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Genetic variability of porang populations (*Amorphophallus muelleri*) in West Java and Central Java based on *trn*L intron sequences

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Key words: *Amorphophallus muelleri*, *trn*L intron, genetic variability.

Abstract

Porang (*A. muelleri*) is herbaceous plant which is included in the family of *Araceae*. Previous studies showed morphological and genetic variation in *A. muelleri*. The suitable molecular marker used to indicate genetic variation is *trn*L intron. It is based on, *trn*L intron is non-coding regions of chloroplast genome which evolved more rapidly than coding region. The present study is aimed to find out genetic variability and phylogenetic relationship among *A. muelleri* populations in Central Java and West Java which is based on *trn*L intron sequences. Porang samples are obtained from central Java (Karangtengah, Wonogiri, Grobogan and Brebes) and West Java (Cisompet Garut). *trn*L sequences were analyzed by maximum parsimony and neighbor joining method. *trn*L intron has singletone variation as many as 13 sites, i.e on the order of squences: 49, 109, 144, 145, 159, 253, 297, 298, 300, 302, 379, 461, 463. In addition, *trn*L intron has 9 parsimony informative sites, i.e on the order of squences: 160, 184, 299, 409, 452, 454, 513, 527, 534. The highest number of haplotypes came from Grobogan populations with values diversity of haplotype is 1, while the least number of haplotypes came from Karangtengah and Wonogiri populations with diversity of haplotype is 0.000. phylogenetic tree shows the clustering of populations into three clade, first clade is *A. variabilis* and second clade are *A. ochroleucus, A. longituberosus and A. sumawongii* as outgroup and thirth clade divided into two subclade porang are clustered based on their population in Central Java and West Java

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Introduction

Porang (*Amorphophallus muelleri* Blume) included in the family *Araceae* is one of 27 species of Amorphophallus in Indonesia and one of the 170 species Amorphophallus in the world (Poerba & Martanti, 2008). Porang is an annual herb that can be reproduced generatively through seeds and vegetatively through tubers and bulbil. bulbil is a brown vegetative reproduction (Yuzammi, 2000) which is a special organ of porang used as modifiers of the other genus Amorphophallus.

In general, geographic distribution of porang is paleotropik (Hetterscheid & Ittenbach, 1996). Jansen *et al.*, (1996) mentioned that porang is distributed from Andaman islands to the east through Burma (Myanmar) and then north to Thailand and to the southeast toward Indonesia and Timor. In Indonesia, distribution of porang is limited to certain areas such as Sumatra, Madura, Bali, West Nusa Tenggara (NTB) and most commonly found on the Java island (Jansen *et al.*, 1996).

In Java porang is only found in certain areas. In East Java porang can be found in Klangon Madiun, Tritik Nganjuk, Gondang Bojonegoro, Brongkos Blitar, Wajak and Lawang Malang (Unpublished). Whereas in Central Java porang can be found in Karangtengah Wonogiri, Grobogan, Brebes, Lebakbarang Tegal and Pekalongan and in West Java porang can be found in Cisompet Garut, Tjikirai, Tjibadak Halimun and Tarogong Garut (Yuzammi, 2000).

The distribution of clumped porang shows that these species are active speciation. It is supported by the fact that certain groups of porang have high value similarity of kinship when they are compared with surrounding porang (Hetterscheid & Ittenbach, 1996). The results of the study with RAPD method show that porang in Java has high value similarity of kinship and have genetic variability between individuals and populations (Poerba & Martanti, 2008).

Kinship and genetic variation of porang with RAPD analysis needs to be strengthened with molecular markers using DNA sequences either chloroplasts, mitochondria or nuclear. From the three genomic DNA, the genomic chloroplast is genomic DNA that is widely used as molecular markers to determine the genetic variation, because it is uniparental (maternal generally in most Angiosperm), it does not undergo recombination and it is small genomic (Provan *et al.*, 2001).

Chloroplast genes evolve faster than nuclear genes (Gabriele *et al.*, 1999). Chloroplast gene that can be used for the analysis of genetic variation is exons (coding regions) and introns (non-coding region). However, since the non-coding region is known having a faster evolution than coding-region, the locus is widely used as phylogenetic marker, the study of evolution and taxonomic studies at lower taxonomic levels (Tsai, *et al.*, 2006).

*Trn*L intron is one of the non-coding regions of the genomic chloroplast which is suitable for phylogenetic analysis of inter-species to inter-family (Kojoma, *et al.*, 2002). In higher plants, intron *trn*L is intron group I which does not undergo self-splicing (Simon *et al.*, 2003). It leads the *trn*L intron in higher plants have high sequence variation when it is compared with *trn*L intron in cyanobacteria (Basendahl *et al.*, 2000). *Trn*L intron has been proven to be used for the phylogenetic analysis of the Amorphophallus BI. Ex (Sedayu *et al.*, 2010; Grob *et al.*, 2004). *Trn*L intron has also successfully demonstrated genetic variability among porang populations in East Java (Rosidiani, 2011).

So far the use of *trn*L intron to determine genetic variation and kinship of porang still confined to the area of East Java, while for Central Java and West Java has not been done. Studies on genetic variability is important, because the demand of porang tuber crops is high. it may encourage the large cultivation which may affect the supply of seeds from the other population that would threaten other local variants (wild) of porang in Java, especially Central Java and

West Java. the objective of this study is aimed to find out the genetic variability and phylogenetic relationship among porang population in Central Java and West Java based on *trn*L intron sequences.

Material and methods

Plant material and DNA Extraction

Samples used in this study were A. muelleri collected from Central Java and West Java. The samples consisted of 6 populations and each population was taken 3 individuals for analysis of genetic variation. These population consisted of A. muelleri (2 population from Cisompet Garut West Java, 1 population from Karangtengah Central Java, one population in Wonogiri Central Java, 1 population from Brebes Pamedaran Central Java and I population from Kemadukbatur Grobogan Central Java). One population of A. variabilis in Wonogiri was selected as outgroup. In addition, it is also used outgrup taken from NCBI (Table 1.).

Table 1. List of species from Genbank as outgroup

Species	Accession number	Author
Amorphophallus ochroleucus	AF387463	Grob dkk (2002)
Amorphophallus longituberosus	AF497050	Grob dkk (2002)
Amorphophallus sumawongii	AF387471	Grob dkk (2002)

About 0.1 g leaves of A. muelleri were frozen in liquid nitrogen and ground with a mortar into powder and then transferred to a microcentrifuge tube. Then the extraction and isolation of genomic DNA of A. muelleri done using DNA Isolation Kit (GenElutet Plant Genomic DNA Miniprep Kit). While the quality of the DNA seen in the 0.8% gel electrophoresis.

TnrL intron was amplified using universal primers "c" (CGAAATCGGTAGACGCT ACG) and "d" (GGGGATAGAGGGACTTGAAC) (Taberlet *et al.,* 1991) (Fig.1.). TnrL intron was amplified in a sample volume of 30 µl with the following composition: 6 µl milliQ, 15 µl of intron Master Mix, 3 µl Primer (c and d respectively 2.5 µM) and 3 µl of DNA template (Sedayu *et al.,* 2010).

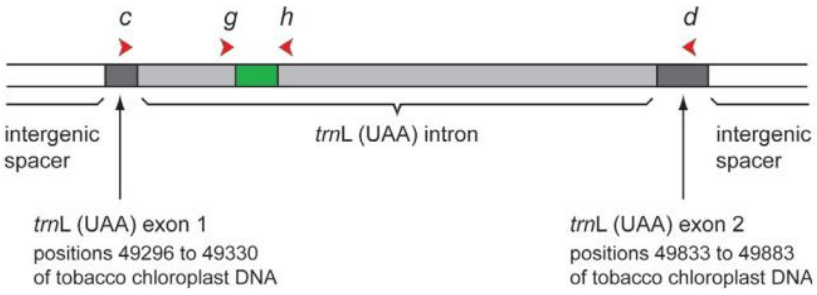

Fig. 1. Primer c and d position on the *trn*l gene (Taberlet *et al*., 2007).

PCR reaction by using a thermocycler (GenAmp PCR System 9700) for 35 cycles. Predenaturation at 95 °C for 5 min, followed by 35 cycles which consist of 45 seconds denaturation at 95 °C, primer annealing at 61.3 for 45 °C seconds, and extension at of 72 °C for 45 seconds. After 35 cycles completed, then followed by extension at 72 °C for 10 minutes. Amplification products were separated on 1.5% agarose gel in TBE buffer (Tris-Borate-EDTA) at 100 V for 45 minutes. Polymerase chain reaction product were separated in agarose gel detected using Gel Documentation (m8 meier UVDI science). 100 bp DNA ladder (Vivantis)

used as a standard to establish the size bands of DNA amplification product. All squences were determined at Macrogen Korea, which used ABI Prism 3730 x1 automate squencer. Squences were edited in Sequence Scanner 1.0.

Squences Alligment

Squences were alligned using MEGA 5.0 and edited with bioedit. Pairwise sequence divergence was calculated using Kimura's (1980) two parameter (K2P) method in MEGA 5.0 (Kumar et.al, 2004), with gap/missing data completely deleted. Squences analysed with dnasp and haplotype Network to determine haplotype variation.

Reconstruction of topology phylogenetic trees performed using maximum parsimony and neigbor joining method and then each generated tree is compared to see the congruence between both methods (Page & Holmes 1998). Reconstruction of topology phylogenetic using maximum parsimony and neigbor joining method based on that maximum parsimony is a character-based algorithms are quite accurate with the computation time is relatively faster than character-based algorithms such as maximum likelihood (Nei and Kumar, 2000) while neigbor joining is a method based on the principle of grouping taxa based on evolutionary distance value pairs operational taxonomy units (Nei, 1987).

Reconstruction of the phylogenetic topology using MEGA 5 program with maximum parsimony method using algorithmic calculation model CNI (Close Neighbor Interchange) on random tree using random addition sequence performed 10 replications while Neighbor Joining with algorithmic computation model of Kimura-2 parameter, tree evaluation is done by using bootstrap analysis on 1000 replicates.

Results and discussion

Genetik variability

TrnL intron amplification using universal primers c and d (Taberlet *et al.*, 1991) successfully performed. This is indicated by the thick band when it electrophoresed on 1.5% agarose gel. TrnL intron of A. muelleri were successfully amplified ranged from 600-700, whereas for outgrup (A. variabilis) ± 600 bp.

After sequencing, the size of sequences are not so different from band of electrophoresis. The trnL intron sequence of A. muelleri ranged from 602-710 bp, while A. variabilis ranged from 570-589. This is different from the trnL intron based on previous study on Amorphophallus i.e ± 900 bp (Sedayu, 2010) and ± 500bp (Rosidiani, 2012). However, the results of BLAST in the NCBI data base shows that the trnL intron squence produced in this study is the trnL intron which has 97% sequence similarity with the intron trnL of Amorphophallus sumawongii.

Based on the analysis of data that come from 17 trnL sequences of porang as ingroup, 3 trnl sequences of A.variabilis as outgroup and trnL sequences of A. ochroleucus, A. longituberosus and A. sumawongii (Table 1.) from Genbank as outgroup, found that trnL intron have singletone variation as many as 13 sites, which is on the order of bases to: 49, 109, 144, 145, 159, 253, 297, 298, 300, 302, 379, 461, 463. In addition, the trnL intron in this study had 9 parsimony informative sites that is on the order of bases to: 160, 184, 299, 409, 452, 454, 513, 527, 534 (Table 1.).

Table 1. the Differences among nucleotide composition of A. muelleri, A. A variabilis, A. ochroleucus, A. longituberosus and A. sumawongii.

	Number of nucleotide squences																				
	0	1	1	1	1	1	1	2	2	2	2	3	3	3	4	4	4	4	4	5	5	5
	4	0	4	4	5	6	8	5	9	9	9	0	0	7	0	5	5	6	6	1	2	3
	9	9	4	5	9	0	4	3	7	8	9	0	2	9	9	2	4	1	3	3	7	4
Population																						
Wonogiri kota 1	T	A	T	T	C	T	G	C	A	T	A	A	T	C	A	G	G	G	G	G	G	G
Wonogiri kota 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Wonogiri kota 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Karang tengah 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Karang tengah 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cisompet I 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	A	A
Cisompet I 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cisompet I 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cisompet II 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	A	A	.
Cisompet II 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	A	A
Cisompet II 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	A	A
Pamedaran 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.
Pamedaran 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Pamedaran 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.		.	.
Grobogan 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	A	A
Grobogan 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A
Grobogan 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. varibilis Brebes	.	.	.	.	.	C	A	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.
A. varibilis Wonogiri	.	.	.	.	.	C	A	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.
A. varibilis Wonogiri	.	.	.	.	.	C	A	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.
A. ochroleucus	.	.	.	.	T	.	.	T	.	.	.	.	.	T	C	.	.	.	.	.	.	.
A. longituberosus	A	.	G	C	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
A. sumawongii	.	G	.	.	.	.	A	.	T	A	.	T	T	.	C	.	.	.	.	.	.	.

Note
gray area = parsimony informative, white area = Singleton variation

Singletone variation is sites where only one taxa have different nucleotides. These sites are invariable sites that can provide information about which trees are parsimonius. So that the sites are informative, informative sites must have at least two types of nucleotides and both must appear at least twice on the site (Yingzhi *et al.*, 2007). These sites which will be used to create the topology of phylogenetic tree by using parsimony methods.

TrnL intron has a GC content of 27.8% to 28.6% and among populations of A. muelleri do not have difference of nucleotide composition. The analysis of trnL intron sequence in each population was found that the highest number of haplotype is from Cisompet and Grobogan population in which the value of haplotype variability is 1, while the least number of haplotype is from Wonogiri and Karangtengah population in which the value of haplotype variability is 0.000 (Table 2.).

Grobogan have the highest haplotype variability because Grobogan population is cultivation area with wide plantations. At the plantation A. muelleri seeds

is possible got from different regions, so its haplotype variability is high. While, Wonogiri and Karangtengah have low of total haplotypes and variability haplotype due to the cultivation area is very small and the area of cultivation is limited. Perhaps, Wonogiri and Karangtengah get the seeds from one area.

Table 2. Haplotype Variability in each population in Central Java and West Java.

Population	GC *content*	Total of haplotype	Haplotype variability
Wonogiri Kota	28.6%	1	0.000
Karangtengah	28.4%	1	0.000
Pamedaran	28.4%	2	0.667
Grobogan	27.9%	3	1
Cisompet II	27.8%	2	0.667
Cisompet I	28.2%	3	1

Haplotype network analysis is performed to find out the distribution, total and variability of haplotype. Haplotype network analysis of 23 samples ingroup and outgroup produced 11 haplotypes which value of haplotype variability is 0.806. 11 haplotypes consist of 5 different species. Haplotype 1 to 6 are *A. muelleri*, haplotypes 7 and 8 are *A. variabilis*, haplotype 9 is *A. ochroleucus*, haplotype 10 is A. *longituberosus*, whereas haplotype 11 is *A. Sumawongi* (Fig. 2.).

Small variation of chloroplast DNA haplotypes in this study are closely related to the characteristics of chloroplast DNA which is conservative, low mutation rate (Provan *et al*., 2001) and maternal inheritance in *A. muelleri* (including groups of angiosperms). The transfer of cloroplast DNA in *A. muelleri* come through gen migration than can happen vegetatively with tubers and bulbil and generatively with seeds. Although porang reproduce by seeds, but seeds formed are apomik thus resulting offspring identical to the parent (Poerba and Martanti, 2008). This is what might make porang haplotype variability is very low.

The highest haplotypes are haplotype 1 which the number of individuals are 10, while the lowest haplotypes are 2, 3, 5, 6, 7, 9,10 and 11. If it is seen from the results of haplotype network (Fig.2.), it can be found that there is a possible *A. muelleri* located in a haplotype 1 is most primitive when compared with the other *A. muelleri*. It can be viewed from the genetic relationship among haplotype 1 to *A. variabilis* (haplotypes 7 and 8) and *A. ochroleucus* (haplotype 9), *A. longituberosus* (haplotip 10), *A. Sumawongi* as outgroup (haplotype 11).

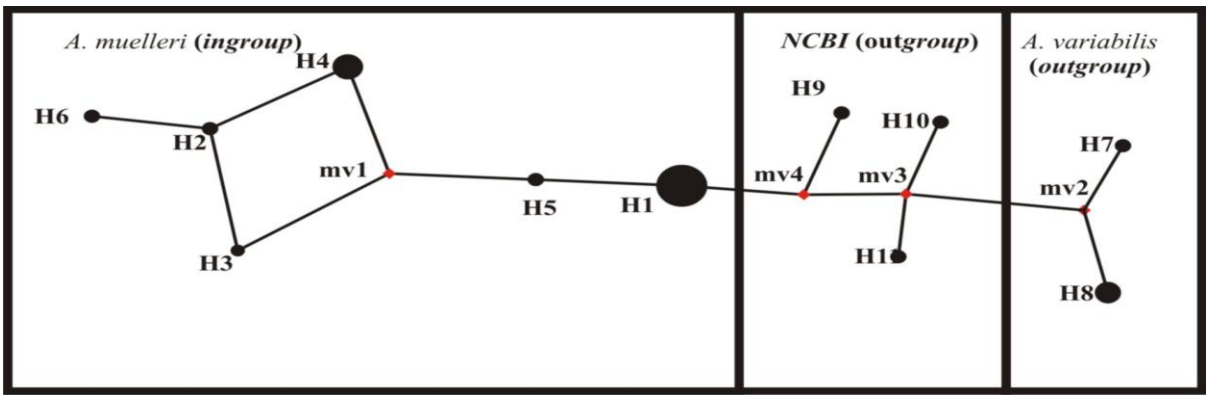

Fig. 2. *Haplotype network of* intron *trn*L squences of *A. muelleri, A. variabilis, A. sumawongi, A. ochroleucus* and *A. longituberosus*.

Note

H1 (haplotype 1) = *A.muelleri* Wonogiri 1, *A.muelleri* Wonogiri 2, *A.muelleri* Wonogiri 3, *A.muelleri* Karangtengah 1, *A.muelleri* Karangtengah 2, *A.muelleri* CisompetI 2, *A.muelleri* Cisompet I 3, *A.muelleri* Pamedaran 1, *A.muelleri* Pamedaran 2, *A.muelleri Grobogan 1*. H2 (haplotype 2)= *A.muelleri* CisompetI 1. H3 (haplotipe 3)= *A.muelleri* CisompetII 1. H4 (haplotype 4)= *A.muelleri* CisompetII 2, *A.muelleri* CisompetII 3, *A.muelleri* Grobogan 1. H5(haplotype 5)= *A.muelleri* Pamedaran 2. H6 (haplotype 6)= *A.muelleri* Grobogan 2. H7 (Haplotype 7)= *A. variabilis* Brebes. H_8 (Haplotype 8)= *A. variabilis* Wonogiri kota 1, *A. variabilis* Wonogiri kota 2. H_9 (Haplotype 9)= *A. ochroleucus*. H10 (Haplotype 10)= *A. longituberosus* H11 (Haplotype 11)= *A. Sumawongi.*

Phylogeny of A. Muelerri

Maximum parsimony

Topology of the phylogenetic tree with maximum parsimony method showed separation accession into 3 major clade. First clade are (*A. ochroleucus, A. and A. longituberosus sumawongii*), second clade are (*A.variabilis* from Wonogiri and Brebes) and third clade with 82 bootstrap support (BS) are divided into 2 subclade by the same ancestor, subclade 1 are (Cisompet II (1, 2 and 3), Grobogan (1 and 2), Cisompet I 1, Pamedaran 2) and subclade 2 are (Wonogiri (1,2 and 3)., Karangtengah (1 and 2), Grobogan 3, Pamedaran (1 and 2) and Cisompet I (2 and 3)) (Fig.2.).

Clade I, II and III consist of individuals from different species. First clade is individual obtained from Genbank and it differs species with *A. muelleri*, while the second clade consists of *A.variabilis* derived from population of Wonogiri and Brebes. Moreover, second clade consists of 2 subclades that it is clustered based on their population (Wonogiri and Brebes population). It strengthens the case that *trn*L intron is one of the non-coding regions of the chloroplast genome that is suitable for phylogenetic analysis from inter-species to inter-family (Kojoma *et al.*, 2002).

In addition, *A. muelleri* in Central Java and West Java are divided into two major clades clustered based on its population. *A. Muelleri* in Wonogiri (clade III subclade 2) and Cisompet II (clade III subclade 2) clustered based on its population, while Cisompet I, Pamedaran and Grobogan are randomly clumped (III subklad 1 and 2) (Fig.2.).

Further analysis can be known that clade III of subclade 1 is dominated by *A.muelleri* derived from West Java, while subclade 2 is dominated by *A. muelerri* from Central Java. *A. muelerri* which is from Grobogan population in Central Java is not clustered according to its geographical location. Grobogan 3 is clustered on Central Java clade, while Grobogan 1 and 2 are clustered on West Java clade. These results show the case that *A. muelerri* of Grobogan population get seeds from the same place with Central Java clade (clade III of subclade 2) and West Java clade (clade III of subclade 1).

The populations of Cisompet I are not clustered based on its geographical location. *A. muelleri* from Cisompet I was *A. muelleri* from one individu (Cisompet I 1 (parent), Cisompet I 2 and 3 {seedling from bulbil}), but Cisompet I 1 is in West Java clade, while Cisompet I (2 and 3) are in Central Java clade (Fig.3.). This suggests that, although *A. muelleri* regenerate vegetatively through bulbil (Jansen *et al.*, 1996) and the resulting offspring should be identical to the parent, but the offspring of bulbil have difference *trn*L intron sequences with its parent.

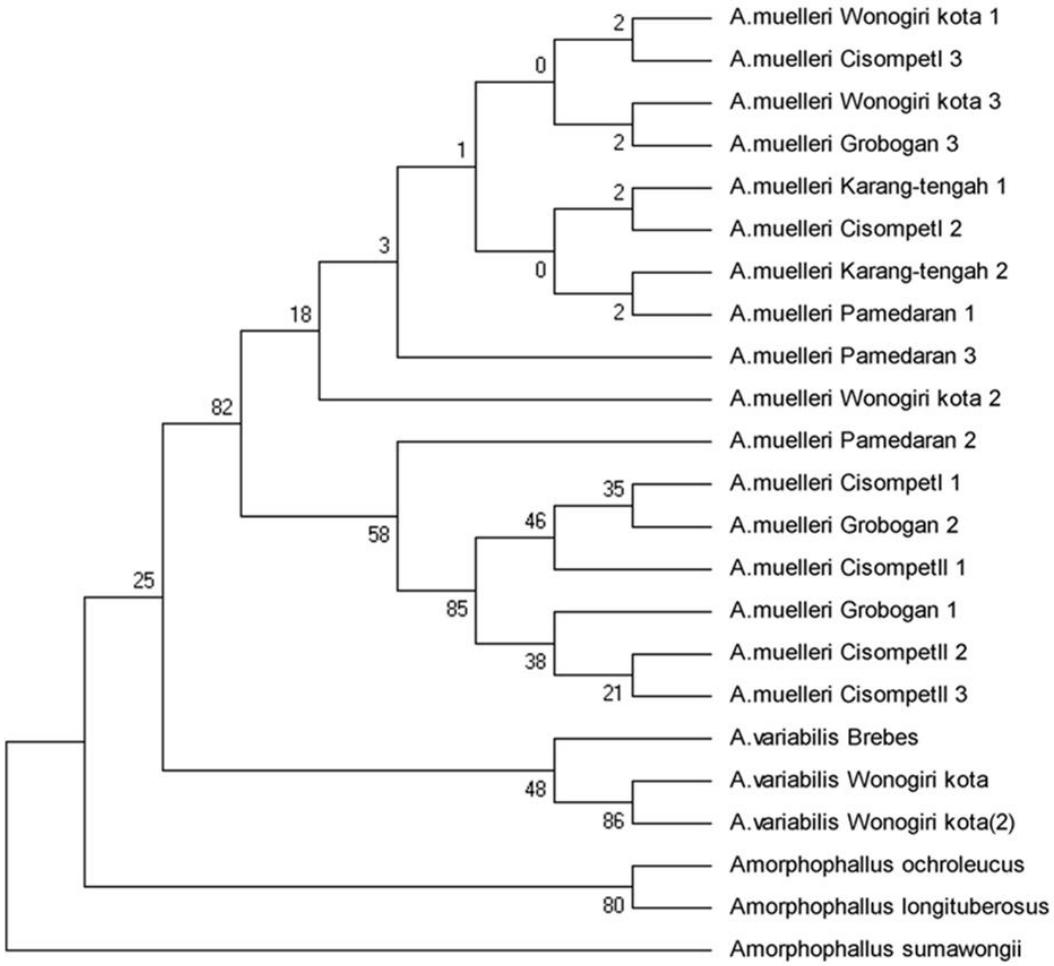

Fig .3. Phylogenetic tree with Maximum parsimony method (bootstrap 1000) of trnL intron sequences.

Neigbor Joining

Topology of the phylogenetic tree with neigbor joining method showed separation of accession into 3 major clades. The first clade used as outgroup composed of *A. variabilis*, *A. ochroleucus*, *A. longituberosus* and *A. Sumawongi*. second clade consists of 2 sub clade. The first sub clade with 93 Bootstrap support (BS) consisting of 2 sisters clade porang derived from Cisompet and Grobogan, while the second sub clade with 61 bootstrap support (BS) consists of *A. muelleri* derived from Pamedaran (Fig.4.). Bootstrap support (BS) between 70-100 shows that the phylogenetic tree branching will not be change. otherwise, if the bootstrap values less than 70 so the chances of branching structure are high, so that when it was reanalysed, the formed phylogenetic tree can change (Simpson, 2006). Therefore there is possibility that subclade 1 and sub clade 2 will be one clade if performed by different analysis

First Clade consists of *A. muelleri* derrived from Wonogiri, Pamedaran, Grobogan and Cisompet. The low bootstrap support (26) allows first clade to join both the Klad 2 subklad pamedaran (Fig.4.). This indicates that the second clade on subclade 2 consists of *A. muelleri* derived from Central Java (Pamedaran, Grobogan and Wonogiri).

The origin of *A. muelleri* parent on the first and second clade above is not certainly known. However, the early allegations on individual ancestor of *A. muelleri* came from Pamedaran Brebes, Central Java. It is based on interviews with chief of LMDH (Near Forest Society Institute) Pamedaran. He stated that *A. muelleri* in Pamedaran is wild *A. muelleri* found in

mountainous areas. Over the last few years the farmers from Pamedaran has been sold wild *A. muelleri* in the form of tubers or bulbil to various regions in East Java and West Java

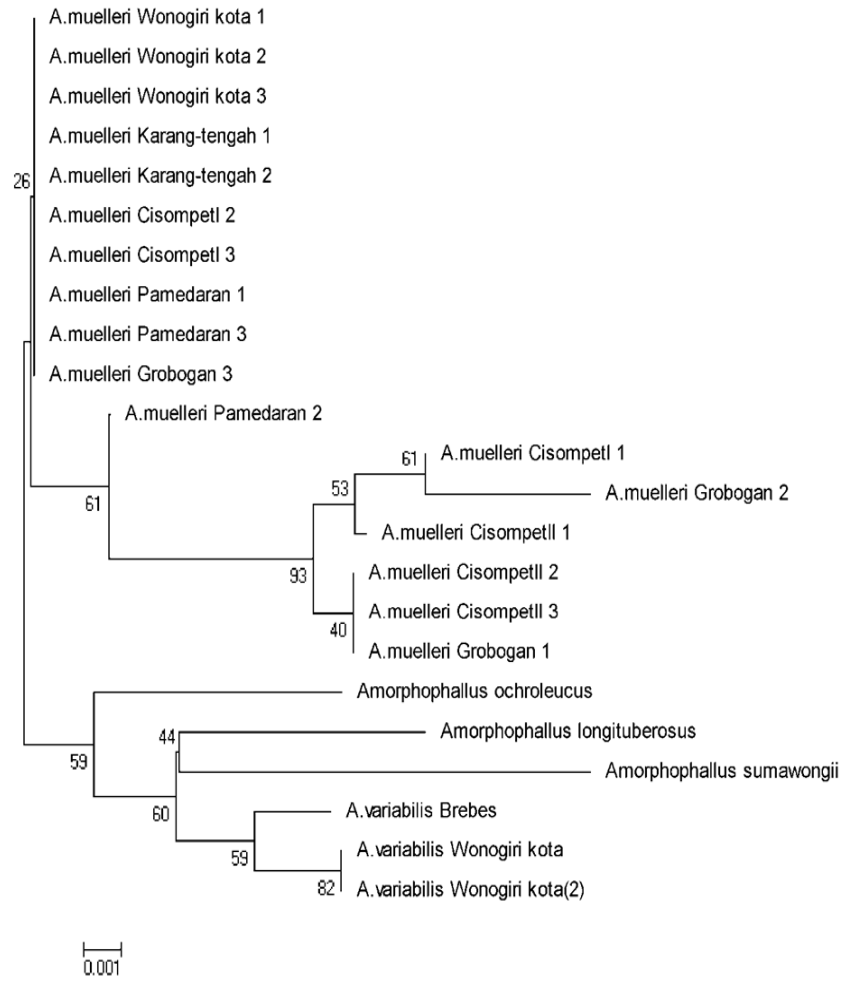

Fig.4. Phylogenetic tree with Neigbor joining method (bootstrap 1000) of trnL intron sequences.

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