumatra, and the accession of the Bogor Botanical Gardens (Figure 1). Samples were in the form of a fresh leaf with *Mangifera indica* and *Anacardium occidentale* as *outgroup*. The accession of genus *Bouea* is presented in Table 1.

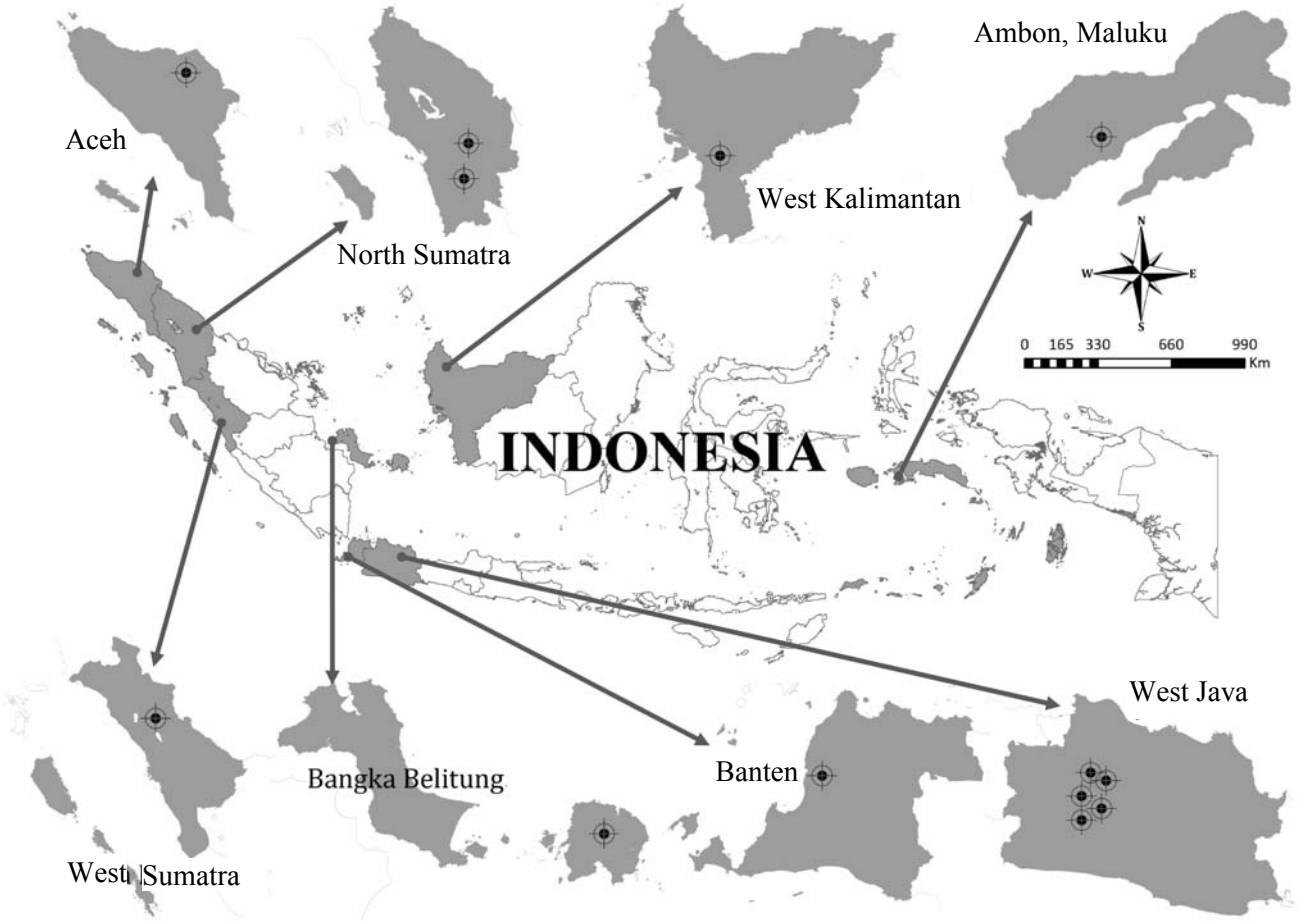

Figure 1. Location of *Bouea* sampling in Indonesia

Table 1. Samples of genus *Bouea* and outgroup used for phylogenetic analysis

Origin of accession	Code	Species	Total	Latitude	Longitude
Aceh	SN	*B. macrophylla*	1	S 04°57'29.7"	E 097°16'32.3"
Sumatera Utara (Gunung Tua)	GT	*B. macrophylla*	1	S 01°02'35.92"	E 099°52'30.56"
Sumatera Utara (Sipiongot)	SP	*B. macrophylla*	1	01°47'14.05"S	099°50'11.68"E
Sumatera Barat	BS	*B. macrophylla*	1	S 00°27'19.3"	E 100°36'19.4"
Bangka Belitung	BL	*B. oppositifolia*	1	2°51'49.11"S	108° 8'50.70"E
Kalimantan Barat	KB	*B. macrophylla*	1	S 01˚21'43.88"	E 110˚10'04.44"
Ambon	AM	*B. macrophylla*	1	S 03˚42'04.32"	E 128˚05'48.27"
Banten	BA	*B. macrophylla*	1	S 06˚17'30.34"	E 105˚50'18.58"
Bogor Botanical Garden	KR1	*B. oppositifolia*	1	06˚35'49.59"S	106˚47'56.19"E
Bogor Botanical Garden	KR2	*B. oppositifolia*	1	06˚35'47.34"S	106˚48'01.80"E
Bogor Botanical Garden	KR3	*B. oppositifolia*	1	06˚35'47.34"S	106˚48'01.80"E
Bogor Botanical Garden	KR5	*B. oppositifolia*	1	06˚35'51.06"S	106˚47'57.46"E
Bogor Botanical Garden	KR4	*B. macrophylla*	1	S 02°57'50.7"	E 104°42'03.1"
Bogor Botanical Garden	MI	*M. indica*	1	06˚35'45.06"S	106˚47'53.46"E
Bogor Botanical Garden	AO	*A. occidentale*	1	06˚35'51.06"S	106˚47'57.46"E
Total			15		

DNA isolation and amplification of cpDNA *trnL-F* intergenic spacer

Isolation of DNA followed the procedure of CTAB (Doyle and Doyle 1987) with some modifications. A total of 0.15 g of leaf samples was crushed using a sterile mortar with the addition of 0.6-0.8 ml of extraction buffer (10% CTAB, 0.5 M EDTA (pH 8.0); 1 M Tris-HCl (pH 8.0); 5 M NaCl; 1% β-mercaptoethanol). The solution was homogenized and then incubated at a temperature of 65°C for 1 hour and then was added by 0.7 ml of purification buffer (chloroform: isoamyl alcohol = 24: 1 v/v), followed by centrifugation at a speed of 11,000 rpm for 10 minutes. The supernatant was transferred into sterile effendorf tube 2 ml and added by 500 mL of cold 2-propanol and then was incubated for 1 night in the freezer. The solution was then centrifuged for 10 minutes at a speed of 11,000 rpm. The liquid phase was discharged and the solid phase (pellet) was air-dried and stored in 100 µL TE solution (1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, and distilled water).

The sequent of *trnL-F* intergenic spacer was amplified with the forward primer pair of 5'-GGT TCA AGT TCT ATC CCC CC -3' and reverse primer pair of 5'-ATT TGA GAC ACG AG ACT GGT -3' (Taberlet et al. 1991) with a total reaction volume of 25 µL (2.5 µL DNA template, 2.5 µL of forward primer, 2.5 of reverse primer; 5 µL of distilled water, 12.5 µL of PCR mix (FastStart PCR Master Mix Roche)). Amplification of DNA sequences of *trnL-F* intergenic spacer used Thermalcycler (Qiagen), the PCR machine, with the condition of predenaturation at temperature of 97°C for 5 minutes followed by 40 cycles with reaction conditions of denaturation at temperature of 94°C for 5 minutes, annealing at temperature of 52°C, and extension at 72°C for 1 minute, then the PCR process was ended with process of post-extension at temperature of 72°C for 5 minutes.

The PCR products were visualized using agarose gel 1% plus 4 µL of SYBR ® Safe DNA Gel Stain. Running process were applied on 6 µL of PCR product added by 1 µL loading dye along with 100 bp DNA ladder marker using electrophoresis machine with mobile phase namely 1X TBE buffer at a voltage of 100 volts for 45 minutes. The appearing of visualization tape used gel documentation. PCR products with visible tape would be sent to 1st Base DNA Sequencing Service for sequencing.

Phylogenetic analysis

Data from the sequencing of *trnL-F* intergenic spacer sequent were analyzed using BioEdit 7.0.1 to determine the consensus sequences based on the conservative sequences and MEGA (Molecular Evolutionary Genetics Analysis) 6: 06 program to construct phylogenetic trees. Sequencing results would be aligned with Clustal W program which was inside MEGA 6: 06 and then the cladogram would be built based on the alignment of sequence data. With the help of MEGA program, the data alignment could be carried out and, based on that alignment, the evolutionary relationship could be predicted. Phylogenetic analyses used maximum parsimony and neighbor-joining method.

RESULTS AND DISCUSSION

The visualization of PCR products using 1% agarose showed single band which means that sequent of *trnL-F* was successfully amplified with forward and reverse primer (Figure 2). The sequencing results were analyzed using Bioedit 7.0.1 to generate a consensus sequence. Consensus sequence as a result of the sequencing consists of 483 characters. From these data, there are 351 characters which were conservative, 8 characters which were potentially parsimony informative and 108 characters which were variable sites. The alignment results showed a gap in the sequence caused by insertions and deletions. These events affect the regulation of gene expression. At the ingroup (*B. macrophylla* and *B. oppositifolia*), deletion occurs in base no. 1-12, 433, and 467-483, the insertion occurs at the base no. 3, 5, 16, 17, 35, 466, 467, 470, and 471, while the insertion-deletion occurs at base no. 3, 5,

467, 470, and 471. Results of alignment showed that both species of genus *Bouea* have a very high degree of homology (98%). This value is higher than the homology level of 14 species of Anacardiaceae family in the area of ITS-1 core genome namely 75% (Hidayat and Pancoro, 2001). The average frequency of nucleotide on sequences *trnL-F* was 32.2% (T), 22.6% (C), 29.3% (A), and 16% (G). These sequences were rich in T/A which was equal to 61.1% while in G/C was 38.8% (Table 2). This was consistent with the statement of Li (1997) which stated that the most composition of nucleotides in a non-coding area of chloroplast DNA is adenine and thymine. On the other hand, the ratio of transition/transversion was quite high (R = 0.92) where the transition/transversion ratio of purine (0.004) and the transition/transversion ratio of pyrimidine (0.258). Variations appear among one species to another in the same genus or different one.

Sequence variations found in cpDNA sequences were generally caused by mutations in a single nucleotide which represents mutation that was present and had happened for a very long period of time (Fitmawati and Hartana 2010). In certain regions, the difference in length sequences of *trnL-F* was caused by mutations (Borsch et al. 2003), although the amount of change in this sequence was very small compared to the changes in the core genome, but was able to provide 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 (Hancock 2003), in contrast to the changes on nucleotide base occurred in the core of DNA which were recombination of both parents.

The phylogenetic tree presented in Figure 3 was built with maximum parsimony and 1000x bootstrap method. Neighbour-Joining method (NJ) was also performed to see differences in genetic distance and analyze the similarity between samples (Figure 3).

Analysis of phylogenetic trees revealed important answers about ancestral traits. Ingroup of genus *Bouea* was separated from outgroup with 100% bootstrap value. This separation showed that the sequence of *trnL-F* Intergenic Spacer was really a sequence of genus *Bouea*. The resulting ingroup tree was a monophyletic tree with three main groups. The first group was a group of one species, namely *B. oppositifolia* consisting of 7 accessions. The second group was also a group of one species, namely *B. macrophylla* consisting of 6 accessions. The third group consisted of outgroup namely M. indica and *A. occidentale* (Figure 3). This was consistent with the statement of Taberlet et al. (1991) which stated that chloroplast DNA was used for the analysis of relationship between species but had not been able to separate the intraspecies grouping. Figure 3 showed that the ramifications of *B. oppositifolia* and *B. macrophylla* were separated explicitly, but it was not happened on *B. macrophylla* accession of Ambonese (AM) that showed unique characteristic by forming separate ramification from both types of *Bouea*. This was presumably caused by the geographic location of *Bouea* accession, which allowed the formation of different and specific ecological niches, allowing the occurrence of significant changes in sequence base of nucleotide -F trnL chloroplast DNA.

A total of 6 accessions of *B. macrophylla* and 7 accessions of *B. oppositifolia* together formed two main branches and were not clustered geographically. Analysis of phylogenetic tree based on the sequences *trnL-F* showed that *B. oppositifolia* was not derived from a common ancestor of the area. The apportionment pattern at various places could explain that genus *Bouea* was a native plant in western Indonesia. The absence of relationship among them was possible because their growing environment was isolated, and this state was associated with genetic factors that lead to the distance relationship. On the other hand, *B. macrophylla* going into single clad showed a high relationship among their members. This can be explained that *B. macrophylla* came from one of the branches derived from *B. oppositifolia*. In this case, *B. macrophylla* was a form of cultivation. This was also supported by morphological characters possessed by *B. macrophylla* such as fruit size, bigger leaf size and brighter colors that were superior in a character of cultivation.

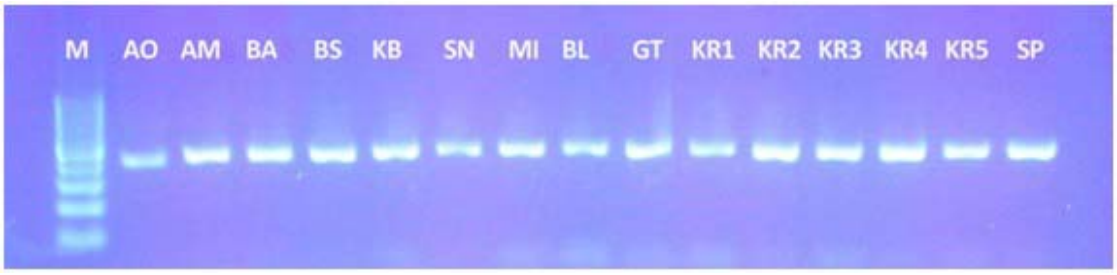

Figure 2. Visualization of PCR products by 1% agarose; AO = *A. occidentale*, AM = Ambon (*B. macrophylla*), BA = Banten (*B. macrophylla*), BS = stone cage, West Sumatra (*B. macrophylla*), KB = Kalimantan Barat (*B. macrophylla*), SN = Lhoksukon , Aceh (*B. macrophylla*), MI = *Mangifera indica*, BL = Bangka Belitung (*B. oppositifolia*), GT = Gunung Tua, North Sumatra (*B. oppositifolia*), KR1, KR2, KR3, and KR5 = Bogor Botanical Gardens (B . *oppositifolia*), KR4 = Bogor Botanical Gardens (*B. macrophylla*), SP = Sipiongot, North Sumatra (*B. oppositifolia*)

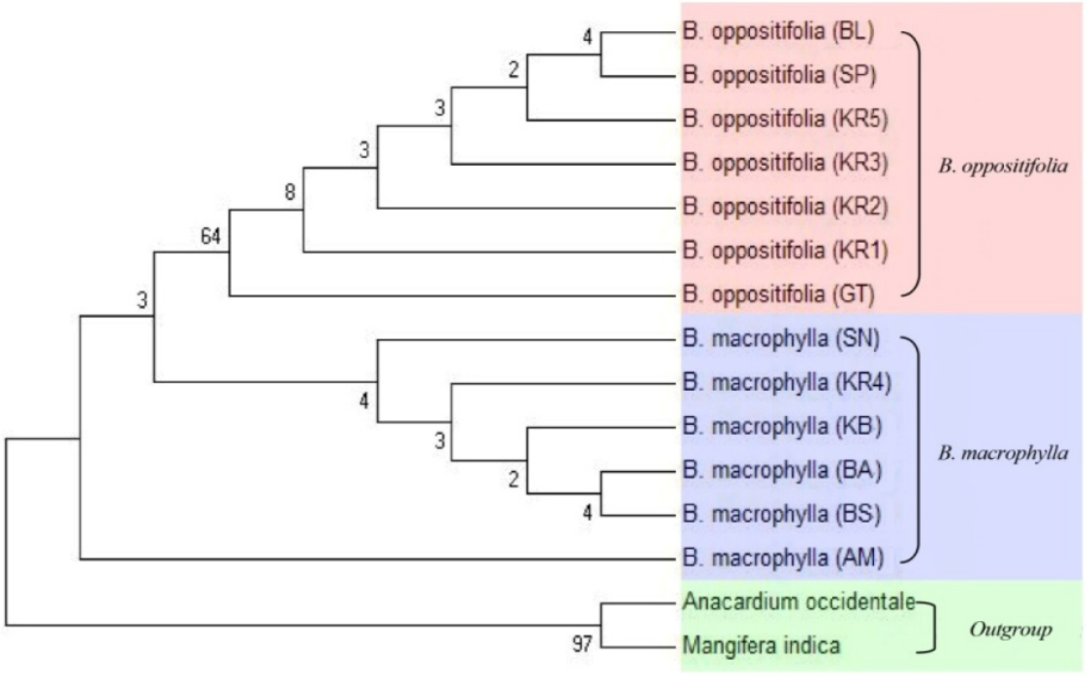

Figure 3. Phylogenetic tree of trnL-F sequences of *Bouea* and outgroup *(A. occidentale* and *M. indica*) which was reconstructed using Maximum parsimony methods based on kimura-2-parameter models. Branching analyzed by bootstrap values> 50% of 1000 replicates.

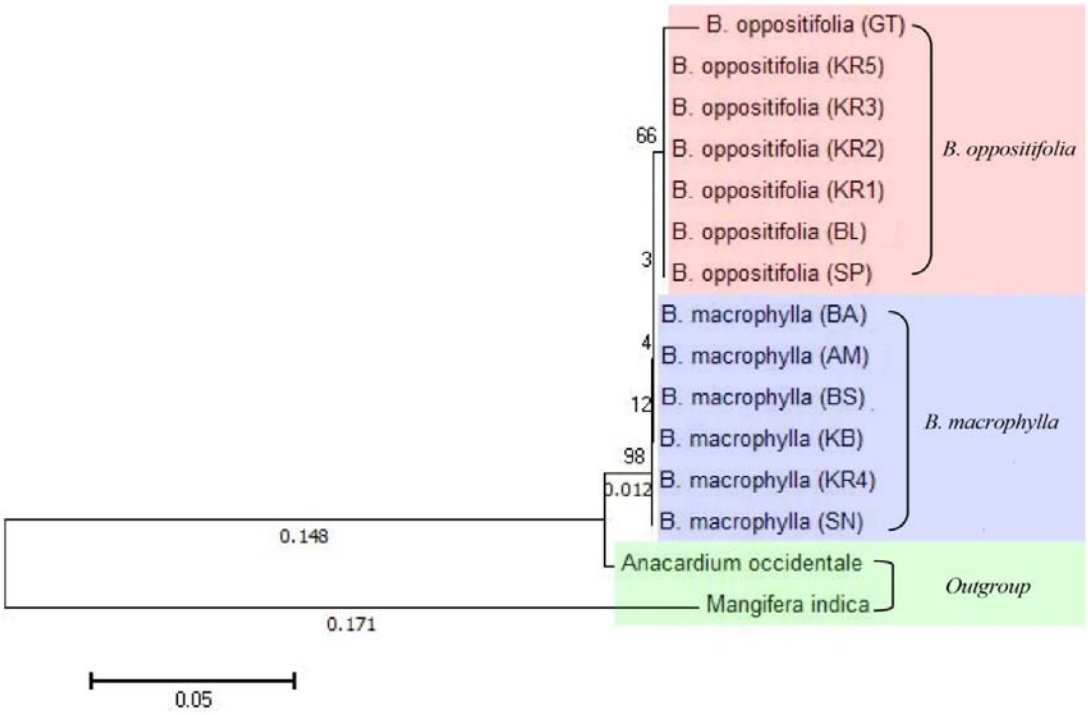

Figure 4. Phylogenetic tree of sequences *trnL-F* of genus *Bouea* and outgroup (*A. occidentale* and *M. indica*) as a result of reconstructions using a Neighbour-Joining method. Branching was analyzed by bootstrap values> 50% of 1000 replicates.

Table 2. Variation in length, AT content, and GC content on *trnL-F* sequences on Genus *Bouea*

Sample	T	C	A	G	Total (bp)	%AT	%GC
B. macrophylla (BA)	32.2	22.6	28.8	16.4	451.0	61.0	39.0
B. macrophylla (AM)	32.4	22.9	28.5	16.2	445.0	60.9	39.1
B. macrophylla (BS)	31.9	22.2	29.4	16.4	445.0	61.3	38.7
B. macrophylla (KB)	32.3	22.6	29.0	16.2	452.0	61.3	38.7
B. macrophylla (KR4)	32.2	22.8	28.9	16.2	426.0	61.0	39.0
B. macrophylla (SN)	32.1	22.2	29.5	16.3	455.0	61.5	38.5
B. oppositifolia (BL)	32.2	22.5	29.1	16.3	454.0	61.2	38.8
B. oppositifolia (GT)	32.1	23.1	28.5	16.3	424.0	60.6	39.4
B. oppositifolia (KR1)	32.1	22.8	28.8	16.4	452.0	60.8	39.2
B. oppositifolia (KR2)	32.1	22.8	29.0	16.2	452.0	61.1	38.9
B. oppositifolia (KR3)	32.2	22.7	28.9	16.1	453.0	61.1	38.9
B. oppositifolia (KR5)	32.1	22.8	29.0	16.2	452.0	61.1	38.9
B. oppositifolia (SP)	31.9	22.8	29.0	16.2	451.0	61.0	39.0
Anacardium occidentale	31.3	22.5	30.2	16.0	457.0	61.5	38.5
Mangifera indica	33.7	21.5	32.0	12.8	413.0	65.6	34.4
Average	32.2	22.6	29.2	16.0	445.5	61.4	38.6

Table 3. similarity coefficient of *B. oppositifolia*, *B. macrophylla*, and two outgroups (*A. occidentale* and *M. indica*) based on cpDNA sequences *trnL-F* intergenic spacer

Accession	BM AM	BM BA	BM BS	BM KB	BM KR4	BM SN	BO BL	BO GT	BO KR1	BO KR2	BO KR3	BO KR5	BO SP	AO	MI
BM AM	1.0														
BM BA	0.995	1.0													
BM BS	0.993	0.988	1.0												
BM KB	0.997	0.997	0.991	1.0											
BM KR4	0.933	0.937	0.931	0.935	1.0										
BM SN	0.991	0.986	0.997	0.989	0.929	1.0									
BO BL	0.986	0.982	0.993	0.984	0.929	0.991	1.0								
BO GT	0.929	0.933	0.927	0.931	0.992	0.925	0.925	1.0							
BO KR1	0.993	0.993	0.991	0.991	0.933	0.989	0.988	0.929	1.0						
BO KR2	0.995	0.995	0.988	0.993	0.933	0.986	0.986	0.929	0.997	1.0					
BO KR3	0.997	0.993	0.991	0.995	0.931	0.989	0.988	0.927	0.995	0.997	1.0				
BO KR5	0.995	0.995	0.988	0.993	0.933	0.986	0.986	0.929	0.997	0.999	0.997	1.0			
BO SP	0.993	0.993	0.986	0.991	0.935	0.984	0.984	0.931	0.995	0.997	0.995	0.997	1.0		
AO	0.969	0.973	0.963	0.971	0.912	0.96	0.956	0.908	0.967	0.969	0.967	0.969	0.967	1.0	
MI	0.6	0.602	0.598	0.601	0.554	0.597	0.592	0.55	0.601	0.599	0.597	0.599	0.597	0.613	1.0

Note: BM = *Bouea macrophylla*, BO = *Bouea oppositifolia*, AO = *Anacardium occidentale*, MI = *Mangifera indica*, AM = Ambon, BA = Banten, BS = Sumatera Barat, KB = Kalimantan Barat, KR1-5 = Bogor Botanical Gardens, SN = Lhoksukon Aceh, BL = Bangka Belitung, GT = Gunung Tua, SP = Sipiongot

Figure 4 confirmed that *B. oppositifolia* derived from Gunung Tua have a longer clad size. Based on analysis of Neighbor Joining can be said that the origin of Old Mountain *B. oppositifolia* older or appear earlier than other *B. oppositifolia*. It also illustrates that *B. oppositifolia* origin Old Mountain is the embryo or the ancestors of *Bouea* in Sumatra. The neighbor-joining tree also revealed that this type of genetic distance *B. oppositifolia* with long age older type than the type *B. macrophylla*. Genus *Bouea* has a closer relationship with *A. occidentale* compared with M. indica is known to have a better resemblance with the genus *Bouea*.

Diversity indicated by markers cpDNA diversity was relatively different from that indicated by morphological markers. The pattern that emerges from cpDNA markers was not always associated with the resulting pattern of morphological markers, and vice versa. This was possible because the expression at the level of morphology was a result of the recombination of two parents and environmental factors. Additionally, gene sequences located on the DNA chloroplast experienced a lower rate of evolution than that on DNA core (Taberlet et al. 1991). A non-coding area had a high mutation rate so it had more variations and more informative than that of the coding area (Taberlet et al. 1991; Hamilton 1999).

The result of Neighbour-Joining analysis on figure 4 showed that *B. oppositifolia* originated from Gunung Tua (GT), North Sumatra had the longest internode and it is showed up earlier so it was suspected that *B. oppositifolia* was the ancestor of *B. macrophylla*. Diversity indicated by cpDNA markers could be very different from the diversity indicated by morphological markers. The pattern that

emerges from cpDNA markers was not always associated with the pattern created by morphological markers, and vice versa. Chloroplast was inherited uniparentally or passed down through the female parent, while morphology was inherited from the two parents through the recombination process and was influenced by the environment. This was why there were differences in morphology and cpDNA grouping.

Table 3 showed the genetic distance and similarity coefficient of *B. oppositifolia*, *B. macrophylla*, and two outgroups (*A. occidentale* and M. indica). *B. oppositifolia* had highest similarity coefficient with 0.999, and it was among *B. oppositifolia* originated from Bogor Botanical Gardens (KR5) and *B. oppositifolia* originated from Bogor Botanical Gardens (KR2). While, *B. macrophylla* had the highest similarity coefficient with 0.997, and it was among *B. macrophylla* from Banten (BA) and Batu Sangkar, West Sumatra (BS) and *B. macrophylla* from West Kalimantan (KB) and also from Lhoksukon, Aceh (SN). The highest similarity coefficient of B. *oppositifolia* with *B. macrophylla* was 0.997 namely *B. macrophylla* originated from Ambon (AM) with *B. oppositifolia* originated from Bogor Botanical Gardens (KR3).

Based on the results of this study, it could be concluded that 6 accessions of *B. macrophylla* together with 7 accessions of *B. oppositifolia* formed two main branches and were not clustered geographically. *B. macrophylla* was a form of cultivation of *B. oppositifolia*. And, *B. oppositifolia* originated from Gunung Tua (GT), North Sumatra had the longest internode and was suspected as the common ancestor of *B. oppositifolia*.

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