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psbA-trnH Intergenic Spacer profile of Wax Apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) Cultivars

(Profil *psbA-trnH Intergenic Spacer* Kultivar Jambu Semarang (*Syzygium samarangense* (Blume) Merr. & L.M. Perry)

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ABSTRACT

The fruit known as wax apple, scientifically named *Syzygium samarangense* (Blume) Merr. & L.M. Perry, is a well-liked agricultural product originating from Demak Regency in the Central Java Province. When it is difficult to distinguish between different forms of an organism, using DNA barcoding to authenticate the identity of species based on their DNA is an important way. The *psbA-trnH intergenic spacer* is a commonly used molecular approach to analyse the genetic characteristics of plant species. This study investigated the *psbA-trnH intergenic spacer* profile of wax apple cultivars from Demak regency. The investigation revealed that the psbA-trnH intergenic spacer sequences of the *S. samarangense* cultivars, namely Madu Thailand and Madu Deli Hijau, had lengths of 535 and 492 base pairs, respectively. Both cultivars demonstrate a greater nucleotide composition of deoxyadenylic acid (A) and deoxythymidylic acid (T) in comparison to deoxycytidilic acid (C) and deoxyguanylic (G). The genetic distance between *S. samarangense* 'Madu Thailand' and 'Madu Deli Hijau' indicates a very tight relationship, with a value of 0.000. The psbA-trnH intergenic spacer proved to be insufficient in differentiating the *S. samarangense* cultivars from Demak regency, mostly because to its low capacity to discern between the wax apple cultivars.

Keywords: Demak, *psbA-trnH intergenic spacer*, *Syzygium samarangense*, wax apple

ABSTRAK

Buah yang dikenal sebagai jambu semarang, secara ilmiah bernama *Syzygium samarangense* (Blume) Merr. & L.M. Perry, merupakan produk pertanian yang digemari, berasal dari Kabupaten Demak di Provinsi Jawa Tengah. Ketika sulit untuk membedakan antara berbagai bentuk organisme, penggunaan kode batang DNA untuk mengautentikasi identitas spesies berdasarkan DNA mereka merupakan cara yang penting. *psbA-trnH intergenic spacer* merupakan pendekatan molekuler yang umum digunakan untuk menganalisis karakteristik genetik spesies tanaman. Penelitian ini menyelidiki profil *psbA-trnH intergenic spacer* dari kultivar jambu semarang dari Kabupaten Demak. Penyelidikan mengungkapkan bahwa urutan *psbA-trnH intergenic spacer* dari kultivar *S. samarangense* yaitu Madu Thailand dan Madu Deli Hijau, masing-masing memiliki panjang 535 dan 492 pasangan basa. Kedua kultivar menunjukkan komposisi nukleotida asam deoksiadenilat (A) dan asam deoksitimidilat (T) yang lebih besar dibandingkan dengan asam deoksisitidilat (C) dan asam deoksiguanilat (G). Jarak genetik antara *S. samarangense* Madu Thailand dan Madu Deli Hijau menunjukkan hubungan yang sangat erat, dengan nilai 0,000. *psbA-trnH intergenic spacer* terbukti tidak cukup dalam membedakan kultivar *S. samarangense* dari Kabupaten Demak, terutama karena kemampuannya yang rendah dalam membedakan kultivar jambu biji.

Kata kunci: Demak, jambu semaran, *psbA-trnH intergenic spacer*, *Syzygium samarangense*

INTRODUCTION

Indonesia is an archipelago with a geographical area of 9 million km^2 and a coastline length of approximately 99,093 km (Kusmana & Hikmat 2015). One of the agricultural cultivation centers in Indonesia is the cultivation of wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry), which is in several regions including in Central Java Province (BPS 2022). Demak was reported by the Statistical Center of Central Java Province in 2021 as the district or city with the highest production of fruits and plant species of water apple in Central Java Province reaching 164,928 quintals.

S. samarangense, one of Indonesia's most promising and highly demanded agricultural commodities (Widodo 2015), belongs to the Myrtaceae. It has many nutrient ingredients, including protein, carbohydrates, calcium, iron, magnesium, potassium, zinc, copper, citric acid, phosphorus, fiber, vitamin C, vitamin A, niacin, riboflavin, thiamine, and

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other beneficial substances (Hariyanto 1993). The fruit is usually used as diarrhea medicine (Ghayur 2006), antibacterial medicine (Ratnam & Raju 2008), and immunopharmacology medicine (Kuo *et al*. 2004). Therefore, many plant breeders cultivate this plant to become a superior product.

According to Mukaromah (2020), *S. samarangense* is highly diverse in cultivar type, so there are difficulties in determining the original fruit type. Many cultivars continuously appeared due to the breeding and selection programs sustained to produce superior *S. samarangense* commodities, such as 'Madu Deli Hijau', 'Demak Hijau', 'Citra', and 'Delima', widely cultivated by the community, especially in Demak Regency. The existence of these characteristics encouraged researchers to conduct authentication on several *S. samarangense* cultivars grown in different areas in Demak Regency.

Authentication is a verification process to prove the identity of a species (Lal *et al.*2016). Authentication in the biology field is a process that tests the authenticity of samples simultaneously because it can be automated. This DNA-based technology is an efficient and accurate approach to species validation. DNA barcoding is a molecular approach using DNA barcode sequences such as *ITS1, ITS2, rbcL, trnL-trnF, psbA-trnH intergenic spacer, matK*, and others (Omelchenko *et al.* 2022*)*. Therefore, it can be used to differentiate the *S. samarangense* cultivars. However, Mukaromah & Ulfah (2021) stated that DNA barcode *trnL-trnF* cannot be used to authenticate *S. samarangense* cultivars Citra and Delima in Demak Regency have not been able to be used comprehensively due to the presence of secondary structures so the electropherogram signal is lost, and the nucleotide sequence cannot be analyzed. Therefore, searching for other DNA barcodes that can be used to authenticate *S. samarangense* cultivars is necessary.

The *psbA-trnH intergenic spacer* is a DNA barcode found in the chloroplast genome of angiosperm plants, known for its significant variety. The DNA barcode mentioned is widely used in species-level phylogenetic analysis and is a good sequence for DNA barcoding research (Štorchová and Olson, 2007). Uncu (2020) has reported that the *psbA-trnH intergenic spacer* is utilized as a DNA barcode for verifying the authenticity of almond, peach, and apricot. Molecular phylogenetic investigations have shown this spacer as a changeable universal sequence. The *psbA-trnH intergenic spacer* barcode is widely utilized as a non-coding universal sequence in plant molecular phylogenetics (Hollingsworth *et al.* 2011).

The *psbA-trnH intergenic spacer* sequence belongs to the intergenic spacer, which is a noncoding sequence in the chloroplast genome for studying species relationships at low taxonomic levels that are currently proliferating (Degtjareva *et al.*2012). Therefore, research to test authenticity, see variation, and identify plants at low taxonomic levels is needed; utilize *psbA-trnH intergenic spacer* as a molecular authentication tool in *S. samarangense* cultivars from Demak Regency so that contribute to biodiversity conservation. Study on *S. samarangense* cultivars is very limited, and it is necessary to analyze them as one of the genetic resources (Rachmah *et al.* 2023). This study examines the *psbA-trnH intergenic spacer* profile of the wax apple cultivars from Demak regency.

MATERIALS AND METHODS

Setting of the Research

This research was conducted in July–December 2022 in the Biotechnology Laboratory, Faculty of Science and Technology, UIN Walisongo, Semarang. Sampling was carried out in Boyolali Village, Gajah District, Demak Regency (6°52'54.7 LS, 110°44'12.0 BT).

Sample Preparation and Preservation

The sampling point's location was identified using data obtained from interviews conducted with farmers that cultivate *S. samarangense*. The sampling location was established using the purposive sampling method on the cultivated field of *S. samarangense* in Boyolali Village, Gajah District, Demak Regency.

The healthy mature leaves samples were taken between the third until fifth leaves order from the tip of the twig. The collected *S. samarangense* leaves were first cleaned by spraying 70% alcohol. Tea bags that had been labeled with the cultivar name and accession code were prepared to store the leaf samples. Next, the *S. samarangense* leaf samples were folded and carefully inserted into the tea bags. Silica gel was inserted into a ziplock plastic and the tea bag was inserted in the previous step. The last step was to label the ziplock plastic with the cultivar name, collection date, and accession code on the surface of the plastic. The cultivars used in this study were Madu Deli Hijau (MG3) and Madu Thailand (MT3).

DNA Extraction, DNA Amplification, and DNA Sequencing

DNA extraction was performed on leaves from *S. samarangense* cultivars using the FAVORGEN DNA Extraction Kit. The *psbA-trnH intergenic spacer* was amplified using the polymerase chain reaction (PCR) technique, with the *trnH* primer sequence as 5'-CGC GCA TGG TGG ATT CAC AAT CC-3' (Tate & Simpson, 2003) and the *psbA* primer as 5'-GTT ATG CAT GAA CGT AAT GCT C-3' (Sang *et al.* 1997). The primers were synthesized by Integrated DNA Technologies, located in Singapore. The barcode region was amplified in reaction mixes of 50 µL, consisting of 2 µL of each primer (10 µM), 25 µL of MyTaq HS Red Mix, 8 µL of template DNA, and 13 µL of ddH_2O. The PCR reaction conditions were carried out following the protocol of Costion et al. (2011) with a modification. This modification involved an initial denaturation step at 94°C for 3 minutes, followed by a denaturation step

at 94°C for 1 minute, an annealing step at 54°C for 30 seconds, and an extension step at 72°C for 40 seconds. The reaction was concluded with a final extension step at 72°C for 10 minutes. The isolated DNA and PCR product were separated by utilizing a 1% agarose gel and observed using a gel documentation system. In addition, the amplicons were sequenced at 1st BASE (Malaysia) using the Sanger DNA Sequencing method, followed by capillary electrophoresis.

Data Analysis

The data were analysed using MEGA11 software, which supports AB1 file format (Kumar *et al.* 2016), and BIOEDIT version 7.2.5 (Hall 1999). The sequencing findings were assembled using the contig approach. The nucleotide content of the Madu Deli Hijau and Madu Thailand cultivars was analysed using the Nucleotide content model in the MEGA11 tool. The DNA sequences were compared to other representative sequences obtained from GenBank (NCBI) using the BLASTn method for alignment. The Clustal W algorithm in MEGA11 was utilised to do a multiple sequence alignment. The phylogenetic tree was reconstructed using the neighbor joining method, employing the Tamura-3 parameter model and a bootstrap replication value of 1000. The genomic distance and genetic variation analysis were conducted using the MEGA11 version 11.0.11 (Tamura 2021).

RESULTS AND DISCUSSION

Environmental factors in the cultivation field of wax apple in Demak Regency

Based on the environmental parameters in Boyolali village (Table 1), the cultivation site is in a lowland area. Istiawan & Kastono (2019) state that the lowland area is below 400 m above sea level. The degree of soil acidity in the cultivated land was neutral, and the measured soil temperature was quite high due to the incoming light intensity, which was also high. The temperature and humidity at the cultivation site were also quite high because the environmental parameters were measured during the day, so the air humidity tended to be higher. Daulay (2022) confirmed the results of measuring environmental parameters at the location of wax apple cultivation land in Boyolali Village based on the theory regarding the requirements for growing wax apples: (a) elevation of the place 0–500 meters above sea level, (b) soil pH 5.5–7.5, (c) rainfall 500–3,000 mm/year, (d) light intensity 40–80%, (e) temperature 18–28°C, and (f) humidity 50–80%). Fiqa *et al.* (2021) state that the environment influences optimum plant growth and development. The environmental conditions greatly affected the productivity of *S. samarangense* cultivation. The temperature at the location of the *S. samarangense* cultivation land was quite high compared to the growing conditions recommended by Daulay (2022) for the growth of *S. samarangense* cultivation plants in the range of 18–28°C.

The high light intensity at the cultivation site could also affect the productivity of wax apple cultivation because light that reaches the bottom of the soil can trigger plant growth, which plants need to survive. In addition, soil pH has a significant influence on plant growth and development (Fiqa *et al.* 2021). Karamina *et al.* (2017) suggested that plants that can adapt to varied soil pH are tolerant. The soil pH measurements at the three sample points of the location had the same value, which is neutral, so it has a positive effect on plant vegetative growth.

DNA sequence characterization of *psbA-trnH intergenic spacer* cultivars

Based on the sequencing results, Madu Deli Hijau and Madu Thailand cultivars were successfully sequenced from both forward and reverse directions. Based on the electropherogram graphs on both samples, Madu Thailand sample has a better graph than Madu Deli Hijau sample in both directions. The electropherogram graph on Madu Thailand had a stable peak seen from the same peak height even though there was still some noise. The contig results for the Madu Thailand cultivar had a sequence length of 535 base pairs (bp), whereas the Madu Deli Hijau cultivar had a sequence length of 492 bp. The Madu Deli Hijau had a shorter sequence length than the Madu Thailand because the electropherogram graph of the sequencing results in the forward section is not good. The Madu Deli Hijau and Madu Thailand *psbA-trnH intergenic spacer* sequences had different nucleotide compositions. However, both have more deoxyadenylic acid (A) and deoxythymidylic acid (T) than deoxycytidilic acid (C) and deoxyguanylic (G) (Table 2). The nucleotide variance of two cultivars is shown on nucleotide numbers 7 and 484 (Table 3).

The appearance of a secondary structure in the Madu Deli Hijau's forward sequence caused various

Table 1 The Environmental parameters in Boyolali Village, Gajah District, Demak Regency

Environmental parameters	Average ± standard deviation
Soil pH	7 ± 0,00
Soil temperature (°C)	27.67 ± 1.15
Light intensity (Cd)	449.33 ± 29.14
Air humidity (%)	66 ± 1.00
Air temperature (°C)	36.06 ± 2.96
Altitude (mdpl)	81 ± 2.64

Table 2 The Nucleotide composition of *psbA trnH intergenic spacer S. samarangense* Madu Thailand and Madu Deli Hijau cultivars

Cultivar	Code	Nucleotide composition (%)				Sequence length (bp)
		A	T	G	C	
Madu Thailand	MT3	33.5	16.3	37.6	12.7	535
Madu Deli Hijau	MG3	33.7	16.7	38.0	11.6	492

Table 3 The Nucleotide variation of *psbA trnH intergenic spacer* in *S. samarangense* Madu Thailand and Madu Deli Hijau cultivars

Sequence	Nucleotide composition		
	trnH	Intergenic spacer	psbA
	7	-	484
Madu Thailand	A	-	T
Madu Deli Hijau	.	-	.

anomalies, inducing inefficient chain elongation and impairing electropherogram analysis. Premature or sharp termination can decrease the sequencing signal during sequencing. The percentage of nucleotide composition of both cultivars is by the composition of the *psbA-trnH intergenic spacer* sequence, which contains many high nucleotides (Degtjareva *et al.* 2012). The BLASTn study considered the following identification parameters: percent identity, expectation value, and query cover. The percent identity represents the furthest degree of resemblance between the compared sequence and the subject.

The query cover value shows the percentage of nucleotides that are the same as the sequences in the GenBank. Meanwhile, the expectation value shows the number of alignment differences with the corresponding score and is expected to be found in the GenBank. The lower the expectation value (*e*-value), the lower the sequence difference (Isda & Sofiyanti 2019). The e-value can be significant if it reaches <0.05 (Frederick *et al.* 2003). If the estimated value is zero (0), then the sequence alignment is very significant. The alignment results show a high level of homology between the observed samples. Many gaps had been presented in the alignment results of both cultivars with 12 comparative sequences (Table 4). Gaps indicated the occurrence of mutation processes in both insertions and deletions.

Genetic distance of Madu Thailand and Madu Deli Hijau

The genetic distance between two organisms has a small genetic distance value (Tallei *et al.* 2016). All sequences had a genetic distance value <1, meaning that the average genetic distance is 0.000, so they were closely related and the same species. The genetic distance value between two species is directly proportional to the distance between the two species (Roslim & Fitriani 2021). The genetic distance between the two cultivars was closely related to *S. samarangense* (NC_060657.1) as 0.002, which tends to be the same species.

According to Irawan *et al.* (2016), the pakoba and jamblang plants also have a genetic distance value of 0.002, suggesting a close relationship and potential shared species. This finding is in line with our own research on the genetic distance between the two *S. samarangense* cultivars, namely the Madu Deli Hijau and Madu Thailand cultivars, which also suggests a very close relationship or the same species. his consensus among different studies adds to the reliability of our findings. Nevertheless, our findings also emphasize the constraints of the *psbA-trnH intergenic spacer* genetic marker in distinguishing *S. samarangense* cultivars, underscoring the necessity for additional investigation in this area.

Phylogenetic tree reconstruction of wax apple cultivars in Demak Regency

Phylogenetic tree reconstruction was performed using the Neighbor-Joining method, 1000 bootstrap replications, and the Tamura-3 parameter substitution model with a 5.00 gamma distribution. These methods and models were used based on the model program in the MEGA11 application, which recommends that these methods and models be applied to reconstructing phylogenetic trees of all sequences. In addition, reconstruction based on the neighbor-joining method is based on the evolutionary distance between species (Darmawan & Fitmawati 2020). Based on the gamma distribution, substitution rates often vary from one point to another in a sequence. The shape of the distribution is determined by a value called the gamma parameter or shape parameter. Evolutionary rates are also modeled by the gamma distribution (Tamura & Kumar 1992).

The results of the phylogenetic tree reconstruction showed that all sequences clustered in the form of clades (Figure 1). Bootstrap value is a value for the confidence of a branch. The smaller the bootstrap value, the lower the level of confidence in the topology of the tree reconstruction results (Syahputra *et al.*2017). Bootstrap with a good value is a value close to 100. Based on the bootstrap value of these *S. samarangense* cultivars, the chance of a change in the

Table 4 The Comparison sequences of *psbA trnH intergenic spacer* based on BLASTn results in NCBI

Scientific name	Accession	*Percent identity* (%)	*E. value*	*Query cover* (%)
Syzygium samarangense	NC 060627.1	99.80	0.0	99
Syzygium jambos	NC 052728.1	99.31	9e-145	96
Syzygium grijsii	NC 065156.1	99.31	9e-145	96
Syzygium forestii	NC 044106.1	99.31	9e-145	96
Syzygium cumini	NC 053327.1	99.28	4e-138	98
Syzygium rehderianum	NC 065261.1	93.81	4e-113	99
Syzygium album	NC 060587.1	96.22	4e-128	99
Syzygium odoratum	NC 059005.1	96.22	4e-128	99
Syzygium acuminatissimum	NC 053640.1	96.22	4e-128	99
Syzygium nervosum	NC 053907.1	96.62	2e-132	99
Syzygium malaccense	NC 052867.1	99.31	9e-145	99
Syzygium aromaticum	MN 746306.1	99,66	2e-146	99

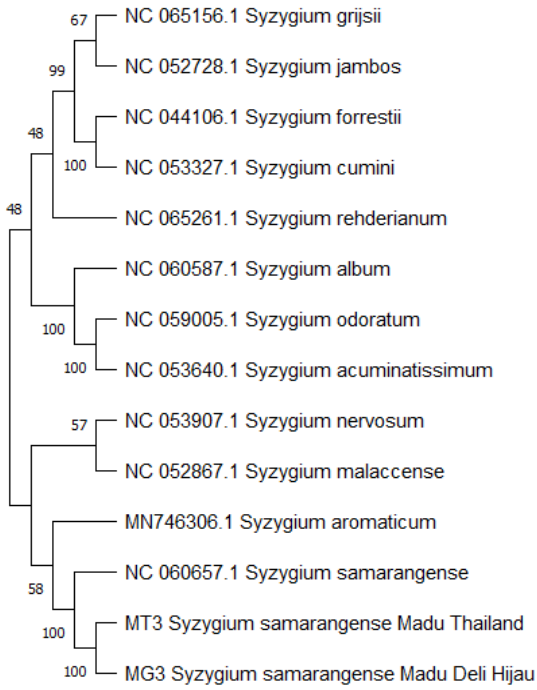

Figure 1 Phylogenetic tree reconstruction of *psbA trnH intergenic spacer* of *Syzygium samarangense* cultivars Madu Thailand and Madu Deli Hijau based on neighbor joining method and bootstrap 1000.

arrangement of the clades was very low. Therefore, the topology of the reconstructed phylogenetic tree in *S. samarangense* cultivars (Madu Deli Hijau and Madu Thailand) was highly confident.

CONCLUSION

Genetic distance analysis shows that the relationship between *S. samarangense* cultivars (Madu Thailand and Madu Deli Hijau) from Demak Regency are closely related or the same species. The *psbA-trnH intergenic spacer* region was unable to verify the authenticity of the *S. samarangense* cultivars from Demak Regency. The discriminatory ability of the *psbA-trnH intergenic spacer* region in distinguishing among wax apple varieties from Demak regency is extremely limited.

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