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# Short Communication: Species identity and taxonomical position of selected species of Annonaceae based on *trn*L molecular marker

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**Abstract.** *Lestari DA, Azrianingsih R. 2019. Species identity and taxonomical position of selected species of Annonaceae based on* trn*L molecular marker. Biodiversitas 20: 1012-1019.* Identification based on morphological characters could be difficult when some characters are not visible. The absence of some morphological characters could effect for identification of species identity and taxonomical position of the species. Confirmation by the DNA data is needed to support species identification. The sequence was used in this research is *trn*L intron as non-coding sequence DNA, based on chloroplast DNA. The research aimed to estimate of species identity and determine of taxonomical position of species of Annonaceae based on *trn*L sequences. Methods were used through steps of DNA extraction, DNA amplification, DNA sequencing, and data analysis to selected species of Annonaceae from Purwodadi Botanic Garden (PBG), the *trn*L intron sequences of 10 Annonaceae species from GenBank database and two species of Magnoliaceae as out-group. Results showed that *trn*L sequence as non-coding gene explains the different groupings with the previous groupings in Annonaceae to this observed species. *trn*L sequence can estimate of species identity as much as 30%, caused by changes of nucleotide bases from mutation and missing data. Polymorphism of DNA sequences showed that 61.18% sites as conserved region, 24.05% sites as polymorphic variation and 14.76% sites as alignment gaps. *Oxymitra* sp. is in group (monophyletic) with *Mitrephora javanica* because they are genetically in close relationship (Uvariae tribes and Annonoideae sub-family), *Popowia* sp. is in group with *Orophea enterocarpa*, because they are genetically in close relationship (Miliuseae tribes and Malmeoideae sub-family).

**Keywords:** Annonaceae, identity, sequence, taxonomical positions, *trn*L

## INTRODUCTION

Annonaceae (custard apple family) is one of the most primitive families of the Angiosperm (Hutchinson 1969) that comprises about 120-130 genera and 2500 species (van Heusden 1992). Based on APG system (Angiosperm Phylogeny Group system)) (1998), APG II system (2003) and APG III system (2009), Annonaceae was placed most closely related to the small Magnoliid family Eupomatiaceae. This family has four subfamilies, there are Anaxagoreoideae, Ambavioideae, Annonoideae, and Malmeoideae. Together, Annonoideae and Malmeoideae comprise the majority of the species and each is further subdivided into some number of tribes. Subdivision of Annonaceae has been problematic, and their classification is far from being comparable with each other, although this family is well characterized and contain valuable elements. Difficulties were shown for classification on tribes level in Asiatic-Australian genera (Kessler 1995; Lestari et al. 2017). Chatrou et al. (2012) was an amendment for Annonoideae and new subfamilies, and some of tribes classification were recognized.

At least 56 species of Annonaceae have been conserved in Purwodadi Botanic Garden (PBG). Some individuals in PBG have not been identified because they have problems in the appearance of flower and fruit. The appearance of flowers never succeeded in becoming a fruit. Unconditional flowering and non-concurrent anthesis between male and female flowers are the main factors that were affecting the reproduction of Annonaceae (Handayani 2016). This problem effects the identification of unidentified species. Whereas, species of Annonaceae can be classified based on morphological characters (Maas and Westra 1984; Westra 1985; Morawetz and Le Thomas 1988; van Heusden 1992; van Setten and Koek-Noorman 1992; Johnson and Murray 1995; Doyle and Le Thomas 1996; Svoma 1998; Johnson 2003; Maas et al. 2003; Tsou and Johnson 2003; Scharaschkin and Doyle 2005, 2006; Su and Saunders 2006; Maas et al. 2007; Couvreur 2009; Huysmans et al. 2010; Surveswaran et al. 2010; Weerasooriya and Saunders 2010a). So that, the identity of species become unclear, and the taxonomical position of this species is doubtful, misidentified and misnamed (Kwon et al. 2016).

Identification keys merely using morphology character could be difficult to use when some features are not visible. Thus, the absence of some morphological features could make the identification impossible (Amandita 2015). Based on these problems, it is needed to confirm the morphological identification by DNA sequence or gene. The emergence of molecular approaches has led to the

formation of several streams within systematics, such as elucidation of phylogenetic relationships and descriptive studies which are combining phylogenetically and phenotypically markers (Stackebrandt and Goebel 1994). Most of species within Annonaceae was confirmed by coding or non-coding of DNA sequence. Molecular phylogenetic analysis of Annonaceae species were studied, such as Zhou et al. (2009; 2010) in *Uvaria* based on sequences of four plastid DNA regions such as *matK*, *psb*A-*trn*H spacer, *rbc*L and *trn*L-F, Tang et al. (2015a; 2015b) in phylogenetic reconstruction of *Goniothalamus* was used nine cpDNA regions, Li et al. (2015) in generic delimitation of *Disepalum* using four chloroplast and two nuclear DNA regions, Chaowasku and Keβler (2013) in molecular phylogeny of *Miliusa* with combination of seven plastid markers, Lestari et al. (2018) in phylogenetic analysis of Annonaceae species collections using three chloroplast DNA, Chatrou et al. (2012) in phylogenetic analysis using DNA molecular marker was known classification of subfamily and new tribes in Annonaceae, and the other studies. Confirmation of species identity on Annonaceae species collections from PBG uses *trn*L intron as one of the non-coding sequence DNA, located on chloroplast DNA (cp-DNA). Uses of this sequence is combined with the other sequences (Chaowasku and Keβler 2013; Chatrou et al. 2012). Applications of this sequence are assumed to be conserved in its evolution in terms of nucleotide substitution with very little rearrangements which permit the molecule to be used in resolving phylogenetic relationships especially at deep levels of evolution (Patwardhan et al. 2014).

The chloroplast of *trn*L (UAA) intron may represent a good target region for many purposes. Its sequences have been widely used for reconstructing phylogenies between closely related species or for identifying plant species. It does not represent the most variable non-coding region of chloroplast DNA, but it bears some unique advantages. Universal primers, subsequently extensively used, have faster rates of evolution as marker for mainly in phylogenetic than the other sequences, evolutionary and taxonomic studies among closely related genera and species, at lower taxonomic level and detecting species level variations (Procaccini et al. 1999; Tsai et al. 2006; Taberlet et al. 2007). The *trn*L gene is part of *trn*L-F region of chloroplast genome that split by group I intron, the intergenic spacer and *trnF* exons and is co-transcribed. This gene can be implied for phylogenetics reconstruction in Dipterocarpaceae (Yulita 2013), consensus between the morphological and molecular character in *Pandanus* (Rachma et al. 2017), genetic variation in *Amorphophallus meulleri* (Wahyudi et al. 2013), suitable for phylogenetic analysis of *Cinnamomum* spp. (Kojoma et al. 2002), genetic variability among porang populations in Eastern Java (Rosidiani 2011) to the identification of orchid species (Kishor and Sharma 2018). The research aimed to estimate species identity and to determine the taxonomical position of selected Annonaceae species based on *trn*L sequence.

## MATERIALS AND METHODS

### Study area

The research was conducted in November 2016 – February 2017 at the Laboratory of Plant Physiology, Tissue Culture, and Microtechnic, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, and PBG – Research Center for Plant Conservation and Botanic Garden, Indonesian Institute of Sciences, Pasuruan, East Java. Materials used were young leaf samples of Annonaceae species collections of PBG as much 5 species (Figure 1) and the *trn*L intron sequences of 10 Annonaceae species were obtained from the GenBank database (http: //www.ncbi.nlm.nih.gov/) with 2 species from Magnoliaceae as the outgroup (Table 1). Magnoliaceae was chosen as the outgroup because Magnoliaceae was the closest sister of Annonaceae. Young leaf material samples were obtained further extracted through steps of DNA extraction, DNA amplification through PCR technique, DNA sequencing, and data analysis. The *trn*L intron sequences of 10 Annonaceae species from GenBank were analyzed together with DNA sequences of 5 Annonaceae species from PBG.

**Table 1.** Material samples of Annonaceae species were observed and outgroup

| Species | No voucher | Subfamily |
|---|---|---|
| *Fissistigma latifolium* (Dunal) Merr. | P19820362 | Annonoideae |
| *Mitrephora javanica* Backer | P19850167 | Malmeoideae |
| *Mitrephora polypyrena* Miq. | P19811116 | Malmeoideae |
| *Oxymitra* sp. | P19850160 | Annonoideae |
| *Popowia* sp. | P19790732 | Malmeoideae |
| | Genbank accession number | |
| *Anaxagorea javanica* Blume | AY580031.1 | Anaxagoreoideae |
| *Anaxagorea luzonensis* A.Gray | AY580032.1 | Anaxagoreoideae |
| *Annona glabra* L. | KX663998.1 | Annonoideae |
| *Annona montana* Macfad. | KX663995.1 | Annonoideae |
| *Annona muricata* L. | KX663992.1 | Annonoideae |
| *Artabotrys hexapetalus* (L.f.) Bhandari | AY231286.1 | Annonoideae |
| *Cananga odorata* (Lam.) Hook.f. & Thomson | KF586710.1 | Ambavioideae |
| *Goniothalamus macrophyllus* (Blume) Hook.f. & Thomson | EU249789.1 | Annonoideae |
| *Goniothalamus malayanus* Hook.f. & Thomson | EU249796.1 | Annonoideae |
| *Magnolia candolli* (Blume) H.Keng* | P19821171 | - |
| *Michelia champaca* L.* | P1997110091 | - |
| *Orophea enterocarpa* Maingay ex. Hook.f. & Thomson | EU249781.1 | Malmeoideae |

Note: *indicated as outgroup

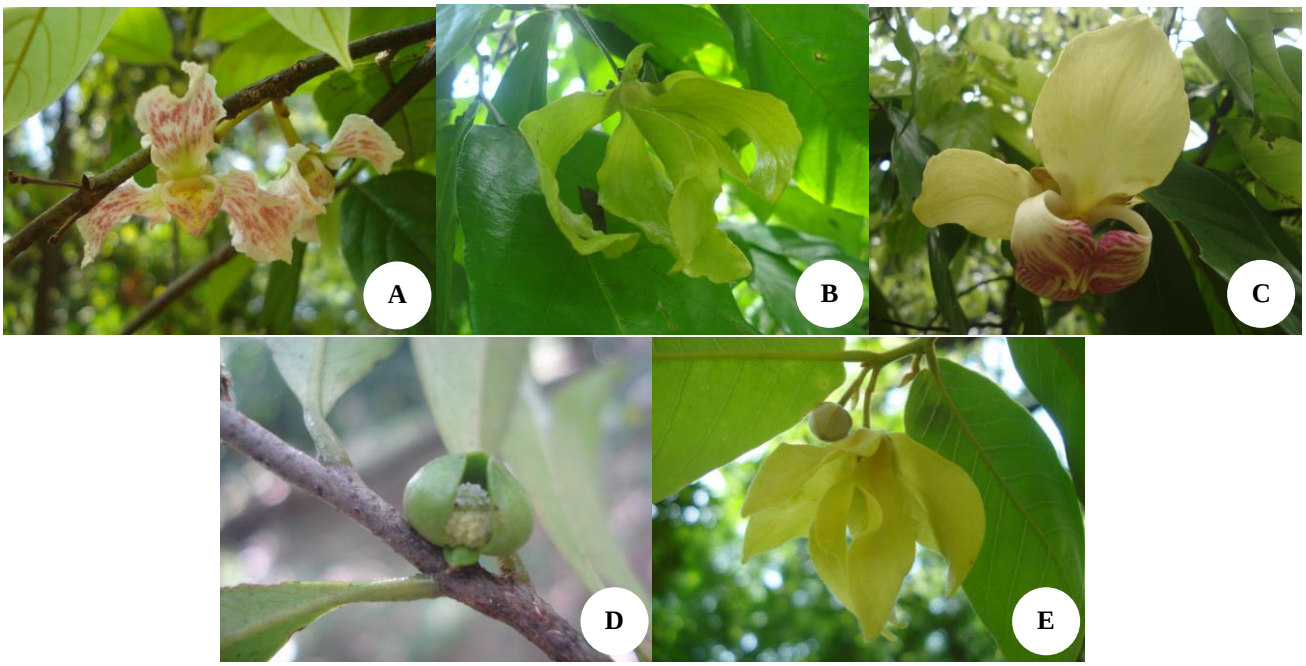


**Figure 1.** Material samples of selected species of Annonaceae. A. *Mitrephora javanica,* B. *Fissistigma latifolium,* C. *Mitrephora polypyrena,* D. *Popowia* sp.*), E. *Oxymitra* sp.*) (*)species identity was confirmed in this study)

**Procedures**

*DNA extraction*

DNA extraction on material samples from PBG was using CTAB (Cethyl Trimethyl Ammonium Bromide) method (Fatchiyah et al. 2011). Long-term use of DNA was stored at -20°C.

*DNA amplification*

DNA amplification was using the molecular marker of *trn*L (non-coding DNA), in each of the DNA samples. Each 10 µL of solution (Fatchiyah et al. 2011), mixed aquabidest ($ddH_2O$) of 2 µL, PCR mix solution (2x concentration) of 5 µL, forward molecular marker (@10 pmole) of 1 µL, reverse molecular marker (@10 pmole) of 1 µL and 1 µL DNA samples. The samples then spin-down for 3 seconds and fed into the PCR thermal cycle tool for amplification. Annealing program on *trn*L molecular marker was initial denaturation at 94°C for 5 minutes; denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 30 seconds (repeated by 35 cycles); final extension at 72°C for 10 minutes with sequence molecular marker (F) 5'-CGAAATCGGTAGACGCTACG-3' and (R) 5'-GGGGATAGAGGGACTTGAAC -3' (Rachma et al. 2017).

*DNA sequencing*

Sequencing was done to know DNA sequences from amplified DNA. DNA samples were sequenced with automatic sequencer machine in 1st Base Laboratory, Malaysia.

**Data analysis**

Sequences data then evaluated by ABI sequence scanner program to know quality of the DNA sequence. Only good quality sequence DNA can be analyzed further. DNA sequence with good quality then tested homology with BLAST on NCBI (a homology level of ≥92%). The result from BLAST was to use estimation of species identity from PBG plant species. Taxonomical position of the species from PBG and 10 Annonaceae species from GenBank with outgroup can be showed by phylogenetic tree (cladogram) was obtained by alignment using Clustal W program and further analysed based on Kimura 2 parameter (K2P) with algorithm methods of Maximum Parsimony (MP) using MEGA 5 program (Pathwardhan et al. 2014; Simpson 2010; Tamura et al. 2011). Comparison between algorithm methods used a bootstrapping value of 1000 replications to test the validity of phylogenetic tree topology. The category of bootstrapping values are high (>85%), moderate (70–85%), weak (50–69%), or very weak (<50%) (Kress et al. 2002). Polymorphism of DNA sequences was analyzed by DnaSP ver. 5.10.01 statistic program. The aim of this analysis to know nucleotide bases changes. Results obtained showed that how much the Annonaceae species mutated.

## RESULTS AND DISCUSSION

Polymorphism of DNA sequences from *trn*L molecular marker alignment shows that from 5 Annonaceae species of PBG, 10 species from GenBank and 2 Magnoliaceae

species as the outgroup, there are 237 sites of nucleotide bases. Whereas, 145 (61.18%) sites as a conserved region, 57 (24.05%) sites as polymorphic variable and 35 (14.76%) sites as alignment gaps or missing data (Table 2).

*Popowia* sp. is in group with *O. enterocarpa*, because DNA sequences of *Popowia* sp. was a transition as much as 3 characters. Nucleotides bases of Cytosine (C) was a transition to Tymine (T) on sites 65, Adenine (A) was a transition to Guanine (G) on sites 145 and G was transition to A on sites 204. *M. javanica* is in group with *Oxymitra* sp. and *F. latifolium* because nucleotide bases of T was insertion on sites 162 and 173, nucleotide bases of C was insertion on sites 174 and 175, and nucleotide bases of A was insertion on sites 172, 176, 177 and 178. Changes of this nucleotide bases cause *M. javanica* genetically in group with *Oxymitra* sp. and *F. latifolium*, although morphologically it has a connate inner petal character where this character should be in group with *M. polypyrena* and *Popowia* sp. This is caused a missing data or alignment gaps.

**Species identity**

Results of DNA sequences show that the length of DNA sequences observed were ranged 524 to 553 bp linear DNA. The highest DNA sequences length was *M. javanica*, and the lowest DNA sequences length was *Oxymitra* sp. The highest and the lowest of GC content percentage was *F. latifolium* (38.2%) and *M. javanica* (36.3%). The range of identity percentage was about 93-99% (Table 3). It means that DNA sequences which were obtained are similar to materials plant species and the possibility of wrong identification in PBG, especially for unidentified species. Species identity of *M. javanica* from BLAST show 93% similarity, this means that BLAST result for this species was not similar to species observed. Results of BLAST sequences DNA was aimed to estimate of species identity from the materials plant species were observed and would be submitted in the GenBank. Samples of DNA alignment was shown in Figure 2.

**Taxonomical position**

Results of cluster analysis from the phylogenetic tree with Maximum Parsimony (MP) analysis method (Figure 3) show that cladogram has a solid and congruent formation of the clade. However, the position of *M. javanica* on the group of a clade is less precise, and should be in the group with *M. polypyrena. Oxymitra* sp. should be in group with Annonoideae sub-family, not in group with *F. latifolium.* This grouping is applied based on the morphological character, due to *M. javanica* and *M. polypyrena* have connate inner petal whereas *Oxymitra* sp. and *F. latifolium* petals are free (Irawan 2002; Moeljono 2009; Weerasooriya and Saunders 2010a; Lestari et al. 2017). According to DNA sequences character, *M. javanica* is in group (monophyletic) with *Oxymitra* sp. and *F. latifolium* because they are genetically in close relationship (Uvariae tribes and Annonoideae sub-family). *Popowia* sp. is in group (monophyletic) with *O. enterocarpa*, because they are genetically in close relationship (Miliuseae tribes and Malmeoideae sub-family).

**Discussion**

Based on Chatrou et al. (2012), plant species materials are not similar identity from results of BLAST sequences DNA. *Mitrephora* is included in Miliuseae tribes and Malmeoideae sub-family, while *Friesodielsia* included in Uvariae tribes and Annonoideae sub-family. *Fissistigma* and *Uvaria* show the appropriate results of BLAST sequence included in Uvariae tribes and Annonoideae sub-family, *Popowia*, *Polyalthia*, and *Mitrephora* are included in Miliuseae tribes and Malmeoideae sub-family. *Oxymitra* is synonym with *Friesodielsia* while *Cyathostemma* is synonym with *Uvaria*. *Friesodielsia* and *Uvaria* are included in Uvariae tribes and Annonoideae sub-family. Incongruity from materials plant species with the results of BLAST DNA sequences is caused by unclear morphological character identification in the field, so was needed confirmation with molecular data and recently name that changes have not been upgraded.

**Table 2.** Polymorphism of DNA sequences from *trn*L molecular marker alignment

| Polymorphism of DNA sequences | Total of nucleotide bases | Position of nucleotides bases |
|---|---|---|
| Conserved | 145 | 1, 2, 4, 5, 6, 7, 8, 11, 12, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 61, 62, 68, 69, 70, 71, 73, 74, 75, 76, 77, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 98, 99, 101, 102, 103, 105, 107, 108, 109, 110, 111, 112, 113, 115, 116, 117, 118, 119, 123, 124, 125, 127, 128, 129, 130, 131, 134, 135, 137, 139, 142, 143, 144, 147, 148, 149, 150, 151, 156, 157, 159, 160, 161, 181, 185, 186, 187, 190, 191, 193, 198, 199, 201, 205, 207, 208, 209, 210, 211, 213, 214, 215, 216, 219, 226, 228, 229, 232, 233, 234, 235, 236, 237 |
| Singleton variabel (2 variants) | 25 | 9, 63, 64, 65, 67, 72, 97, 100, 120, 121, 133, 138, 179, 182, 183, 184, 189, 197, 200, 202, 204, 206, 212, 217, 227 |
| Parsimony informative | | |
| - 2 variants | 25 | 10, 13, 15, 60, 66, 104, 106, 114, 122, 126, 132, 136, 140, 141, 146, 152, 155, 158, 188, 192, 194, 203, 218, 220, 231 |
| - 3 variants | 6 | 3, 145, 153, 154, 196, 230 |
| - 4 variants | 1 | 180 |

**Table 3.** Results of BLAST DNA sequences from PBG plant species

| Species | Species identity from BLAST | % identity |
|---|---|---|
| *Fissistigma latifolium* (Dunal) Merr. | *Uvaria wrayi* (King) L.L. Zhou, Y.C.F. Su & R.M.K. Saunders | 99 |
| *Magnolia candolli* (Blume) H. Keng | *Magnolia liliifera* (L.) Baill. | 99 |
| *Michelia champaca* L. | *Magnolia laevifolia* (Y.W. Law & Y.F. Wu) Noot | 99 |
| *Mitrephora javanica* Backer | *Friesodielsia desmoides* (Craib) Steenis | 93 |
| *Mitrephora polypyrena* Miq. | *Mitrephora keithii* Ridl. | 99 |
| *Oxymitra* sp. | *Cyathostemma viridiflorum* Griff. | 99 |
| *Popowia* sp. | *Polyalthia littoralis* (Blume) Boerl. | 99 |


**Figure 2.** Samples of DNA alignment of material samples

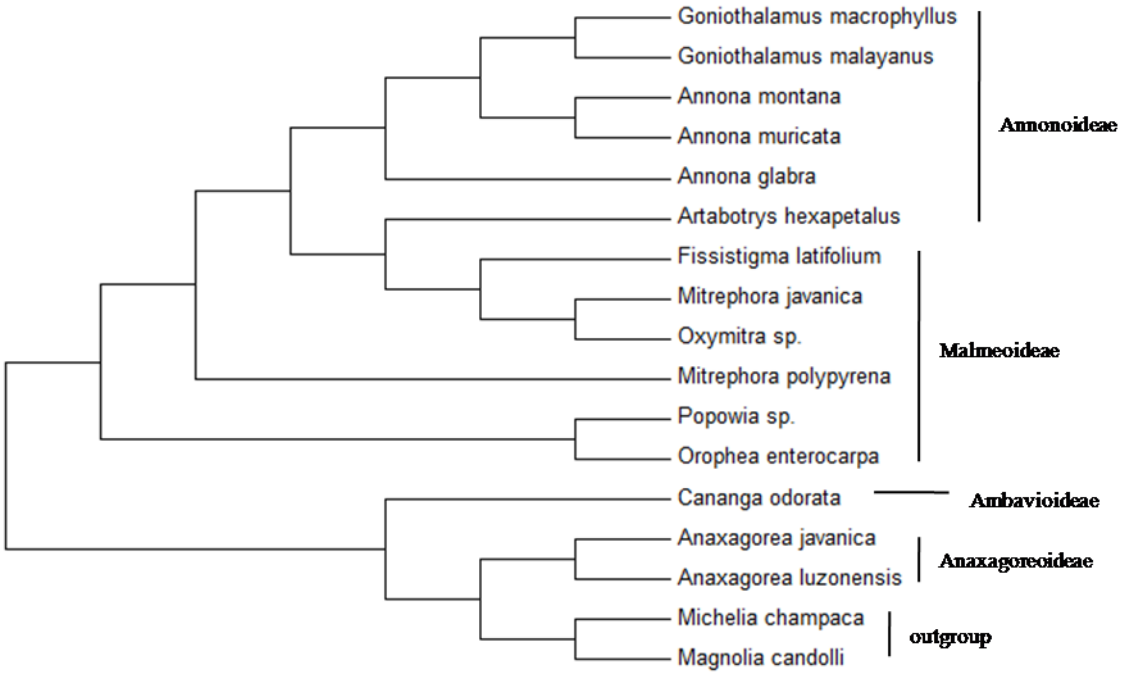


**Figure 3.** Taxonomical position based on *trn*L molecular marker with algorithm method of *Maximum Parsimony* with bootstrap value 1000 replications

*Friesodielsia desmoides* is a small tree or shrub that is distributed in Africa and Asia, and grown as an ornamental plant. This plant has two synonym names, i.e. *Goniothalamus desmoides* and *Oxymitra desmoides.* Colour of the flower is yellow with connate inner petal and size of the outer petal is longer than inner petal (Meesakul et al. 2017). In PBG, this species is identified as *M. javanica.* Morphologically, *M. javanica* has a connate inner petal, the color of inner petal is pale yellow and the color of flower is white with the purple spot (Figure 1). The material sample of *M. javanica* is not matched with the results of BLAST DNA sequences. In the same case, *F. latifolium* is different to *U. wrayi* based on morphological character. *F. latifolium* has simple hairs and inner petals slightly equal or smaller than outer but *U. wrayi* has stellate hairs and inner petal has equal with outer (Irawan 2002; Irawan and Guhardja 2003; Zhou et al. 2009). Same as *M. javanica,* this species is not matched with the results of BLAST DNA sequences. The other species, genus of *Popowia* strongly supports its monophyly with *Polyalthia.* The asymmetrical leaf base usually characterizes the species of *Popowia* and *Polyalthia*, but they are differentiated in patterns of secondary leaf venation and the numbers of ovules per carpel (Xue et al. 2012; Ngoc et al. 2016). Results of BLAST DNA sequences are correctly identified to species name for *Popowia* sp.

Another example, *Cyathostemma* is synonym with *Uvaria.* This genus is characterized by small petals, globose buds, and petals not expanding or reflexing (Utteridge 2000). *Cyathostemma* and *Oxymitra* have similar habitus which is a woody climber but differentiated in hairs of petiole or midvein in the leaves. *Cyathostemma* has stellate hairs but *Oxymitra* has glabrous (no hairs). Same as *M. javanica* and *F. latifolium,* this species is not matched with the results of BLAST DNA sequences. The last example is *M. polypyrena,* which has cream turning bright yellow of the outer petal with cream, reddish or purplish lines of the inner petal, but *M. keithii* has yellow of outer petal and yellow with pink margins of the inner petal (Weerasooriya and Saunders 2010b). This shows that *trn*L sequence was suitable for genus level and not for species level, especially for genus of *Mitrephora*.

The *trn*L sequence presents a perception of other groupings that may differ from previous groupings, because this sequence contains genes that are not directly related to the petal expressions in which petal characters are morphological characters that have been used as taxonomic markers for the classification of the Annonaceae group. Based on that, only 30% are correctly identified species name from 5 species were observed based on this sequence. This indicates that more sequences were needed to confirm the species name identification, because certain sequence did not applicate to the other species in one family. So, the success of species name identification not only in taxa that observed but also in the utilized marker (Amandita 2015).

Monophyletic groups are considered to have very close relationships and assumed to carry the same genetic or biochemical traits or patterns (Hidayat and Pancoro 2008; Rahayu and Nugroho 2015). Alignment gaps or missing data are the presence of mutations insertion-deletion interspecies and the most influential to identity of the

species (Hollingsworth et al. 2011). Variable polymorphic sites consist of singletons variable (25 sites) and parsimony informative (25 sites). Singleton variable is variations where there is only one position of a different nucleotide from one OTU (Operational Taxonomical Unit) (Hidayat and Pancoro 2008). Singleton variable is autopomorphic character, where DNA sequences character only belongs to certain species. Generally, these species are more adaptive to the specific habit than the other species. Parsimony informative is the character of DNA sequences that at least has 2 types of nucleotide bases and both of them must appear at least twice in that position (Yingzhi et al. 2007).

The DNA sequence of *trn*L as non-coding gene presents a perception of other groupings that may differ from previous groupings in Annonaceae, especially species on this study. This sequence can estimate the identity of species as much as 30% correctly. The taxonomical position of 5 species of Annonaceae are observed was influenced by changes in nucleotide bases.

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