Al-Hayat: Journal of Biology and Applied Biology
Volume 6, No 1 (2023): 77-89
DOI. 10.21580/ah.v6i1.18713

Authentication of Wax Apple (*Syzygium samarangense* (Blume) Merr. & L.M Perry) Delima and Citra Cultivars by Morphological and Molecular Approach

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Abstract

Wax apple Delima and Citra Cultivars are two superior non-climacteric tropical fruit commodities from Demak Regency which have similar morphological characters but have different fruit characteristics. Identification wax apple cultivars from Demak regency using DNA barcoding approach has not been researched yet. The aims of this research are to analyze the morphological similarities of wax apple between Delima and Citra cultivars and to identify genetic variations of wax apple Delima and Citra using *trnL-trnF* intergenic spacer for molecularly authentication. The results shown that there were no differences in the environmental parameters of the wax apple two cultivated area in Demak Regency. According to the morphological approach, the Delima and Citra cultivars in Demak Regency were grouped separately into Delima and Citra clusters with a similarity index of 61.5% (Citra cultivars) and 60.5% (Delima cultivars) and separated based on the origin of their cultivation area. Genetic variations between wax apple Delima and Citra in Demak Regency consisted of deletions (9delA & 17delA) and conserved P6 loop in all compared cultivars. The possibility of heteroplasmy R (A,G) found at 73 nucleotide number in Delima Betokan cultivar and Citra cultivars (Betokan and Jungpasir). Meanwhile, Delima Jungpasir cultivar is K (G,T). There were P8 stem-loops with different lengths between Delima cultivars (Betokan and Jungpasir) and the same length in Citra cultivars (Betokan and Jungpasir). Therefore, the *trnL-trnF* intergenic spacer has not been thoroughly used in wax apple Delima and Citra authentication due to the presence of a secondary structure which causes the loss of the electropherogram signal so that the nucleotide sequence cannot be read.

Keywords: Citra, Delima, DNA barcoding, *trnL-trnF*, wax apple

Introduction

The wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry is a tropical fruit plant from the Myrtaceae classified into non-climacteric fruit. The wax apple originally grown from Southeast Asia, is widely cultivated in the lowlands and has sweet taste and striking fruit color (Mukaromah, 2020). The wax apple has many cultivars with different colors, flavors and various forms. The breeding and selection process influences the occurrence of speciation so that the original type of wax apple (*Syzygium samarangense* var. *Samangese*) is difficult to determine. Wax apple cultivars like Citra and Delima are the most widely cultivated by society. These two cultivars have similar morphological characters but have different fruit characteristics (Widodo, 2015). *Syzygium* species identification using

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traditional methods such as morphological approach takes a long time, and only reveal phenotypic similarities but cannot describe the relationship and authenticity of wax apple cultivars. This is consistent with studies by Ubaidillah & Sutrisno (2009) reveal that the similarity and difference values will not represent true evolutionary relationships. Therefore, a genotypic approach is needed to support phenotypic data (genotypic and environmental interactions) to trace the relationship and authenticity of the two cultivars. This is positively correlated with a review conducted by Mukaromah (2020) that described that research on wax apple is mostly in phytochemistry and post-harvest technology but there are still few who carry out molecular authentication of Indonesian wax apple cultivars.

DNA Barcoding utilizes specific parts of DNA that are useful in the species identification process. DNA Barcoding is used for all groups of organisms and is available to aid the understanding, conservation and use of biodiversity. DNA barcode markers for plant groups are two coding sites in chloroplasts, part of the genes, *rbcL* and *matK* (Vere, Rich, Trinder, & Long, 2015). Six primers for amplification of three non-coding regions of chloroplast DNA using Polymerase Chain Reaction have been developed. This primer is universal and can be used to amplify DNA from various plant species such as algae, bryophytes, pteridophytes, gymnosperms and angiosperms. Therefore, *trnL-trnF* primers can be utilized to population analysis and evolutionary biology (inter- and intraspecies phylogeny) of plants studies (Taberlet, Gielly, Pautou, & Bouvet, 1991). The DNA metabarcoding approach using the *trnL* marker is suitable for quantitative analysis in food composition quality testing. Food product authentication is useful for preventing food adulteration, providing correct labeling, food safety, protecting consumers, sustainability of the economic value of quality food (Bruno et al., 2019).

Based on the analysis conducted by DeSalle & Goldstein (2019), the utilization of DNA Barcodes in the identification process, taxonomic decision making, forensics, and discovery of cryptic species is growing rapidly. DNA barcoding utilization for authenticating the wax apple cultivars such as Delima and Citra is very appropriate to do. Selection of the *trnL* chloroplast DNA barcode is one of the easiest solutions to authenticate wax apple cultivars quickly and accurately. Thefore, this research is very important to support food, agriculture and health sector.

The aims of this research is to analyze the morphological similarities of wax apple Delima and Citra and to identify genetic variations of wax apple Delima and Citra using *trnL-trnF* intergenic spacer for molecularly authentication.

Research Methods

Wax apple cultivars sampling

The sampling location points was determined based on data from Demak agricultural government information services and results of interviews with wax apple farmers from two villages. The sampling location for wax apple cultivars was determined using the purposive sampling method on wax apple cultivation land in Betokan Village, Demak sub-disctrict and Jungpasir Village, Wedung sub-district, Demak regency. Details of the accessions taken in this study include the wax apple Delima Betokan cultivars (DB A1, DB A2, DB A3, DB A4, and DB A5), the Citra Betokan cultivars (CB A1, CB A2, CB A3, CB A4, and CB

A5), Delima Jungpasir cultivars (DU A1 and DU A2), and Citra Jungpasir cultivars (CU A1, CU A2, CU A3, CU A4, CU A5).

Environmental parameter analysis

Abiotic factors such as air temperature using thermometer, soil temperature measured by soil tester, light intensity and humidity measured by thermohygrometer, and altitude measured by altimeter have been taken at each sampling location. Furthermore, soil chemical properties such as pH, C organic, N total, P total, exchangeable K, exchangeable Na, Ca and Mg as well as measurements of soil physical properties such as porosity, field capacity, texture and water content analyzed at physic and soil conservation Laboratory, Sebelas Maret University, Surakarta

Wax apple morphological identification

Morphological characters identification of wax apple is based on Plant Morphology by Gembong Tjitrosoepomo and Wax Apple and Water Guava by Widodo. All morphological characters have been identified and recorded in detail consisted of stature, crown shape, branching type, stem bark color, stem diameter, stem texture, leaf structure, leaf tips, leaf bases, leaf edges, leaf ribs, leaf length, leaf width, petiole length, color of the upper surface of mature leaves, color of the lower surface of mature leaves, distance between leaf nodes per stalk, color of flower petals , ovary color, ovary height, ovary width, fruit surface, fruit surface color, fruit taste, fruit texture, water content, fruit flesh color, fruit tip circumference, fruit base circumference, fruit length, fruit flesh thickness, fruit weight , number of seeds per fruit, seed shape, seed color when split, seed circumference, and seed length. The morphological identification data will be made into a binary table (0/1) to continue with the phenetical analysis.

DNA isolation, amplification and sequencing

DNA isolated from wax apple cultivars leaves followed Genomic DNA Mini Kit Plant GP 100 (Genaid) protocols. The *trnL-F* DNA barcode amplification was carried out using the polymerase chain reaction (PCR) technique using the primer pair *trnL-trnF c*-5'-CGAAATCGGTAGACGCTACG-3' and *trnL-trnF f*-ATTTGAACTGGTGACACGAG-3' (Taberlet et al., 1991). Primers were synthesized by Integrated DNA Technologies, Singapore. PCR was carried out in a 50 µL volume consisting of 25 µL MyTaq HS Red Mix 2X (Bioline), 13 µL sterile ddH_2O, 2 µL of each primer (10 pmol/µM) and 8 µL DNA template. Amplification conditions in the PCR program include pre-denaturation (94°C for 5 minutes), followed by 35 amplification cycles as follows denaturation (94°C for 1 minute), annealing (52°C for 1 minute), extension (72°C for 2 minutes) and final extension (72°C for 5 minutes and stored at 15°C (Taberlet et al., 1991). DNA isolation and PCR products were carried out using 0.8% agarose gel and observed using gel documentation set (Biorad). Sequencing was carried out at 1st BASE (Malaysia) by the Sanger DNA Sequencing by using Capillary Electrophoresis.

Data analysis

Environmental parameters analyzed by the independent t-test using SPPS 22 with a confidence level at 95%. Similarity index and phenetic dendogram have been recontructed using the MVSP 3.1A software based on distance matrix method with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method and Nei & Li coefficients. The DNA sequencing results in the AB1 file format analyzed by BIOEDIT (Hall, 2011) and MEGA

X software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). The sequencing results trimmed using the contig method until they have the same length as the comparison sequences from NCBI genebank.

Research Results and Discussion

Wax apple cultivated environment

Chemical and physical soil parameters of Betokan Village and Jungpasir Village shown similar condition and suitable for wax apple cultivation. Jungpasir Village had relatively hotter air temperatures, lower air humidity, lower soil water content, higher nutrient content of phosphorus, Na and Ca compared to Betokan Village (Table 1.). The soil pH is important parameters contributed in soil fertility. Soil pH of Betokan Village and Jungpasir Village suitable for growing crops in the range slightly acidic to neutral soil pH (6-7) (Fazekasova, 2021). Betokan village had organic mater higher than Jungpasir village. It might influence soil buffering capacity inducing the minerals and organic material protonation in the soil or is deliberately gained to the soil (Weaver et al., 2004). Sand texture and clay texture affect both soil non-capillary pores shared and contributed in gravitational water releasing proccess and permiting good air exchange between soil and atmosphere (Fazekasova, 2021). However, the sand texture in two villages were not sufficiently support the ideal non-capillary pores caused by low sand texture percentage. Therefore, wax apple cultivars phenotype might not be influenced by environmental factors.

Phenetical analysis of wax apple Delima and Citra cultivars

According to Fig. 1, it can be seen that the wax apple Delima and Citra cultivars classified separately into two clusters with similarity index of 61.5% (Citra cultivars) and 60.5% (Delima Cultivars). Meanwhile, wax apple cultivar accessions from two different cultivation sites (Betokan Village and Jungpasir Village) were obviously separated based on cultivation sites origin. Wax apple cultivars cultivated in Jungpasir Village (DU A1 and DU A2) have a high similarity index as 88.9%, while Delima Betokan cultivars (CB A3 and CB A4) also had a high similarity as 92.6%. Therefore, the morphological characteristics of the Delima and Citra wax apple cultivars were more influenced by cultivar type than by cultivation sites.

Table 1. Environmetal parameters of wax apple cultivation area

Environmental parameters	Soil	
	Betokan Village	Jungpasir Village
Air temperature (°C)	33[a]	40[a]
Humidity	57[a]	39[a]
Soil pH (location)	6[a]	7[a]
Soil pH (Laboratory)	6.63[a]	6.54[a]
Light intensity (Cd)	658[a]	635[a]
Soil temperature (°C)	26[a]	29[a]
Altitute (mdpl)	79[a]	72[a]
N Total (%)	0.2[a]	0.17[a]
P_2O_5 total	7.36[a]	8.14[a]
Exchangeable K	0.15[a]	0.32[a]
Exchangeable Na	0.24[a]	0.47[a]
Exchangeable Ca	2.76[a]	3.95[a]
Exchangeable Mg	0.53[a]	0.42[a]
C. Organic	2.81[a]	1.63[a]
Organic material	4.85[a]	2.81[a]
Field capacity	62.17[a]	59.42[a]
Water content	10.18[a]	9.35[a]
BV	1.18[a]	1.23[a]
BJ	2.16[a]	2.09[a]
Dush texture	29.37[a]	31.9[a]
Clay texture	47.84[a]	53.93[a]
Sand textture	22.79[a]	14.17[a]

a: no statistically significant at 95% confidence level

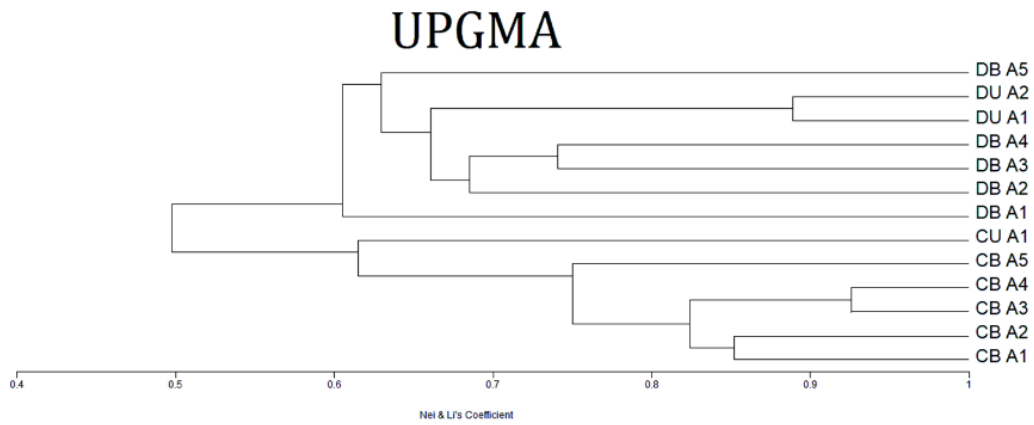

Figure 1. Dendrogram of wax apple accessions the Delima and Citra cultivars from Betokan Village and Jungpasir Village, Demak District.
(DB: Delima Betokan Cultivar; CB: Citra Betokan; DU: Delima Jungpasir; CU: Citra Jungpasir)

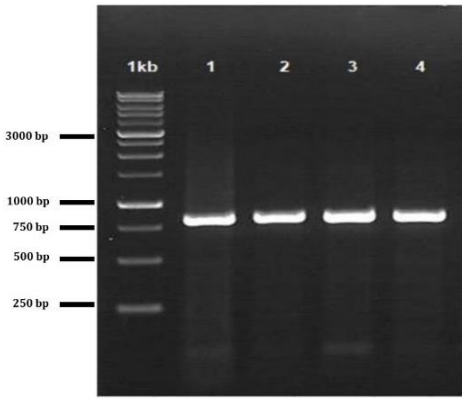

Figure 2. *trnL-trnF* amplification product of wax apple 'Delima' dan 'Citra' from Demak regency. 1: Citra Betokan; 2: Delima Betokan; Citra Jungpasir; 4: Delima Jungpasir

According to the amplification of the *trnL-trnF* DNA barcode (Fig. 2), the *trnL-trnF* fragment was successfully amplified at 800 bp. This result was correlated with Nguyen et al. (2020) revealed that the *trnL-trnF* amplified nucleotides length in NCBI genbank around 700 bp. It might be reduced because sequencing result quality. According to the electropherogram sequeuncing result, it

good signal **bad signal**

Figure 3. *trnL-trnF* sequeuncing result of wax apple Delima and Citra cultivars from Demak regency

found a secondary structure in the four samples characterized by the loss of the sequence reading signal (Fig. 3). The presence of a secondary structure caused difficulties in the nucleotide sequence reading process so that the nucleotide sequence that could not be read. Taberlet et al., (2007) explained that the *trnL-trnF* DNA barcode has a secondary structure, namely P6 and P8 loops. It was shown that the P6 loop of *trnL-trnF* region in wax apple cultivars located at nucleotide number 61 – 124 bp (65 bp) (Fig.4). The P6 loop in the four samples were conserved and were also found in

comparative sequeunce of wax apple *trnL-trnF* region in NCBI genebank.

Based on multiple sequence alignment (Fig.4), both wax apple Delima and Citra cultivars originally from both cultivations (Betokan Village and Jungpasir Village) shown the following deletions found in 9delA and 17delA. When compared with the wax apple *trnL-trnF* region from the NCBI genebank, the wax apple Delima and Citra cultivars from Demak regency closely related to the wax apple cultivar originating from the Fairchild Tropical Botanic Garden USA, Florida (*Syzygium samarangense* Voucher FTG: Flickinger 108) (Flickinger et al., 2020). Deletions were also found in the cactus family (Cactaceae) subfamily (Cactoideae) with the amplicon length from *trnT – trnF* region only detected around 550 bp out of 900 bp because there was a deletion of around ± 300 bp (Applequist & Wallace, 2002). It was possible that this could also be found in wax apple cultivars. However, this finding should be confirmed with many species in the genus *Syzygium* and wax apple cultivars.

Insertions and deletions of *trnL* introns in Dipterocarpaceae do not have homoplasy. The *trnL* DNA sequence contains phylogenetic signals that can be used to reconstruct the phylogenetics of the Dipterocarpoideae subfamily (Yulita, 2013). According to Fig.4, there might be found heteroplasmy (the presence of a mixture of maternal and paternal copies of the genome in the chloroplast) in *trnL-trnF* region of wax apple cultivars. Chloroplast heteroplasmy is a variation in chloroplast sequence within individual plants reported in many species. Heteroplasmy in chloroplasts may be due to sequence contamination from other genomes such as the nuclear or mitochondrial genome during chloroplast preparation or amplification of specific sequences from these genomes (Hoang, Furtado, McQualter, & Henry, 2015). Therefore, the presence of heteroplasmy needs to be checked again regarding the chloroplast inheritance pattern in the wax apple species because generally the inheritance pattern is maternal but does not rule out the possibility of it being paternal or contaminated with mitochondrial DNA. Heteroplasmy in the wax apple Citra (Betokan and Jungpasir) cultivars and the Delima Betokan cultivar was found at nucleotide number 73 {R(A,G)}. Meanwhile, heteroplasmy in wax apple cultivar Delima Jungpasir was found at nucleotide number 73 {K(G,T)}. According Fig.4, heteroplasmy was also found in *Syzygium samarangense* Voucher DMB 188 and DMB 190 (Srilanka) at nucleotide number 682 {R(A,G)}. The heteroplasmy also appear in chloroplast genome of 24 Thai durian varieties and Musang King (Shearman et al., 2020).

Based on Fig.4, the existence of a stem loop P8 secondary structure is possible with a length of 410 – 445 bp. Wax apple Citra (CUA2 and CBA3) cultivars had a stem loop P8 secondary structure at 194 – 602 bp. Meanwhile, Delima (DUA2) cultivar had the stem loop P8 secondary structure in the region 186 – 602 bp and Delima (DBA4) cultivar the stem loop P8 secondary structure at 159 – 602 bp. Kishor & Sharma (2018) explained that the stem loop P8 secondary structure is the part of the *trnL-trnF* DNA Barcode region had hypervariability in size with several repetitive motifs in plants and creating difficulties in alignment. Four types of restriction endonuclease enzymes (*ApoI, AgsI, TspDTI,* and *VspI*) can be used for further analysis of the trnL intron P8 sequence in orchids either by in silico or PCR-RFLP. Therefore, the P8 region of the *trnL* intron has the potential to be developed as a DNA barcode due to the presence of specific restriction enzyme cutting points in each species.

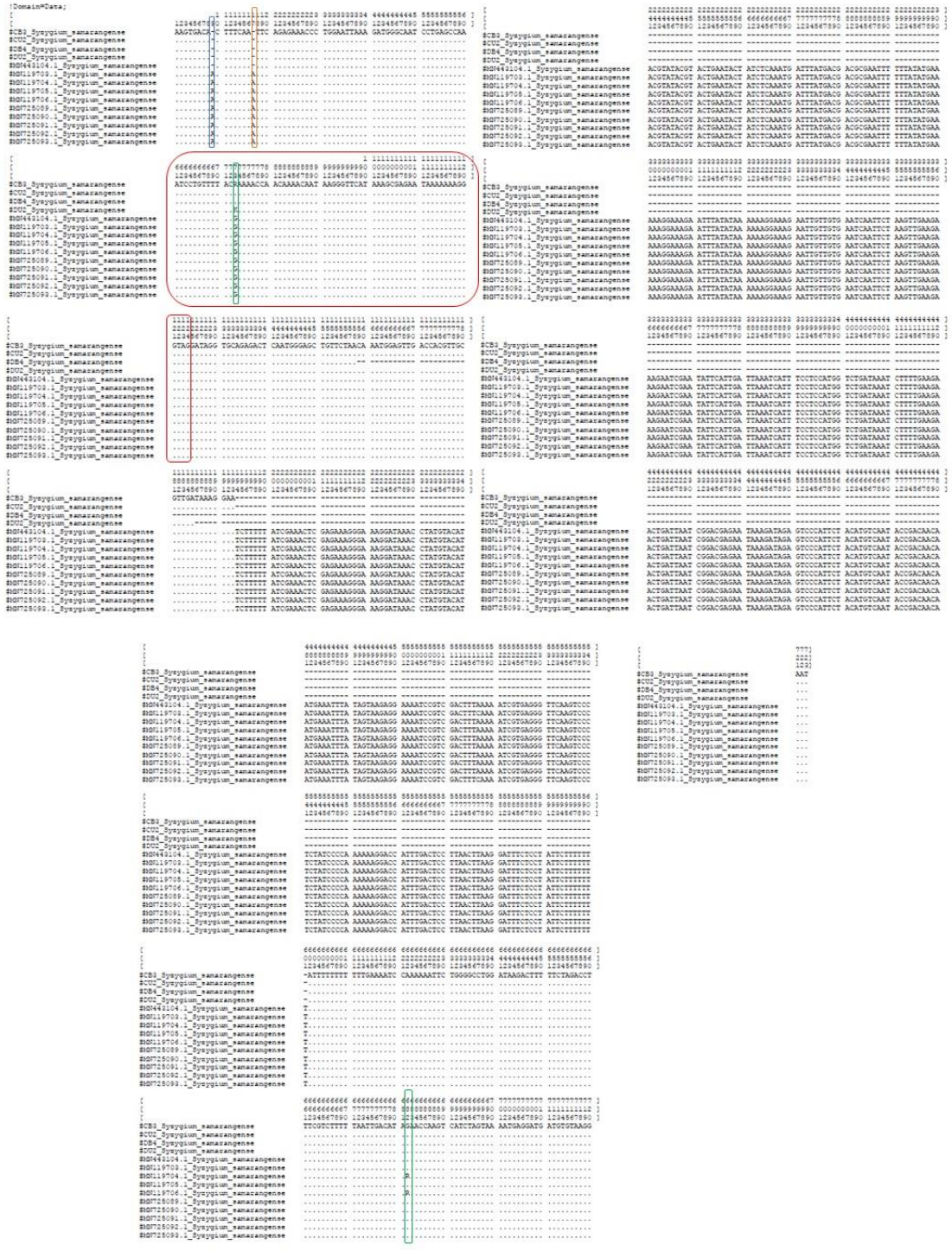

Figure 4. Alignment of *trnL-trnF* barcode DNA sequences of wax apple Delima and Citra cultivars in Demak regency compared to ten of wax apple *trnL-trnF* DNA barcodes from NCBI Genebank (a) P6 loop in the red box; (b) deletion of nucleotide sequences (9 and 17) in blue and orange box; (c) heteroplasmy at nucleotide (73 and 682) in the green box

Therefore, part of the P8 loop can be removed during phylogenetic analysis.

The *trnau*" UAA (*trnL*) intron is widely used in phylogenetic reconstruction of angiosperm tribes. The two main secondary structure strand elements of the *trnL* intron are the stem loop regions P6 and P8 which contain sequences that vary widely across taxa. In Dipterocarpaceae tribe, there are four stem-loop structures located in the *trnL* secondary structure in the P6 and P8 loops. This structure can be used as a marker for genera in Dipterocarpaceae family. In addition, there are mutations at the species level so that the P6 and P8 loops have the potential to be used as DNA markers in types of Dipterocarpaceae. The failure of the stem loop structure in the secondary structure of the *trnL* intron can be caused by errors in the replication process and pairing errors in repeated nucleotide bases (Yulita, 2007).

Five wax apple varieties from Dong Thap Province, Vietnam show a wide range of morphological characteristics including size, color and fruit shape. Based on *ITS* and *trnL-F* sequence analysis, four wax apple varieties (Xanh Duong, Hong Dao, Hoa An and Sua) are identical with a similarity level as 100%, while the An Phuoc variety shows one position difference in the *ITS* sequence and five positions in the *trnL-F* sequence. The results of phylogenetic analysis show that four wax apple cultivars (Xanh Duong, Hong Dao, Hoa An and Sua) are 100% identical to two varieties from China (KC815987, KC800610) and from Sri Lanka (MN104146). Meanwhile, the An Phuoc variety shows high similarity with the variety grown in Sri Lanka (MN104142).

Phylogenetic inference of the Myrtaceae tribe was carried out using maximum parsimony and Bayesian methods by aligning nuclear barcode DNA sequence data (*ITS*) and three chloroplast DNA barcode regions (*psbA-trnH, ndhF-rpl32, trnL-trnF*). The results of this analysis were congruent with each other but a conflict was found between the nuclear and chloroplast DNA barcode regions involving congeneric species (Flickinger et al., 2020). Congeneric species are a good model to investigate the relative importance of ecological processes that maintain high species diversity because they tend to exploit the same limiting resources and/or have similar tolerance limits to the same environmental conditions due to close phylogenetic relationships (Ribero et al ., 2021). *ITS2* is an efficient marker used in identifying banana species and also has the potential to be used as a candidate for calculating phylogenetic relationships between subspecies and cultivars (Dhivya et al., 2020). Therefore, the identification of wax apple cultivars from Demak regency can be continued using another DNA barcode so that genetic variations and phylogenetic relationships can be identified.

Conclusion

The research conclusions are wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) Delima and Citra cultivars grouped separately into two clusters (Delima and Citra clusters) with a similarity index at 61.5% (Citra Cultivar) and 60.5% (Delima Cultivars), and divided based on cultivated region. Genetic variations between wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) Delima and Citra cultivars include deletions (9delA & 17delA) and conserved P6 Loop in all compared cultivars. Heteroplasmy appears at nucleotide number 73 {R(A,G)} in Delima Betokan cultivar and Citra cultivars (Betokan and Jungpasir) while {K(G,T)} shown in Delima Jungpasir cultivar, there are P8 stem-loops with different lengths between the Delima (Betokan and Jungpasir) cultivar and the same length in Citra cultivars (Betokan and Jungpasir). According to the results, of *trnL-F* cannot be used in wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) Delima and Citra cultivars from Demak Regency due to the presence of a secondary structure which causes the loss of the electropherogram signal so that the nucleotide sequence is not can be read.

Acknowledgement

We express our gratitude to Dr. Hamdan Hadi Kusuma, M.Sc. as research grant reviewer and this research is funded by DIPA BOPTN LP2M UIN Walisongo Semarang 2021.

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