

## Moderate Level of Genetic Diversity in *Anthocephalus Macrophyllus Roxb*, an Endemic Tree of Sulawesi and Its Implication in Conservation

Siti Halimah Larekeng<sup>1\*</sup>, Muh. Restu<sup>1</sup>, Gusmiaty<sup>1</sup>, Syamsuddin Millang<sup>2</sup>, Budirman Bachtiar<sup>2</sup>

<sup>1</sup> Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Indonesia.

<sup>2</sup> Silviculture and Tree Physiology Laboratory, Faculty of Forestry, Hasanuddin University, Indonesia.

\* Corresponding author E-mail: sitih5h.82@gmail.com

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### ABSTRACT

*Anthocephalus macrophyllus* Roxb is an endemic tree to Sulawesi having high economic value. It has rarely been investigated, either in genetic population or genetic consevation aspects. The information regarding genetic diversity is very important in order to gain more understanding for breeding and conservation strategies. Genetic diversity is used as material selection of expected genotype. The study was to determine genetic diversity of *A. macrophyllus* from Sulawesi provenances that would be used in further development of this species. Leaf samples were collected from 108 families of *A. macrophyllus* belong to Sulawesi provenances. Four out of ten microsatellite markers that had high polymorphism were used for amplifying the 108 DNA samples. Results showed number of allele and mean of allele were 3 to 4 alleles and 3,5, respectively. Moreover, PIC mean of the evaluated loci was 3,7. The analysis of genetic relationship showed that the 108 families had moderate level of genetic diversity. This research suggest to establish germplasm nursery via either seed from different provenance. In situ and ex situ approaches have to be applied together for conserving genetic resources of *Anthocephalus macrophyllus*.

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### Keywords:

*Anthocephalus macrophyllus* Roxb; endemic tree; genetic diversity; ex situ conservation; microsatellite marker

### 1. Introduction

Indonesian tropical forest plays an important role in reducing emission and maintaining climate and forest biodiversity. The tropical forest species like mangrove (Sarno *et al.*, 2015), *Ocotea* species (Martins *et al.*, 2014), *Gmelina arborea* (Wee *et al.*, 2012) has developed research about biodiversity. *Anthocephalus macrophyllus* has rarely been investigated, either in genetic population or genetic conservation aspects. Other the population distribution of its native habitats (provenances) will affect the genetic diversity of this species. Different provenances will show varied genetic diversity among the provenances. The information about genetic diversity is very important in

order to achieve more understanding for further development of *Anthocephalus macrophyllus*. Genetic diversity is used as material selection of expected genotype. One of the approaches to estimating genotype variability is by new method based on molecular analysis.

The advancement of marker molecular in DNA analysis, such as microsatellite, has been utilized for characterizing genetic variation and relationship within genus, species, cultivar and accession. Microsatellite markers have widely been applied in forensic identification, diagnose and identification of diseases, study of genetic population, bottlenecks effect and biological conservation in order to observe the changes in population, the impacts of fragmentation and interaction at different populations as well as the identification of new population formed (Zulfahmi, 2007). Liang *et al.* (2015) used microsatellite markers for evaluating genetic diversity of *Diosphyros kaki*. Poncet *et al.* (2007) also assessed genetic diversity of different *Coffea* using microsatellite markers. Larekeng *et al.* (2017) used microsatellite markers for *Diosphyros celebica* an endemic tree in Sulawesi. The benefits of SSR analysis are 1) accurately identifying polymorphism, 2) co-dominant nature, and 3) highly reproducible. Due to the capability of SSR markers for distinguishing individuals, they have currently been applied in identifying and analyzing parent coconut trees (Larekeng *et al.*, 2015) and eboni (Restu *et al.*, 2016), evaluate xenia effect in two types kopyor coconut (Maskromo *et al.*, 2016).

The development and implementation of conservation strategies must take into account the species current genetic diversity to recognize priority areas for both in situ and ex situ conservation (Martins *et al.*, 2015). The purpose of this study was to determine genetic diversity of *Anthocephalus macrophyllus* in Sulawesi for improving the understanding of this species.

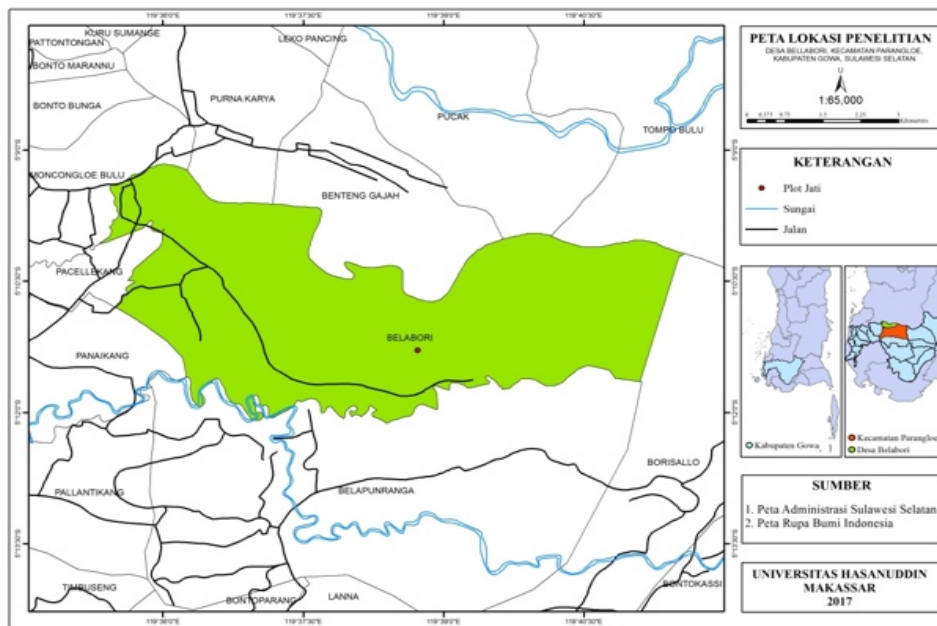
## 2. Materials and Methods

### 2.1. Study area

Sample collection was conducted in Juni 2016. The area is administratively located mostly in Bellabori village, Parangloe subdistrict (previously known as Camba subdistrict), Gowa, South Sulawesi, Indonesia (Figure 1). The location of the all samples were plotted in the map of adult individuals generated by Arcgis 10.1 software. DNA and molecular analysis was carried out in Laboratory of Biotechnology and Tree Breeding Laboratory, Department of Forestry, Faculty of Forestry, Hasanuddin University, Makassar, South Sulawesi, Indonesia.

### 2.2. Plant Materials

As many as 108 individuals of *Anthocephalus macrophyllus* from 108 mother tree (family) were evaluated in this study. All plant materials were collected at progeny test of Regional Tree Seed/Seedling Office (BPTH Region II Sulawesi). One to two young leave of each family were cut and used as DNA sources. The leaves collected were wrapped according to their family in a transparant plastic wrap with detail family identification on it and then temporary stored in cool box. They were transferred to laboratory and kept at -20°C in freezer until extraction.



**Figure 1.** Map of Study Site of *Anthocephalus macrophyllus* Roxb stands at Bellabori village, Parangloe subdistrict (previously known as Camba subdistrict), Gowa, South Sulawesi, Indonesia

### 2.3. DNA Extraction

DNA was extracted from 100 mg of leaf tissue. Extraction steps were carried out using Genomic DNA Mini Plant kit (Geneaid) protocol. The quality of extracted DNA was then assessed on 0,8% of agarose. The DNA amplification procedure used in this study was previously reported in *Coffea canephora* by Poncet *et al.* (2007). Those loci were M306, M309, M321, and M326 which had been earlier screened from ten selected SSR loci conducted by Restu *et al.* (2017a).

### 2.4. Amplification, Separation and Microsatellite Genotyping

Amplification reaction mixture consisted of PCR mix Kapa 2G fast, DNA template, SSR primer (10 ng/ $\mu$ L of each primer), and ddH<sub>2</sub>O. The amplification process were conducted by the following steps: one cycle of pre-amplification at 95 °C for 3 minutes, 35 cycles of amplification steps at 95 °C for 30 seconds (template denaturation), primer annealing for 30 seconds at 54,5 °C to 58 °C (specific temperature of each primer), and 72 °C for 60 seconds (primer extension), and one cycle of final extention at 72 °C for 5 minutes. A Labcycler® Thermocycler (Sensoquest, Göttingen, Germany) was used for performing amplifications with PCR protocols following KAPA Biosystem kit.

PCR products were subsequently separated using horizontal electrophoresis with 3% of Super Fine Resolution (SFR) agarose and TAE 1x buffer. The flourescent stain GelRed was added once SFR agarose dissolved. Electrophoresis separation was done for 90 minutes at 100 volt (Seng *et al.*, 2013). The electropherograms were visualized and documented using geldoc (Biostep).

## 2.5. Data analysis

The genetic data were obtained in the form of PCR amplification bands using certain primer pair. Those bands were later interpreted as genotype data and then analyzed using molecular softwares. GeneAlex ver 6.5 software (Peakall and Smouse, 2012) was applied for analyzing number of alleles (Na), number of effective alleles (Ne) as well as expected heterozygosity (He). Individuals clustering was done by Neighbor Joining (NJ) method using DARWin ver 6.0 software (Perrier *et al.*, 2003). In addition, PIC value was calculated using *Polymorphic Information Content Calculator an Online Program* (Naggy *et al.*, 2012).

## 3. Results and Discussion

The four evaluated loci shown in Table 1 and was able to generate a total of 14 alleles. Each marker locus generated a range of 3 to 4 alleles (Table 2). Mean number of effective alleles was 1,76. The lowest Ne was observed in M306 locus (1,35), whilst the highest one was 1,94 (M321 locus). The M306 locus showed the lowest PIC value, 0,23, whereas M321 locus had the highest (0,44). Mean PIC value of these loci was 0.38. Moreover, the lowest He observed was 0,25 which generated using M305 locus, while that of the highest one was 0,42 (M321 locus). Genetic relationships among individuals were clustered using UPGMA method. The clustering showed all evaluated individuals were distributed into 5 clusters (Figure 2), and the fourth cluster had the highest individuals number. On the other hand, the first cluster had the shortest genetic distances among its individuals.

**Table 1.** Primer Name, Repeat Motif and Primer Sequence (5' to 3') of Four Microsatellite Marker Loci From Rubiaceae Family Used in Amplification

No	Locus	Primer (5'-3')
1	M306	F: CTCGTTTGCTCTTTTTG R: TTTGTTAGTTTCTCACCACCA
2	M309	F: AGCAACATTTCCCAGTCAA R: GACCGCAATTTTCTTGTTTC
3	M321	F: TCGATTGGTTTTGCATACATCT R: GCCAAGATAATGGTTGTGTGG
4	M326	F: GCTTTCTTGCTTTCTTTTCC R: CATCCACTTACCCTCCTCCAAA

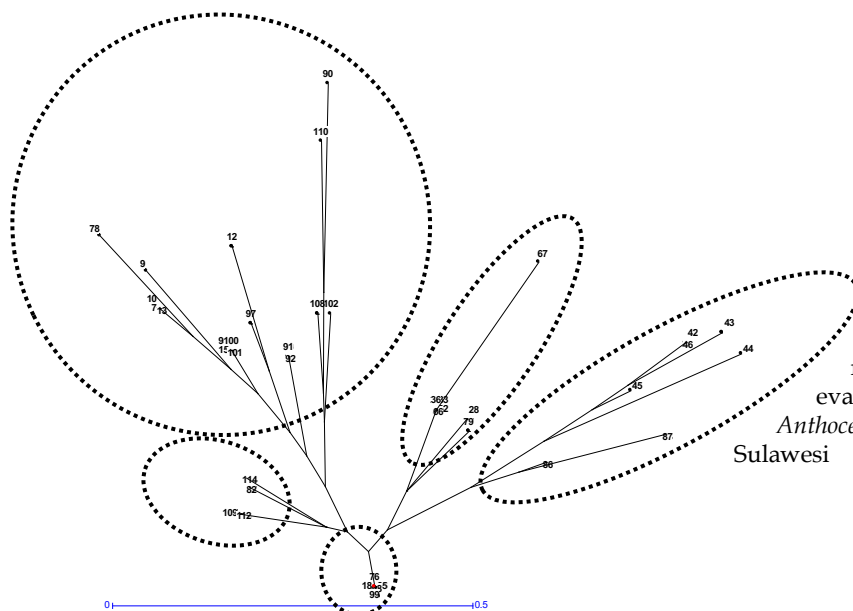
SSR marker loci developed from *C. Canephora* were able to generate the evaluated individuals of *Anthocephalus macrophyllus*. It is suggested due to these both species are clasified into same family (Family Rubiaceae). The developed SSR loci of a certain species could be used to amplify DNA of other species, whose close taxonomical relationship to it (same family or genus). According to previous study by Wang *et al.*, (2011), species categorized into same family have the same SSR loci, thus they can also be used as other species's SSR loci.

**Table 2.** The parameters of genetic diversity observed in each locus

No	Locus name	Tm (°C)	Na	Ne	PIC	He
1	M306	54,5	3	1,35	0,23	0,26
2	M309	55	4	1,83	0,42	0,45
3	M321	58,5	4	1,94	0,44	0,49
4	M326	58	3	1,91	0,41	0,48
Mean			3,5	1,76	0,37	0,42

*Tm*: Melting temperature

Ten screened SSR marker loci in the earlier study by Restu *et al.* (2017a) showed only 4 loci could be used in genetic analysis of *Anthocephalus macrophyllus*. The remaining six unselected loci were due to monomorphic bands (2 loci), could not amplify DNA fragments (3 loci) and unclear amplified products (1 locus). Manurung *et al.* (2017) also used four microsatellite loci (M3, M64, M81, and M98) to characterize the genetic diversity of *Avicennia marina*.



**Figure 2.** Dendrogram of relationship among 108 evaluated individuals of *Anthocephalus macrophyllus* in Sulawesi

The SSR loci used in this study produced 3,7 of mean PIC value. The PIC values represents the ability of loci in detecting alleles. This PIC value indicates that SSR loci are informative enough for analyzing the genetic diversity of *Anthocephalus macrophyllus*. Bostein *et al.* (1980) stated PIC values are categorized into 3 classes,  $PIC > 0,5$  (highly informative),  $0,25 < PIC < 0,5$  (moderately infomative), and  $PIC < 0,25$  (less informative). M306 locus generated the same number of alleles as M326, yet it had much higher PIC value. It proves PIC value does not depend on the number of alleles but the frequency of alleles. One of alleles at M306 locus had low frequency (data not shown). Thus, although M306 and M326 loci showed same number of alleles, they had different PIC values. It is supported by DeVicente and Fulton (2003) who stated PIC value of a locus is determined by the frequency of alleles at the locus.



The number of alleles was 3 to 4 alleles with a mean of 3,5 alleles. It was almost as many as reported by Poncet *et al.* (2007) in *Coffea canephora* dan *Coffea pseudozanguebariae* and Jestrow *et al.* (2016) in *Coccothrinax jimenezii* which their mean of alleles were 3,3 and 3, respectively. Result of this study was higher than in previous study by Irmayanti *et al.* (2015) in Mindi (2,75), but lower than in Sayekti *et al.* (2015) and Win *et al.* (2015) (5,1 and 13, respectively). The few number of loci (4 loci) and DNA samples only collected from one region, Sulawesi, cause low number of alleles found in this analysis. In contrast to this study, Sayekti *et al.* (2015) used samples from different partial regions in Angola, and Win *et al.* (2015)'s were from various places in Myanmar. Total number of samples and where samples collected are the main affecting factors how many alleles number will be detected (Nurtjahyaningsih dan Rimbawanto, 2015).

The understanding of genetic diversity of *Anthocephalus macrophyllus* forms a significant platform on which further studies can build upon. These highly polymorphic microsatellite markers could be further used to construct a genetic map, followed by marker assisted breeding for *Anthocephalus macrophyllus*. Some information, polymorphic microsatellite markers can be used to study mating system of *G.arborea* and to clarify if inbreeding indeed happens for *G.arborea* (Wee *et al.*, 2012), *D. celebica* (Restu *et al.*, 2017b). Paternity analysis of *Moringa oleifera* populations used eight microsatellite loci and showed the maximum pollen dispersal distance between two trees estimated in this experimental stand is highly localized at current  $3 \times 3$  m planting density (Wu *et al.*, 2018).

Conservation is mainly aimed at preserving species biodiversity. Orchid in Bolli forest are rare plants based from the analysis of vegetations, there was as much as 37 species of orchids found mostly in riparian zones. The small distribution and low specific density of this orchid showed to be very susceptible to local extinction if their habitat is disturbed. This population pressure will threaten the sustainability of wild orchids, demanding the attention of those concerned for their much needed conservation efforts in the future (Sjahril *et al.*, 2013). The similar of this study proves that the moderate level of genetic diversity has significant implication in providing the information of species extinction in order to improve tree breeding program. Furthermore, ex situ conservation is essential for protecting vulnerable even endangered species (Thomas *et al.*, 2014) so that we recommend to establish germplasm nursery via either seed from different provenance. In situ and ex situ approaches have to be applied together for conserving genetic resources of *Anthocephalus macrophyllus*.

#### 4. Conclusion

The analysis of genetic relationship *Anthocephalus macrophyllus* showed that moderate level of genetic diversity. In situ and ex situ approaches have to be applied together for conserving genetic resources of *Anthocephalus macrophyllus* and we recommend to establish germplasm nursery via either seed from different provenance.

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