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Phylogenetic Relationship of Indonesian Water Yam (*Dioscorea alata* L.) Cultivars Based on DNA Marker Using ITS-rDNA Analysis

Purnomo[1], Budi Setiadi Daryono[2] & Hironobu Shiwachi[3]

[1] Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada, Indonesia

[2] Laboratory of Genetic, Faculty of Biology, Universitas Gadjah Mada, Indonesia

[3] Graduate School of Agriculture, Tokyo University of Agriculture, Tokyo, Japan

Correspondence: Purnomo, Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada, Indonesia. E-mail: nomo@ugm.ac.id

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Abstract

Research on genetic diversity and intra-species classification of Indonesian *Dioscorea alata* L. based on morphological characters has been done, and the result shows that there are 4 sub-groups of green cultivars group, and 5 sub-groups of purplish-red cultivars group. The objectives of this study are to determine the phylogenetic relationship cultivars of *D. alata* Indonesia compared to *D. bulbifera* as nearest species as well as 3 cultivars from GenBank. The young leaves of 18 water yam cultivar accessions were collected from Java, Madura, South Sumatera, South Kalimantan, Centre Celebes (Sulawesi), Ternate, West Papua, and Nusa Tenggara islands of Indonesia. DNA Isolation was conducted using Phytopure reagent. DNA amplification was conducted using thermocycler, predenaturation at 95 ^{o}C 5 minutes, denaturation at 95 ^{o}C 1 minutes, annealing at 60 ^{o}C 3 minutes, and elongation at 72 ^{o}C 2 minutes along with 30 cycles. The PCR products were electrophoresed in 1.5% agarose and visualized under UV transiluminator. Fifty-five µl of PCR products with positive targeted band between 700-800 bp were sent to 1st Base Singapore for purification and sequencing of 18S, ITS-1, 5.8S, ITS-2, and 28S rDNA. The DNA sequences were compared and aligned by BioEdit program (version 7.0.5.2) and MEGA programs. Comparison of entire sequences of the tested samples were aligned by software ClustalW (version 1.83). Phylogenetic trees were based on hierarchical clustering of the alignments of 18S, ITS-1, 5.8S, ITS-2, and 28S rDNA and produced by Neigbor-Joining using MEGA 5 software of the bootstrap values (1000 replicates). The study result shows that *D. alata* cultivars have high genetic variability on ITS-1, 5.8S, and ITS-2 rDNA region. Groups of green and purplish red cultivars formed based on morphological characters are not formed based on ITS-rDNA markers. Sub-groups were formed based on ITS-rDNA molecular markers derived from both the green and purplish-red cultivar groups. This result revealed that two cultivar groups are not similar with RAPD and morphological characters.

Keyword: *Dioscorea alata* L., cultivars, relationship, molecular, ITS-1 & ITS-2 rDNA

1. Introduction

There are many cultivars of Indonesian water yam (*D. alata*), all of the cultivars have edible tuber with many kinds of shapes and colors. Based on morphological and molecular characters by RAPD analysis, Purnomo et al. (2012) classified Indonesian water yam cultivars into *D. alata* cultivar group 'green' and 'purplish-red'. *D. alata* cultivar group 'green' has 4 sub-groups, *i.e.* (1) white tuber flesh rounded to oblong tuber shape (uwi beras, uwi elus, uwi alas, uwi putih, uwi kemayung cultivars), (2) white and sweetish tuber flesh (uwi legi), (3) white tuber flesh ovate to oblong tuber shape (uwi putih cultivars from Ternate and Lombok), and (4) yellow ovate to oblong tuber shape (uwi butun, uwi kuning). *D. alata* cultivar group 'Purplish red' has 5 sub-group cultivars, i.e. sub-group of (1) white to yellow long cylindrical tuber shape (uwi luyung putih, uwi luyung kuning, uwi ulo, uwi ular, uwi kuning from Merauke), (2) purplish-red long cylindrical tuber shape (uwi luyung senggani), (3) white with purple ring tuber flesh (uwi bangkulit from many place in Indonesia), (4) Purple irregular tuber shape (uwi senggani, uwi ungu, uwi merah from many place in Indonesia), (5) yellow tuber flesh with purple tuber skin (uwi kuning from Pelaihari, Kalimantan).

Morphological characters and molecular by RAPD analysis can be conducted to the similarity relationship only. Therefore, the region of ITS-rDNA is helpful to phylogenetic analysis that can determine the ancestral, origin, and phylogenetic relationship analysis among cultivars. Currently, ITS-rDNA is widely used in taxonomy and molecular phylogenetics (Lin et al., 2007). ITS-1 and ITS-2 regions are variable and useful as a source of polymorphisms for distinguishing genetic variation among species within the genus or among populations.

The region of ribosomal DNA (rDNA) is the sources of valuable characters as *Angiospermae* phylogeny on *Internal Transcribed Spacer* (ITS) is located between 18S-28S nuclear rDNA genes. ITS-1 is located between 18S and 5.8S gen, while ITS-2 is located between 5.8S and 28S gen. The regions of 18S, 5.8S, and 28S rDNA are the conserved area, and the regions of ITS-1 and ITS-2 are the variable area. The variables area is used for genetic variability interspecies on the same genus or among the population because it has a high polymorphism. The regions of ITS 1 and ITS 2 are commonly used as character sources to determine the phylogenetic relationship. Amplification of target band on PCR product the region of ITS-1 and ITS-2 rDNA on more than 500 base pair (Baldwin et al., 1995; Baldwin & Markos, 1998; Lin et al., 2007, 2011).

ITS-1 and ITS-2 regions of rDNA analysis are used to identify molecular variability and phylogeny of *Colletotrichum* species from *Almond* and the other fruits (Freeman et al., 2000). ITS-1 and ITS-2 regions of rDNA analysis are used to determine the phylogeny of *Cercospora* and *Mycosphaerella* (Goodwin et al., 2001). The geographical distribution of *Waitea circinata* var. *circinata* on *annual bluegrass* in America can be detected by the sequence of ITS-rDNA region (Chen et al., 2009). Determination of short and long term of DNA barcodes is required for phylogeny analysis of terrestrial plants (Chase et al., 2005).

In the present study, ITS-1- and ITS-2-based analyses were used to ascertain the genetic relationship among 18 cultivar accessions of *D. alata* (water yam), 1 accession of *Dioscorea* sp., 3 water yam cultivars from GenBank, and 1 species of *D. bulbifera* as out group. The objectives of this study were to determine the phylogenetic relationship cultivars of *D. alata* Indonesia compared to *D. bulbifera* as nearest species and cultivars from GenBank.

2. Materials and Methods

2.1 Plant Materials

The young leaves of 18 water yam cultivar accessions from many places in Indonesia were collected from Java, Madura, South Sumatera, South Kalimantan, Centre Celebes (Sulawesi), Ternate, West Papua, and Nusa Tenggara islands (Table 1). Nucleotide series of 3 water yam cultivars from GenBank *Dioscorea alata* L. cultivar *Guangxi Yulin shanyao clone* 2 (FJ860067.1), *D. alata* L. bio-material *Hainan shanyao* (FJ860070.1), dan *D. alata* L. cultivar Guangxi *Shatianzhen shanyao* (FJ860065.1) were collected from China.

Table 1. Accession numbers, cultivar names, accession origin, and morphological characters of *Dioscorea alata*

Accession number	Cultivar name (Indonesia)	Accession origin	Morphological characters (Stem nodes, wing stem, leave nerves color), (tuber shape), and (tuber flesh color)
06	*Uwi beras*	Rembang, Central Java	Green, rounded, white
10	*Uwi putih*	Purwodadi, Central Java	Green, rounded, white
17	*Ubi putih*	Buon, Luwuk, Central Sulawesi	Green, rounded-cylindrical, white
21	*Uwi legi*	Sewon, Bantul, Yogyakarta	Green, oblong, white
25	*Uwi butun*	Gunung Kidul, Yogyakarta	Green, ovate, yellowish white
29	*Uwi Luyung putih*	Sewon, Bantul, Yogyakarta	Green, length cylindrical, white
35	*Uwi Luyung kuning*	Kulon Progo, Yogyakarta	Green, length cylindrical, yellow
37	*Uwi ulo*	Karebet, Sendangsari, Bantul, Yogyakarta	Green, length, white-yellow
42	*Uwi kuning*	Sleman, Yogyakarta	Green, ovate-oblong, yellow
48	*Uwi bangkulit*	Serongga, Batulicin, South Kalimantan	Purplish red, ovate, white with purple ring
50	*Uwi Luyung senggani*	Karebet, Sendangsari, Bantul, Yogyakarta	Purplish red, length cylindrical, purple
52	*Uwi senggani*	Karebet, Sendangsari, Bantul, Yogyakarta	Purplish red, irregular, purple
55	*Uwi ungu*	Pelaihari, South Kalimantan	Purplish red, irregular, purple
62	*Obi item*	Pamekasan, Madura, East Java	Purplish red, oblong, purple with blackish dot
64	*Obi violet*	Bangkalan, Madura, East Java	Purplish red, oblong, violet
70	*Ubi ungu*	Banggai, Central Sulawesi	Purplish red, oblong-cylindrical, violet with white ring
78	*Gembolo* (*D. bulbifera*)	Wonosadi, Gunung Kidul, Yogyakarta	Dark green, rounded with 3-6 branches, grayish white
107	*Ubi hutan (Dioscorea sp.)*	Kinton-Toili, Luwuk, Central Sulawesi	Light green, length cylindrical, white-white ash
138	*Ubi ungu*	Lombok, Nusa Tenggara	Purplish red, ovate-rounded, purple
142	*Ubi ungu*	Merauke, West Papua	Purplish red, oblong-cylindrical, purple

2.2 DNA Extraction

DNA Isolation is conducted by *Phytopure* (Daryono & Natsuaki, 2002). The fresh young leaf is sliced and then grounded into powder with liquid nitrogen. Powder is brought in 1.5 µl tube, 400-500 µl of phytopure I reagen is added and shaken by hand, and then 75-100 µl phytopure II reagen is added and shaken by hand, and then incubated at 65 ^{o}C for 10 minutes on waterbath, and then on ice for 20 minutes, and then 400-500 µl cold chloroform is brought in, and then 50-70 µl phytopure resin was added in the centre carefully, and then centrifuged at 3000 rpm for 10 minutes. The supernatans in the new tube 1.5 ml is removed, cold isopropanol with the same volume with supernatans is added and shaken well by hand, centrifuged at 10.000 rpm for 10 minutes, white DNA pellet on the bottom. Supernatans was thrown and DNA pellet washed with 100 µl ethanol 70% added and centrifuged at 10.000 rpm for 5 minutes. Ethanol and dried DNA pellet were eliminated with the wind, and then 1xTE buffer was added and it was kept in the freezer at -12 ^{o}C.

The purity of DNA was determined with *GeneQuant* (Life Science, Ltd., UK) with ratio on spectrophotometer at 260 nm (absorbance optimum detection for DNA) and 280 nm from DNA samples, DNA purity refers to standard DNA purity 1.8-2.2 on 260/280 nm (Sambrook et al., 1989; Googwin et al., 2001).

2.3 Polymerase Chain Reaction (PCR) Amplification

Each PCR reaction (25 µl) on tube 200 µl consisted of Mega Mix Blue 22 µl, forward primer 5-GATCGCGGCGGCGACTTGGGCGGTTC-3 1 µl, reverse primer 5-GGTAGTCCCG CCTGACCTGGG-3 1 µl, and DNA template 1 µl. The use of primer was according to Li et al. (2011) and Muellner et al. (2008). PCR reaction in the tube was centrifuged at 8000 rpm for 30 seconds. Amplification was conducted by thermocycler, predenaturation at 95 ^{o}C for 5 minutes, denaturation at 95 ^{o}C for 1 minute, annealing at 60 ^{o}C for 3 minutes, elongation at 72 ^{o}C for 2 minutes. PCR reaction was conducted on 30 cycles. The PCR products were electrophoresed in 1.5% agarose with good view (modification of ethidium bromide) staining and visualized under UV transilluminator compared to DNA ladder (marker). Photography is done with digital cameras.

2.4 Nucleotide Sequencing

Fifty-five (55) µl PCR products with targeted band positive between 700 and 800 bp were sent to 1st Base Singapore for purified and sequencing of 18S, ITS-1, 5.8S, ITS-2, and 28S rDNA.

2.5 Sequence Alignment and Phylogenetic Tree

The DNA sequences were compared and aligned by BioEdit program (version 7.0.5.2) and MEGA programs and further verified by comparison with sequences of other cultivars and *D. bulbifera* by BLAST (version 2.2.2.4) search on the website of the National Centre for Biotechnology Information (NCBI). A comparison of the entire sequences of the tested samples was made by software ClustalW (version 1.83). Phylogenetic trees were based on hierarchical clustering of the alignments of 18S, ITS-1, 5.8S, ITS-2, and 28S rDNA and produced by Neigbour-Joining using MEGA 5 software of the bootstrap values (1000 replicates). For outgroup sequences *D. bulbifera* was chosen because morphologically this species has a high morphological similarity (closed relationship).

3. Result and Discussion

3.1 ITS rDNA Band Target

Visuals of electrophoresis of PCR product under ultraviolet (UV) are shown in Figure 1. Figure 1 shows the size of ITS target band of 19 water yam (*D. alata*) cultivars and *D. bulbifera* species. Accession numbers 01, 05, 23, and 40 in Figure 1 are without a real band and also they have no representative sequence because of the number of nucleotides less than 400. On a number of 20 accessions composed of 18 cultivars, *D. alata, Dioscorea* sp., and *D. bulbifera*, in the ITS-rDNA region by electrophoresis the target bands are detected on 700-800 bp size. Those results are suitable to the last ITS-rDNA research, especially on *Angiospermae* (Baldwin & Markos, 1998; Balwin et al., 1995; Hidayat & Pancoro, 2001; Chase et al., 2005). The tick band indicates that those regions are the optimal amplification of ITS-rDNA.

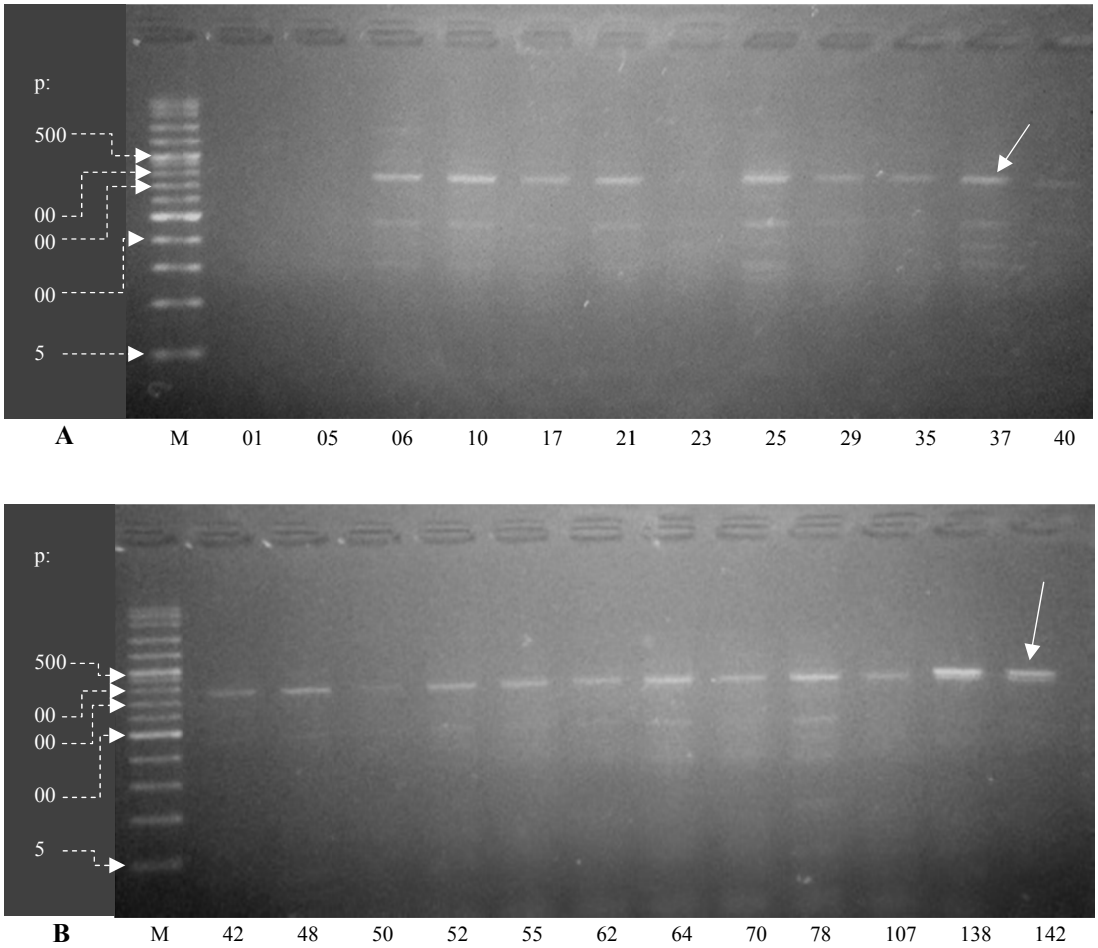

Figure 1. Electrophoresis product PCR for ITS target band on between 700 and 800 bp (single arrow shows the ITS bend). Legend: A = accession number 01-40, B = accession number 42-142. M = DNA marker 1 Kb (Fermentas), series number of 01, 05 until 142 = accession number of Indonesia *D. alata* cultivars

3.2 rDNA Sequence Analysis

ITS-rDNA forward primer 5-GATCGCGGCGGCGACTTGGGCGGTTC-3 and reverse primer 5-GGTAGTCCC GCCTGACCTGGG-3 producing nucleotide series of 19 water yam cultivars and *D. bulbifera* species. 18S, ITS-1, 5.8S, ITS-2, and 28S rDNA sequences of 19 *D. alata* cultivars, *D. blbifera*, and 3 *D. alata* cultivars from GenBank have 457-831 nucleotides. From the longest 831 nucleotides conserve region are 6.38% and variable region are 93.62%. Almost all nucleotides in the variable region experience the kind mutation, *i.e.* deletion, insertion, transition, and transverse. All cultivars have 100% homology compared with water yam from GenBank and 99.98% with *D. bulbifera*.

3.3 Sequence Alignment/Nucleotide Variation

Commonly in the species of *Angiospermae*, the mutation occurred on in ITS-1 and ITS-2 only, but in all Indonesian *D. alata* cultivars mutation occurred on ITS-1, ITS-2, also in 5.8S, 18S, and 28S genes. Almost all nucleotides in the variable region experience the kind of mutation, *i.e.* deletion, insertion, transition, and transverse mutation. Intra-species classification into 2 groups of monophyletic is more supported by a mutation in the insertion and deletion type, and into sub-groups is more supported by transition and transverse.

3.4 Sequence Coefficients of Identity

The analysis on the sequence identity matrix using ITS-1 and ITS-2 sequences showed that the identity percentages (genetic similarity) among 20 accession of Indonesia *D. alata* and 3 cultivars from GenBank had a range of 72.92 to 99.01 (Table 2). The genetic distance in ITS among 20 accession of Indonesia *D. alata* and 3 cultivars from GenBank was 0.00 to 0.18. According to the data, a cultivar of *uwi luyung putih*, *uwi luyung kuning*, and *uwi ulo* (genetic similarity = 0.00; genetic distance = 98.40-98.80) has the highest relatedness to all cultivars, and on both the 3 cultivars morphologically has a long cylindrical tuber shape. There is also the cultivar of *uwi senggani* and *uwi violet* (Madura) (99.01; 0.00) and also the cultivar of *uwi legi* and *uwi violet* (98.89; 0.00) (Table 2).

3.5 Phylogenetic Trees

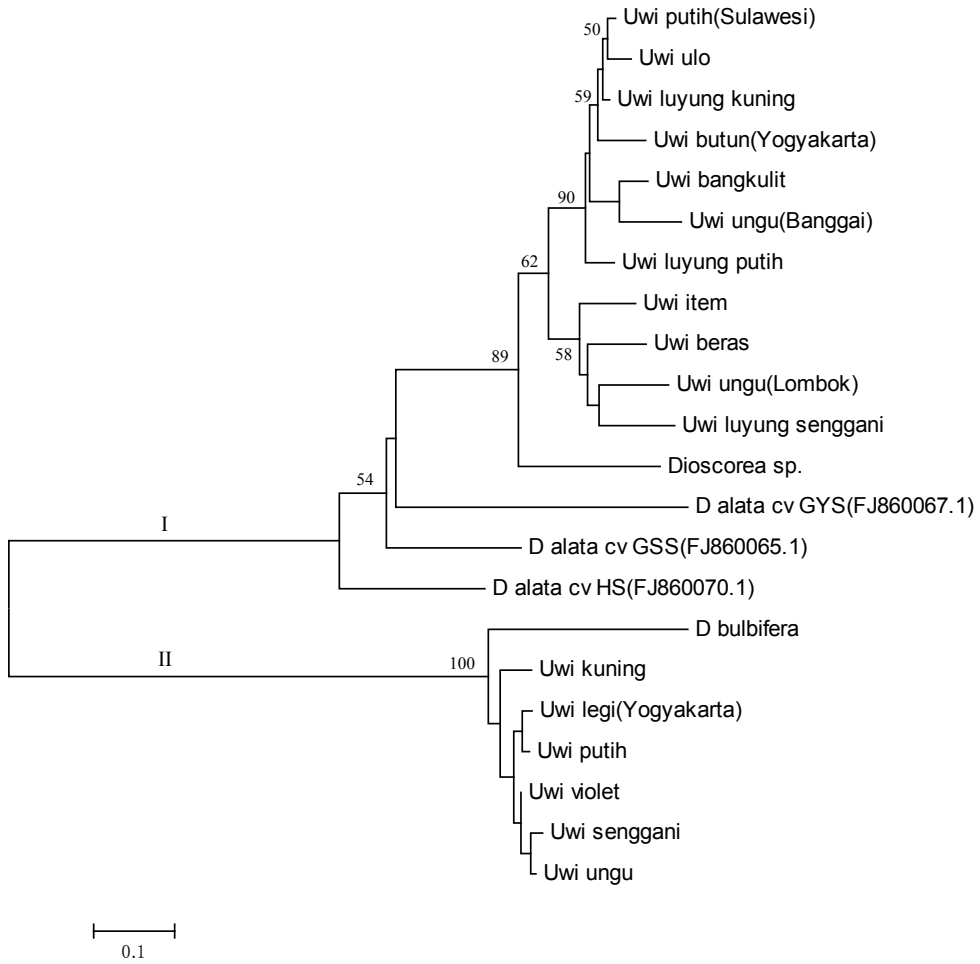

Figure 2. Phylogenetic tree of Indonesia *D. alata* cultivars compared with *D. bulbifera* based on ITS-rDNA marker generated by Neighbor-Joining

Phylogenetic tree based on ITS-1 and ITS-2 (Figure 2) was generated by Neighbor-Joining (NJ). Bootstrap values are determined by Kimura 2 method, bootstrap value > 50 indicate the reliable grouping so that the relationship between accession and their evolution history can be created. Relationships are indicated by the length clade of the phylogenetic tree that is illustrated genetic distance and similarity between accessions (Tamura et al., 2011).

Figure 2 shows that *D. bulbifera* have a close relationship with 23 *D. alata* cultivars, including *Dioscorea* sp., and *D. alata* cultivars have high genetic variability on 18S, ITS-1, 5.8S, ITS-2 and 5.8S rDNA regions. There are 2 monophyletic clades that occurred on the phylogenetic tree; clade I was a group composed of 11 cultivars; *D. alata* Indonesia has a close relationship with 3 *D. alata* cultivars from GenBank and *Diosocrea* sp., and clade II, composed of 7 cultivars of *D. alata* Indonesia, had a closer relationship with *D. bulbifera*. Clade I has 92.28-93.33% genetic similarity, and 0.06-0.07 genetic distances. Clade II has 74.63-77.72 genetic similarity and 0.16-0.18 genetic distances. The phylogenetic relationship on clade I is to prove that *Dioscorea* sp. includes the species of *D. alata*.

The genetic variability in *D. alata* is dominantly caused by natural gene flow rather than vegetative reproduction and by a natural hybrid between species of *Dioscorea*. Water yam may be domesticated in the Indochina region with *D. hamiltonii* (J. D. Hook) and *D. persimilis* Prain & Burk. The cultivation of water yams was started using wild cultigens (Lebot et al., 1998) and then continued using vegetative reproduction or clone (soma clonal) from tubers. The genetic distance between *D. alata* cultivars is very closed (nearest); it was known that each cultivar

is the result of soma clonal reproduction from a primitive form. It was in accordance with the research of van den Brouche et al. (2015) and Chair et al. (2016), which states that *D. alata* cultivars in Vanuatu is soma clonal engineered and selected as cultivars.

Phylogeny tree branches (clades; lineages) that form the two branches I and II have a far relationship due to mutational events in each cultivar. All of the mutation types such as insertion, deletion, transition, and transvertion occurred, and the insertion and deletion dominantly. The difference of nucleotide sequence of ITS-rDNA region is expressed to the basic tuber shape of *D. alata* cultivars; clade I has a short to long cylindrical tuber shape, and clade II has ovate to oblong or irregular tuber shape.

The phylogeny tree branches and sub-branches showed no grouping based on geographic region, such as the clusters that occur based on molecular characters with RAPD analysis. Establishment of branches I and II based on molecular characters with ITS-rDNA analysis does not have the pattern of group and sub-group cultivars, such as the grouping based on morphological characters (Purnomo et al., 2012) and RAPD (Purnomo et al., 2016). Distinguishing between branches I and II based on analysis of rDNA ITS regions is predominantly due to the insertion and deletion mutation types. The results show compliance with the research of identification of *Durio* spp. in Kalimantan with the same marker (Mursyidin & Qurrohman, 2012), and the difference of sub-branches on each branch are dominantly due to the insertion, deletion, transition, and transvertion mutation types. These results are consistent with the research on the identification of 10 strains of algae *Porphyra haitanensis* experiencing the same type of mutation (Chen et al., 2010).

Sub-groups are formed based on ITS-rDNA molecular markers derived from both the green and purplish-red cultivar groups based on the results of clustering by morphological characters and RAPD analysis so that the samples used in ITS-rDNA analysis represent each cluster that was formed. Hence, the two branches are polyphyletic.

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