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GENETIC VARIATION OF WAX APPLE (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) CULTIVAR USING INTERNAL TRANSCRIBED SPACER 2 (ITS2)

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ABSTRAK

Syifara Chika, Tiara Dwi Meilina, Muhammad Ramdhani Arfan, Asri Febriana & Arnia Sari Mukaromah 2024. Variasi genetik kultivar jambu Semarang (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) menggunakan internal transcribed spacer 2 (ITS2). Floribunda 7(4): 153–165 — Jambu semarang (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) merupakan tanaman buah tropis, memiliki kultivar yang beragam, dan dikelompokkan dalam buah non klimaterik. Tujuan penelitian ini adalah mengidentifikasi variasi genetik kultivar *S. samarangense* pada wilayah *DNA Barcode* ITS2, menganalisis hubungan kekerabatan filogenetik di antara kultivar *S. samarangense,* dan menentukan keberhasilan penanda ITS2 untuk menggambarkan variasi genetik kultivar *S. samarangense* dari Kabupaten Demak, Jawa Tengah, Indonesia. Langkah-langkah pada penelitian ini terdiri dari pengambilan sampel daun, analisis parameter lingkungan, isolasi DNA, amplifikasi DNA, elektroforesis, DNA sekuensing di First Base Malaysia, analisis data sekuensing, prediksi struktur sekunder ITS2 dan rekonstruksi pohon filogenetik menggunakan MEGA 11 serta basis data ITS2. Hasil menunjukkan bahwa terdapat sedikit variasi nukleotida di antara kultivar *S. samarangense* dan terdapat variasi nukleotida di antara *S. samarangense* dan *Syzygium aqueum* pada daerah ITS2. *S. samarangense* kultivar Citra Wonosari dan kultivar Delima Wonosari memiliki struktur sekunder ITS2 yang identik. Namun, terdapat sedikit perbedaan di antara struktur sekunder *S. samarangense* cultivar Citra Botorejo yang terdiri hanya dua lingkaran pada untai I dan lingkaran kedua pada untai I dari cultivar Citra Tempuran lebih besar dari semua sampel. Pohon filogenetik menggambarkan hubungan yang dekat di antara *S. samarangense* cultivar Citra dan telah memisahkan *S. samarangense* cultivar Delima pada cabang yang berbeda. Hubungan genetik di antara kultivar *S. samarangense* lebih dipengaruhi jenis kultivar daripada lokasi budidaya. Oleh karena itu, ITS2 dapat digunakan untuk analisis variasi genetik kultivar *S. samarangense*.

Kata kunci: Citra, Delima, Variasi genetik, Internal Transcribed Spacer 2, *Syzygium samarangense*

Syifara Chika, Tiara Dwi Meilina, Muhammad Ramdhani Arfan, Asri Febriana & Arnia Sari Mukaromah 2024. Genetic variation of wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) cultivar using internal transcribed spacer 2 (ITS2). Floribunda 7(4): 153–165 — Wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) is a tropical fruit, has diverse cultivars and classified as a non-climacteric fruit. The study objectives were to identify the genetic variation of the *S. samarangense* cultivars in the ITS2 DNA barcode region, analyze the phylogenetic relationships among *S. samarangense* cultivars and determine successfulness of ITS2 barcode to describe genetic variation of the *S. samarangense* cultivars from Demak Regency, Central Java, Indonesia. Furthermore, the stages in this study consisted of sampling, environmental parameters analysis, DNA isolation, DNA amplification, electrophoresis, DNA sequencing in the First Base Malaysia, analysis of DNA sequencing data, secondary structure prediction of ITS2, and reconstruction a phylogenetic tree using MEGA 11 and ITS2 database. The result shown appearance of few nucleotide variation among *S. samarangense* cultivars and there were nucleotides variations between *S. samarangense* and *Syzygium aqueum* in the ITS2 region. *S. samarangense* cultivar Citra Wonosari and cultivar Delima Wonosari had the identical secondary structure of ITS2. However, this was slightly different between *S.*

samarangense cultivar Citra Botorejo secondary structure of ITS2 which consisted of only two loops in helix I and cultivar Citra Tempuran second loop in helix I which bigger than all samples. The phylogenetic tree described the closely relationship between *S. samarangense* Citra cultivars, and had separated *S. samarangense* cultivar Delima in the different clade. The genetic relationship of *S. samarangense* cultivars were more influenced by cultivar type rather than cultivated location. Therefore, ITS2 could be utilized for genetic variation analysis of *S. samarangense* cultivars.

Keywords: Citra, Delima, Genetic variation, Internal Transcribed Spacer 2, *Syzygium samarangense*

Indonesia has a very high diversity of plants. One of the biodiversity that has the potential to be developed is the diversity of tropical fruits such as the wax apple. The wax apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry) is a tropical fruit plant from the *Myrtaceae* which is classified as a non-climacteric fruit. It exhibits high diversity in cultivars, metabolite content, and its biological activities (Mukaromah, 2020). Demak regency is one of the center of *S. samarangense* cultivation. *S. samarangense* cultivars such as Citra and Delima have high economic value. However, Demak regency government have not been collected the genetic variation of *S. samarangense* that grows in that area. Genetic variation data in plants is crucial as a reference for germplasm genetic diversity. According to Widodo (2015), there are still few researchers who research the morphological characters of the wax apple. Meanwhile, the emergence of wax apple new cultivars, especially *S. samarangense*, made the identification process difficult. Cultivar variation in the wax apple is a form of genetic diversity that can affect the morphological characters displayed (Rachmah *et al.*, 2023). The most widely cultivated *S. samarangense* cultivars in Semarang are Citra and Delima cultivars. The Citra and Delima cultivars have similar morphological characteristics but differ in the character of the fruit. The morphological approach can only answer the phenotypic similarities but has not been able to answer the relatedness and genetic variation of *S. samarangense* plant cultivars (Mukaromah *et al.*, 2023).

Data on genetic variation of *S. samarangense* is important to study because it can predict the future of *S. samarangense* so that it remains sustainable. In addition to maintaining the number of species populations, conservation activities also need to pay attention to genetic aspects. Genetic conservation aims to preserve maximum genetic diversity, allowing species to adapt and evolve effectively (Coker, 2017; Widyatmoko, 2019). A molecular approach is an alternative in conserving genetic, and it is commonly achieved by isolating a particular organ to obtain genetic material (Octavia *et al.*, 2021). Genetic conservation in plants requires the involvement of molecular genetics such as DNA barcoding. The identification process using the DNA barcoding method is consistent, fast, and can be accounted for, so it is very useful in the process to accelerate the species identification, especially in the genus *Syzygium* (Irawan *et al.*, 2016; Roslim & Fitriani, 2021).

Each DNA barcode used in the DNA barcoding process has advantages and can be adapted to the species to be studied. According to Mukaromah *et al.* (2023) the DNA barcoding method using trnL-trnF is not applicable for authenticating the Citra and Delima cultivars of *S. samarangense* in Demak Regency due to the presence of a secondary structure, hindering the readability of nucleotide sequences. Therefore, it is necessary to use other types of DNA barcodes which are expected to be used to distinguish genetic variations between cultivars of *S. samarangense* plant from Demak Regency. Internal Transcribed Spacer 2 (ITS2) in particular has received recognition in DNA barcoding because of its ability to differentiate species with a success rate of up to 92.7% in a plant (Buys *et al.*, 2016). ITS2 has been used to analyze genetic variation of 23 accessions of Indonesian banana cultivars (Meitha *et al.*, 2020). Therefore, the ITS2 DNA barcode is predicted to be suitable for the analysis of genetic variation of *S. samarangense* cultivars. Analysis of genetic variation of *S. samarangense* has an important role, namely as a strategy for plant breeding, genetic conservation, and producing superior quality *S. samarangense* (Ilmi, 2021). The results of this study are expected to be useful in the preservation and breeding of *S. samarangense* and can be useful as a reference in selecting effective DNA barcodes for identification of *S. samarangense*. The study objectives were to identify the genetic variation of the *S. samarangense* cultivars in the ITS2 DNA barcode region, analyze the phylogenetic relationships among *S. samarangense* cultivars and determine successfulness of ITS2 barcode to describe genetic variation of the *S. samarangense* cultivars from Demak Regency, Central Java, Indonesia.

MATERIALS AND METHODS

The materials utilized in this study included the FavorPrep Plant Genomic DNA Extraction Mini Kit (Favorgen,Taiwan), ITS2 degenerate primer (Forward and Reverse), MyTaq HS Red Mix 2X (Bioline, UK), 1X Tris-borate EDTA

Buffer solution (Merck), 1.5 mL microtube, microtube 200 µL, pipette tip, agarose powder (GeneDirex), ethanol absolute, isopropanol, loading dye (6X), GelRed (Biotium Inc, USA), Nuclease Free Water (Thermo Scientific), 100 bp DNA ladder (Genaid Biotech Ltd., Taiwan), 1kb DNA ladder (Geneaid, Taiwan), 70% ethanol, liquid nitrogen, tea bag, gloves, tissue, alumunium foil, plastic, and sterile distilled water.

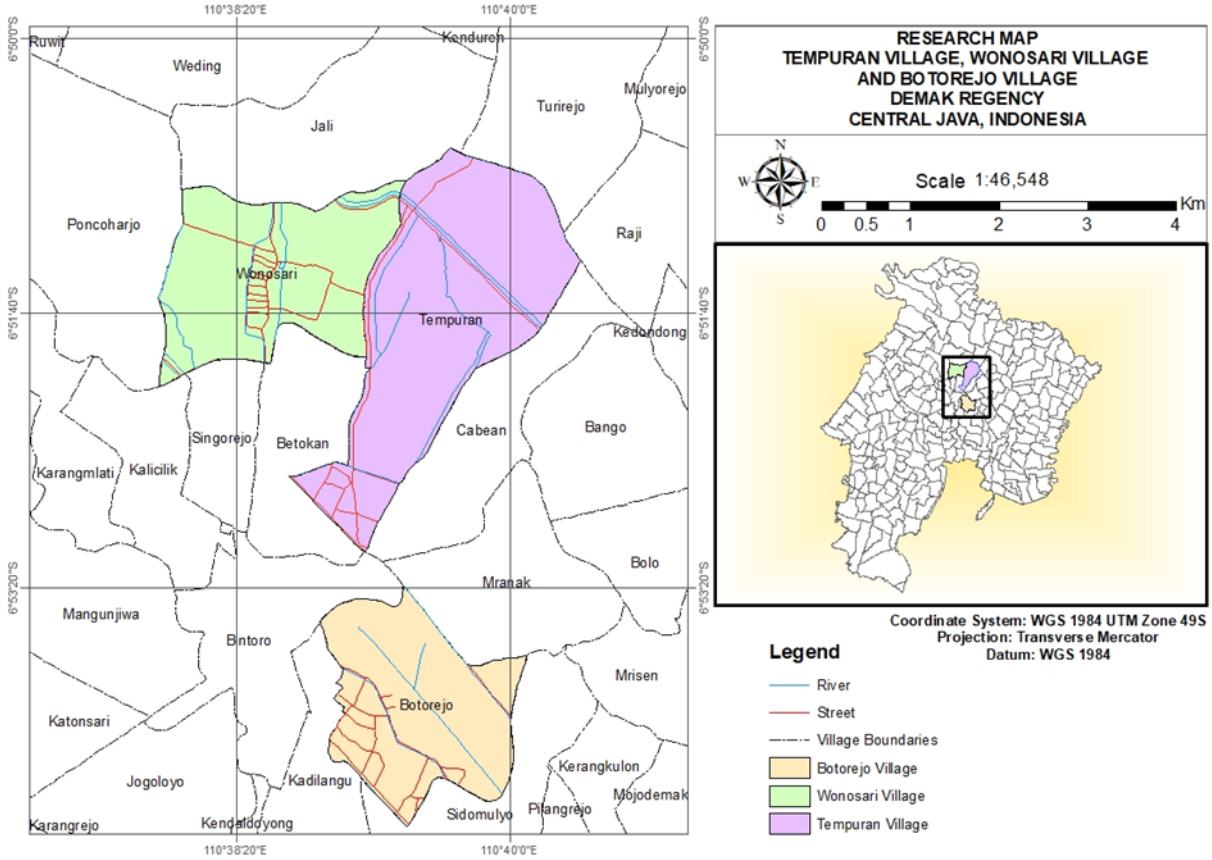

Figure 1. Map of Demak Regency, showing sampling locations of *S. samarangense* cultivars were collected and utilized in this study

Environmental Parameters

Environmental parameters had been measured consisted of the degree of soil acidity using a pH meter, altitude, and air temperature using an altimeter, and humidity measured using a thermohygrometer. Environmental parameters were analyzed for differences in significance using the OneWay ANOVA test using SPSS 22 with a 95% confidence level.

Sampling Location

The location of the sampling points was determined based on data from the Agriculture Office of the Demak Regency and the results of interviews with *S. samarangense* farmers in the three target villages. The sampling locations were determined using purposive sampling method. Sampling was carried out in July 2022. The sampling locations for *S. samarangense* cultivars were located in Demak Regency such as (1) Wonosari village, Bonang district, (2) Tempuran village, Demak district, (3) Botorejo village, Wonosalam district (Figure 1.).

Sample collection

The distinguishing morphological characters of *S. samarangense* cultivars such as stem circumference, fruit surface, exocarp color, fruit shape, fruit size (fruit tip diameter, fruit base diameter and fruit height), fruit weight, mesocarp thickness, fruit taste, fruit texture, number of seeds and seed size could be utilized to ensure cultivar type in each location (Rachmah *et al.*, 2023) (refer to Figure 2). *S. samarangense* cultivar Citra leaves were collected from Tempuran village, Wonosari village and Botorejo village. Meanwhile, *S. samarangense* cultivar Delima leaves were collected from Wonosari village Five leaves from each *S. samarangense* cultivar were taken, cleaned with 70% alcohol, and placed in tea bags containing silica gel. Samples were prepared based on the cultivar and cultivation location to facilitate the analysis process.

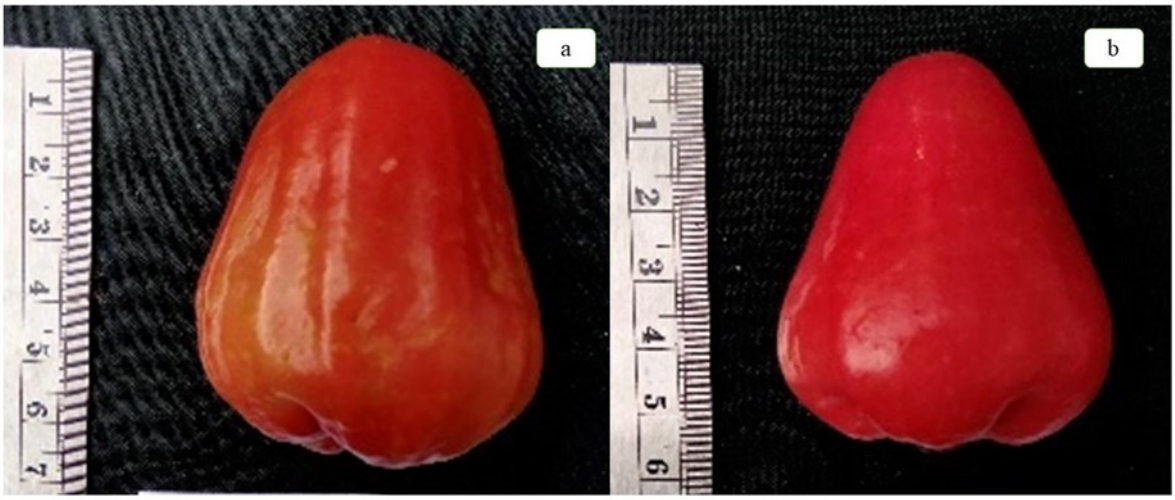

Figure 2. Fruit of *Syzygium samarangense* cultivars were collected from Betokan village, Demak Regency and utilized as guidance throughout sampling. (a)Citra; (b)Delima (Mukaromah & Ulfah *et al.*, 2021)

DNA Isolation and Amplification

The DNA isolation method was carried out according to the working protocol of the FavorPrep Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). ITS2 Barcode DNA amplification was carried out by polymerase chain reaction (PCR) technique using a primer pair designed by Cheng *et al.*, (2016), namely the forward primer ITS-u3F (5'-CAW CGA TGA AGA ACG YAG C -3') and reverse primer ITS-u4R (5'-RGT TTC TTT TCC TCC GCT TA-3'). The PCR reaction was carried out in a total volume of 50 µL consisted of 25 µL MyTaq HS Red Mix (Bioline, UK), 13 µL sterile ddH_2O, 2 µL forward primer, 2 µL reverse primer, and 8 µL DNA template. In the PCR process amplifying ITS2, the pre-denaturation used a temperature of 95°C for 3 minutes, the denaturation stage at 94°C for 30 seconds, the annealing stage at 55°C for 30 seconds, the extension stage at 72°C for 1 minute, post-extension stage for 10 minutes at 72°C, and storage stage at 15°C before the amplicons were removed from the PCR machine. The denaturation, annealing, and extension stages were repeated 35 cycle (Modification of Cheng *et al.*, 2016).

Amplification Visualization and Sequencing

The agarose gel was prepared with a concentration of 1% and was immersed in 1x TBE buffer solution on the electrophoresis apparatus. The electrophoresis machine was turned on and set at 100 volts for 30 minutes. Furthermore, the electrophoresis results were visualized using Gel Documentation System. The sequencing process was carried out at 1st BASE (Malaysia) using the Sanger sequencing method (Mukaromah *et al.*, 2023).

Data analysis

DNA sequencing results in the AB1 file format were read using the BioEdit 7.0.5.3 and MEGA 11 software (Hall, 1999; Tamura *et al.*, 2021). Sequencing data of *S. samarangense* samples in the form of forward and reverse data were processed through the BioEdit application to obtain contig sequences. The sequence had been trimmed using the trimming method until it had the same length as the reference sequence. Accession Number Registration at NCBI GenBank via the link: https://www.ncbi.nlm.nih.gov/. Genetic variation analysis were carried out through the BLAST process for each contig sequence on the NCBI page. The selection of BLAST result sequences is based on the query cover and percent identity values with the highest values. Furthermore, the four samples of *S. samarangense* from Demak Regency and the comparison sequence from NCBI were aligned using the Clustal-W program on the MEGA 11 software to identify the genetic variation in all sequences.

Prediction of ITS2 Secondary Structure

Annotation, motif prediction, secondary structure and phylogenetic tree was analyzed by https://ITS2.bioapps.biozentrum.uni-wuerzburg.de/. Combination ITS2 sequence and secondary structure was utilized to phylogenetic tree reconstruction based on Neighbor Joining (Merget *et al.*, 2012).

Reconstruction phylogenetic tree

The phylogenetic tree of *S. samarangense* cultivars was reconstructed and compared with sequences from NCBI using the MEGA 11 software with the Maximum Likelihood method with 1000 bootstrap and Jukes and Cantor, 1969;

Tamura *et al*., 2021; Felsenstein et al., 1985.

RESULT AND DISCUSSION

Environmental Parameters at *S. samarangense* Cultivation

Environmental parameters at the cultivation site of *S. samarangense* shown no significant differences in elevation (Table 1). The soils of the three cultivation location villages had a pH that tends to be neutral with almost the same soil temperature, air temperature, and relative humidity. However, in Wonosari Village, the light intensity tend to be low due to the dense area of *S. samarangense* cultivation. Joko (2014) revealed that the *S. samarangense* plant has considerable adaptability in the tropics, both in the lowlands and in the land with an altitude of 1,000 m above sea level. This plant can grow well with humidity around 50 to 80%. The ideal soil pH for growth is 5.5 to 7.5 and is very suitable for growing on flat ground. Therefore, the environmental characteristics in Wonosari, Botorejo, and Tempuran Village in Demak Regency were suitable for *S. samarangense* growth and development.

Table 1. Environmental Parameters of *S. samarangense* in Demak Regency

Environmental Parameters	Location		
	Wonosari Village	Tempuran Village	Botorejo Village
Air Temperature (°C)	36±1,47[a]	34±0,05[a]	34±0,50[a]
pH	7.0±0[a]	7.0±0[a]	7±0,28[a]
Light Intensity (Cd)	63±24,1[a]	93±56,5[a]	394±69,0[a]
Soil Temperature (°C)	31±1,73[a]	29±1,73[a]	32±2,30[a]
Height (mdpl)	99±1,15[a]	97±0[a]	95±0,57[a]
Humidity	57+4,61[a]	65±3,78[a]	50±0[a]

a: there is no statistically significant difference at the 95% confidence level

Based on statistical analysis using OneWay ANOVA test, it was necessary to pay attention to the p-value (sig). If the p-value (sig) is greater than α (0.05) it indicated that each group is homogeneous. The three *S. samarangense* cultivation locations in Demak Regency had a sig value as 0.535, which mean greater than α (0.05). Therefore, there were no differences in environmental parameters from Botorejo, Wonosari, and Tempuran Village.

ITS2 *S. samarangense* Amplification

The four samples of *S. samarangense* from Demak Regency were successfully amplified using ITS2 with a target size of around 400 bp.

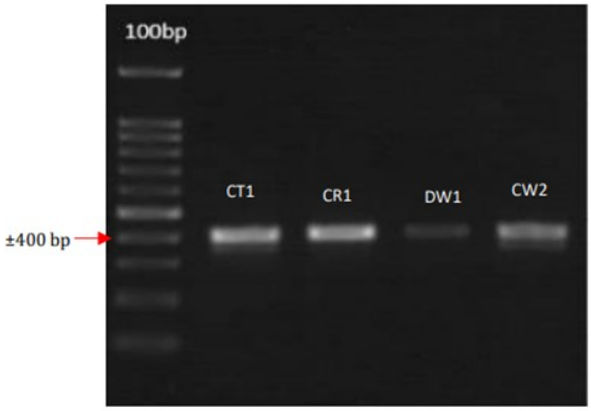

Figure 3. Electrophoretic visualization of PCR product samples of *S. samarangense* cultivars. Citra Tempuran (CT1), Citra Botorejo (CR1), Citra Wonosari (CW2), and Delima Wonosari (DW1)

The amplification results of *S. samarangense* cultivars included Citra Tempuran (CT1), Citra Botorejo (CR1), Citra Wonosari (CW2), and Delima Wonosari (DW1) shown good results, namely having a single band that was large, thick and obvious (Figure 3). Cheng et al (2016) primer pairs used in this study were degenerate ITS2 (ITS u-3 and ITS u-4). This primer pair could be used to

amplify the ITS2 region of the *S. samarangense* cultivars. The DNA barcode had been successfully amplified. The visualized DNA had been appropriate the ITS2 target, which was around 400 bp. According to Chen et al, (2010), the ITS2 region has a fairly short size, which is around 160-400 bp.

NCBI GenBank Accession Number Registration

All of *S. samarangense* sample sequences from Demak Regency had been submitted in NCBI GenBank and managed to obtain accession numbers. The four samples had been registered with details according to the ITS2 gene organization, namely 5.8S ribosomal RNA gene (partial sequence); internal transcribed spacer 2 (complete sequence), and large subunit ribosomal RNA gene (partial sequence). List of accession numbers for the four samples of *S. samarangense* from Demak Regency had been presented in Table 2.

Table 2. List of Accession Numbers for the Four Samples of *S. samarangense* from Demak Regency

***S. samarangense* Cultivar**	**Location**	**Sampling Code**	**Accesion Number**
Citra	Tempuran	CT1	OQ581467
Citra	Botorejo	CR1	OQ581466
Citra	Wonosari	CW2	OQ581468
Delima	Wonosari	DW1	OQ581469

Genetic Variation of DNA Barcode ITS2 *S. samarangense* Cultivars

All of *S. samarangense* cultivar samples from Demak Regency and the reference sequences from NCBI were characterized to determine the nucleotide composition of each sequence. The sequences of *S. samarangense* cultivars and referenced sequences from the BLAST results had different percentages of nucleotide composition. The nucleotide composition of these sequences had been presented in Table 3. The most abundant nucleotide was deoxycytidylic acid (C). This is in line with the research by Meitha *et al*. (2020) which explained that the nucleotide composition of the ITS2 region sequence contains the most deoxycytidylic acid (C) and deoxyguanylic acid (G) nucleotides.

Table 3. Percentage composition of the nucleotide sequences of the genus *S. samarangense* from Demak Regency

No.	**Sequence**	**Nucleotide Composition (%)**				**Total (bp)**
		T	**C**	**A**	**G**	
1.	OQ581469 *Syzygium samarangense* cultivar Delima Wonosari	23.0	27.8	21.7	27.3	397
2.	OQ581468 *Syzygium samarangense* cultivar Citra Wonosari	23.0	27.8	21.4	27.6	399
3.	OQ581466 *Syzygium samarangense* cultivar Citra Botorejo	22.5	28.2	21.5	27.6	396
4.	OQ581467 S*yzygium samarangense* cultivar Citra Tempuran	22.3	28.3	21.5	27.7	403

All of *S. samarangense* samples from Demak Regency, one *S. samarangense* sequence from NCBI namely the Nongke cultivar, and *S. aqueum* were selected to represent all the data and analyzed for genetic variation. Data on nucleotide variations in the ITS2 barcode DNA gene organization had been presented in Table 4.

Table 4. Nucleotide Variation in ITS2 Barcode DNA Gene Organization

Sequence	Nucleotide Composition												
	5.8S				ITS2								26S
	54	67	69	133	146	152	171	188	238	262	264	273	341
KC815987.1 *S. samarangense* cult. Nongke	G	C	T	G	Y	T	C	T	A	C	T	Y	C
OQ581467 *S. samarangense* cult. Citra Tempuran	.	.	.	.	.	.	.	C	.	.	.	.	.
OQ581466 *S. samarangense* cult. Citra Botorejo	.	.	.	.	.	.	.	C	.	.	.	.	Y
OQ581469 *S. samarangense* cult. Delima Wonosari	S	.	.	.	.	.	.	C	.	.	.	.	.
OQ581468 *S. samarangense* cult. Citra Wonosari	.	.	.	.	.	.	.	C	.	.	.	.	.
OK052940.1 *S. aqueum*	.	.	.	.	T	.	.	.	.	.	.	C	.

Table 4. described the nucleotide variations of the 5.8S, ITS2, and 26S regions in *S. samarangense* and *Syzygium aqueum*. It could be seen that ITS2 DNA barcodes revealed low intraspecific variation and had interspecific diversity. Intraspecific variation, namely among the four samples of *S. samarangense* from Demak Regency in the ITS2 region, only had one nucleotide variation, namely C188T. The 5.8S region had one variation, namely S54G in the *S. samarangense* cultivar DelimaWonosari sequence, while the 26S region had one variation, namely Y341C. The four *S. samarangense* samples from Demak Regency and referenced sequences had high nucleotide variations in the ITS2 region when compared to the 5.8S and 26S regions. The ITS2 region of *S. samarangense* cultivars were highly conserved. This was in line with the research of Peltier *et al.*, (2017) which stated that the ITS2 region is an area that is highly conserved intraspecifically. The ITS2 sequence has high intraspecific homogeneity and interspecific variability so it can be used as a marker in the process of identifying closely related species (Wilkerson *et al.*, 2014). Duan *et al.*, (2019) which stated that ITS2 has interspecific diversity and low intraspecific variation in *Rehmannia* identification. The study also explained that the combination of ITS2+psbA-trnH DNA barcodes showed good results in the process of classification and identification of *Rehmannia* species.

The unique genetic variation found in the four samples of *S. samarangense* from the Demak Regency were the presence of a double peak. The presence of the double peak represented two different nucleotides (Figure 4.).

Figure 4. Genetic variation in the form of double peaks in the four samples of *S. samarangense* from Demak Regency (red: deoxythymidylic acid, green: deoxyadenylyc acid, black: deoxyguanylic acid, blue: deoxycytidylic acid)

The identified double peaks included the symbol 'Y' in all four samples and the symbol 'S' only found in the *S. samarangense* cultivar Delima Wonosari. One type of genetic variation found in the four samples of *S. samarangense* from Demak Regency was heterogeneous (double peak). Heterogeneous, namely the presence of two peaks (double peak) with different nucleotides in the same place. Heterogeneous was the variation in nuclear sequences within individual plants that had been reported in many species (Feng *et al.*, 2016). The heterogeneous found in the four samples of *S. samarangense* from Demak Regency is coded 'Y' which represents deoxycytidylic acid (C) and deoxythymidylic acid (T) nucleotides. *S. samarangense* cultivar Delima Wonosari had a variation with the code 'S' which represents deoxycytidylic acid (C) and deoxyguanylic acid (G) nucleotides. Code 'Y' was found in the four samples of *S. samarangense* at nucleotide numbers 147 and 268. In the Citra Tempuran sample, a variation with code 'Y' was found at nucleotide number 273.

Prediction of ITS2 Secondary Structure

Internal Transcribed Spacer 2 sequence in this study had been annotated and could be seen in Figure 5., Figure 6., and Figure 7. ITS2 sequence result of *S. samarangense* cultivars had successfully been annotated which consisted of 5.8 S motif, ITS 2 and 28S.

Figure 5. Annotation of ITS2 Sequeunce of *S. samarangense* Cultivars
a: Citra Botorejo; b: Citra Tempuran; c: Citra Wonosari; d: Delima Wonosari

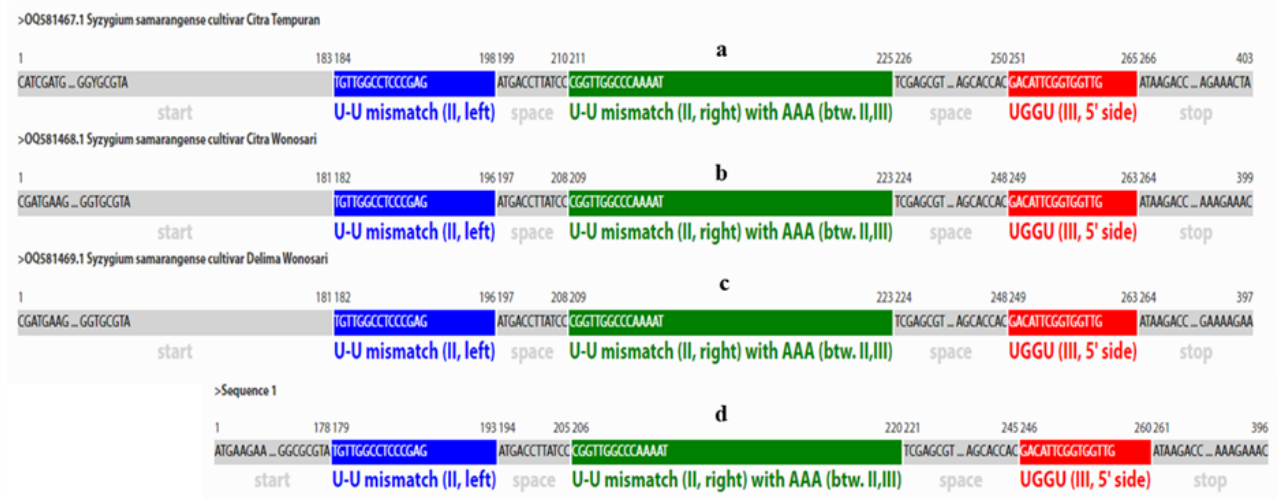

Figure 6. ITS2 Secondary Structure Motif Prediction of *S. samarangense* Cultivars
a: Citra Tempuran; b: Citra Wonosari; c: Delima Wonosari; d: Citra Botorejo

Furthermore, the prediction of secondary structure of ITS2 region in *S. samarangense* cultivars from Demak Regency shown at Figure 7. It consisted of four conserved motifs of U-U mismatch in between helix I and II, and U-U mismatch between Helix II and III, and UGGU in helix III, 5' edge. *S. samarangense* cultivar Citra Wonosari and cultivar Delima Wonosari had the identical secondary structure of ITS2, arranged by three loops in helix I, three in helix II, five in helix III dan one in helix IV. However, this was noticeably difference with cultivar Citra Botorejo which consisted of only two loops in helix I and second loop in helix I of cultivar Citra Tempuran which bigger than all

samples. It was caused by insertion of one nucleotide in the second loop of helix I. Meitha *et al.* (2020) explained that the loop formation in the ITS2 secondary structure is effected by mismatch pairing of the nucleotide in the ITS2 RNA, after helices formatted. These mismatch pairings also found in the four specific and conserved motifs of ITS2 RNA, which were found in *S. samarangense* cultivars. Helix I to IV, is stated to be highly conserved in Eukaryotes (Schultz *et al.*, 2005). Helix III appears the longest helix among all helices and is special having a UGGU motif (TGGT sequences for DNA) in the 5' side. Helix II consists of a U-U mismatch motif in Eukaryotes (Schultz *et al.*, 2005) and having a pyrimidine-pyrimidine bulge (Coleman, 2003). Meanwhile, helix I and IV are less conserved than other helices, with helix IV being more rapidly developing helix in plants and algae (Coleman, 2003). These secondary structure will strengthen the phylogenetic analysis of *S. samarangense* cultivars.

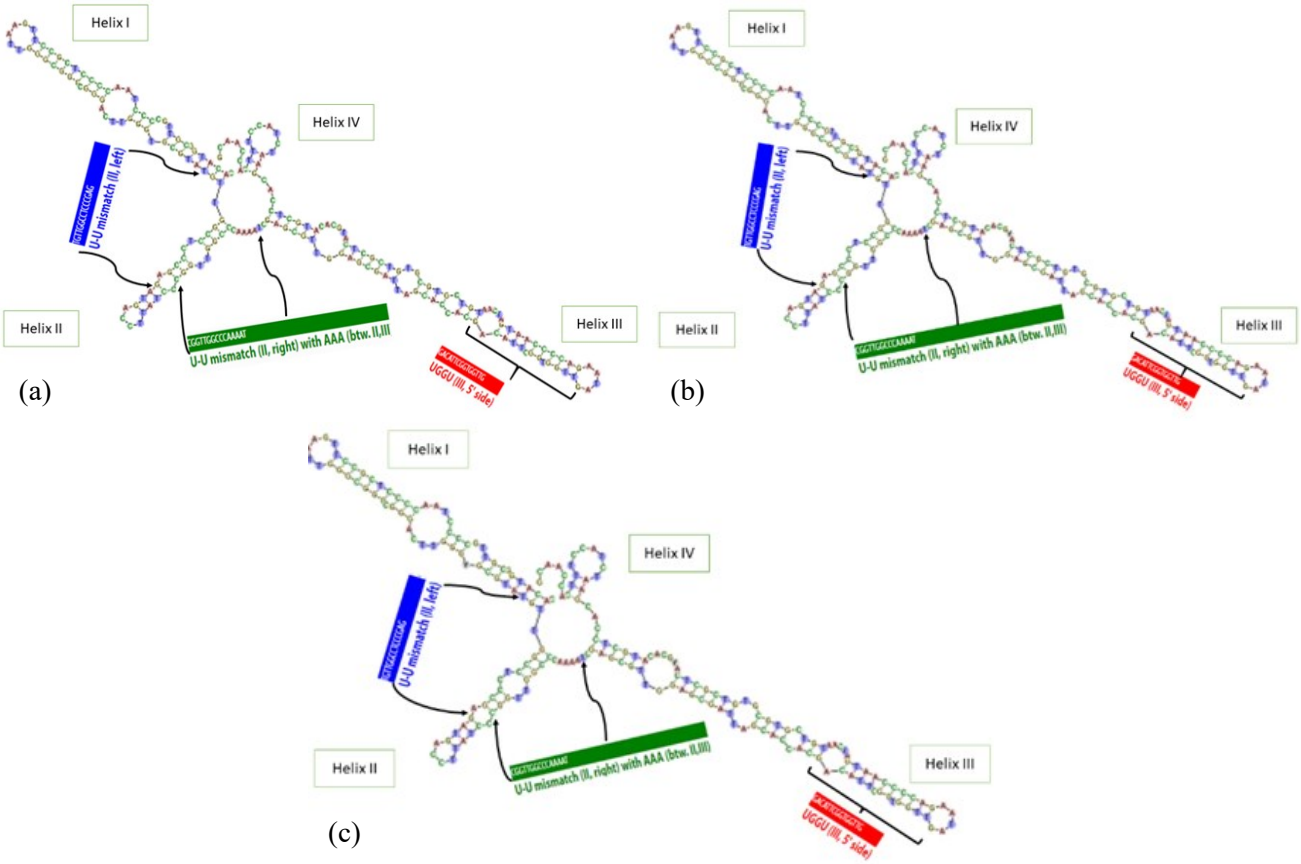

Figure 7. The prediction of secondary structure of ITS2 region of *S. samarangense* cultivars from Demak Regency. (a) *S. samarangense* cultivar Citra Wonosari and Delima Wonosari; (b) *S. samarangense* cultivar Citra Botorejo; (c) *S. samarangense* cultivar Citra Tempuran

Phylogenetic relationship of *S. samarangense* Cultivars

Based on the phylogenetic tree (Figure 8. and Figure 9.), the sequences of *S. samarangense* were separated according to the cultivar differences. Samples of *S. samarangense* cultivar Citra Botorejo are closely related to *S. samarangense* cultivar Citra Tempuran and *S. samarangense* cultivar Citra Wonosari. *S. samarangense* cutivar Delima Wonosari were separated into different branches because it was a different cultivar so it had nucleotide differences from the other *S. samarangense* Citra cultivars.

All samples of *S. samarangense* cultivars from Demak Regency is separated from the compared sequence because they have genetic variations at different nucleotide numbers. Sequences of *S. samarangense* with different cultivars will split into different branches. This indicates that the ITS2 DNA barcode could be used to analyze genetic variation at the cultivar level. This is in line with research by Meitha *et al*. (2020) who have successfully used ITS2 DNA barcodes to analyze genetic variation in 23 accessions of banana cultivars in Indonesia.

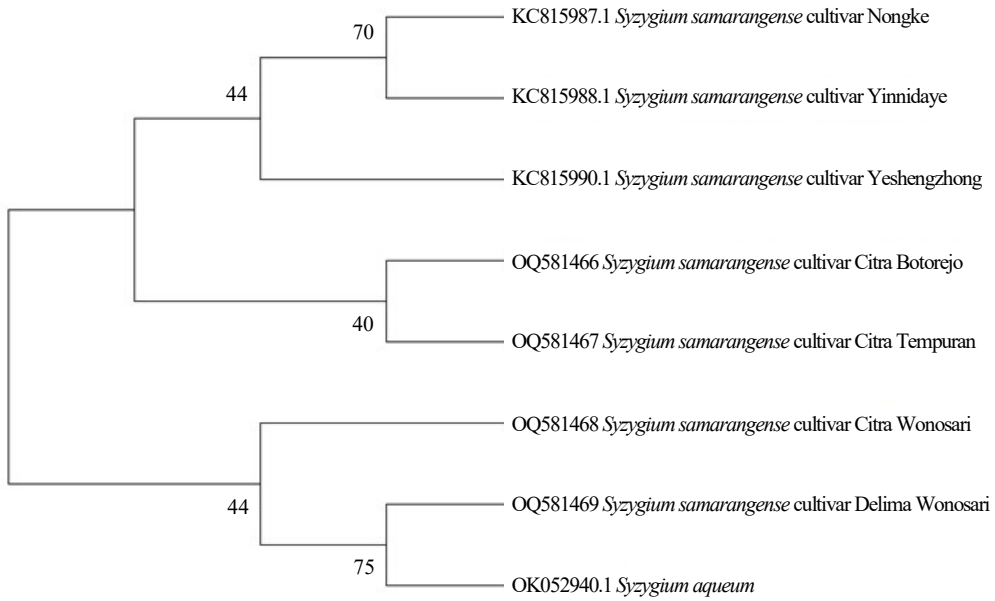

Figure 8. Reconstruction of a phylogenetic tree based on sequences using the Maximum Likelihood method and Jukes-Cantor Model using a bootstrap value of 1000 repetitions based on sequence

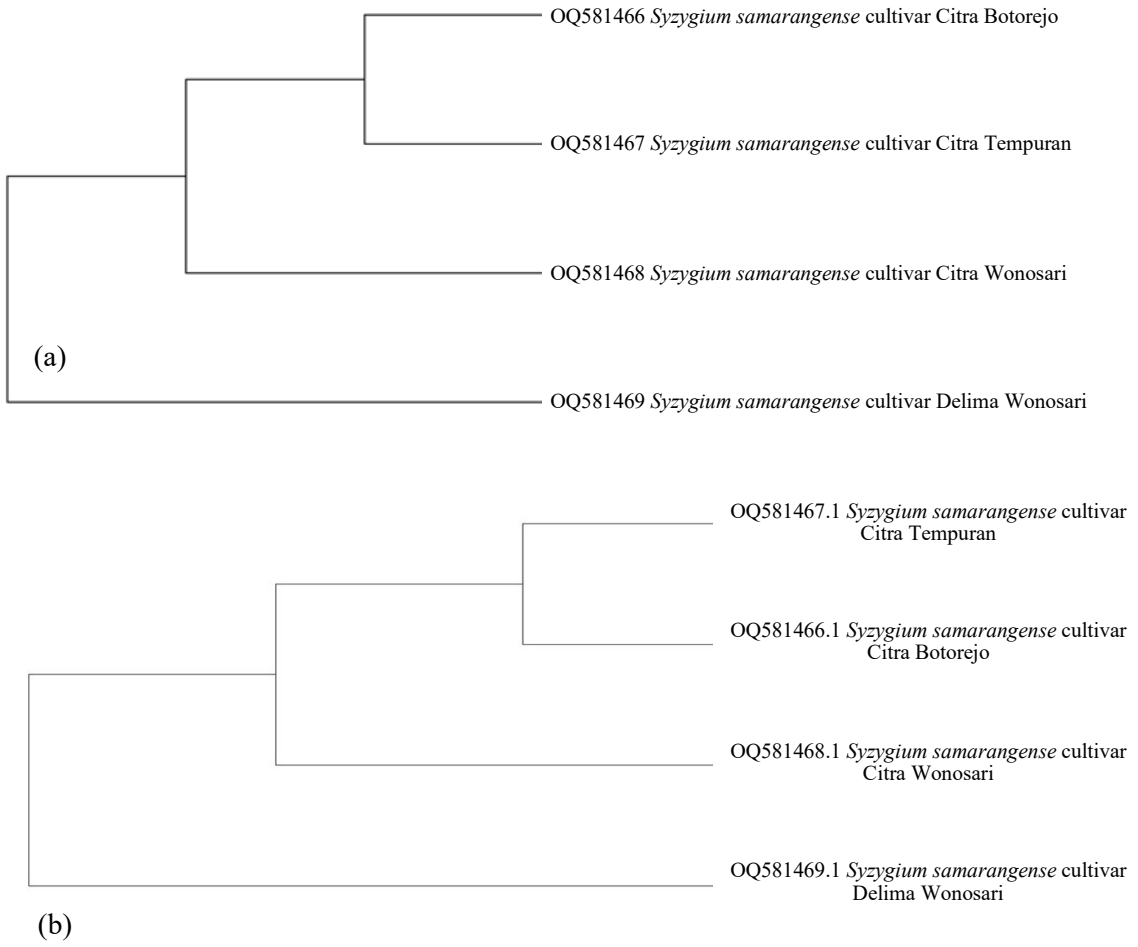

Figure 9. Reconstruction of phylogenetic trees *S. samarangense* cultivars from Demak Regency based on sequence of ITS2. (a) based on ITS2 sequence only; (b) based on ITS2 sequence and secondary structure

The phylogenetic results based on the ITS2 had been appropriate with the phenetic study based on morphological characters from a previous study by Mukaromah and Ulfah (2021) which stated that *S. samarangense* Citra and Delima cultivars from Demak Regency were grouped separately into two clusters. Therefore, the morphological characters of *S. samarangense* Citra and Delima cultivars were more influenced by cultivar types than by cultivation locations. According to Ananto (2022), who observed the morphology of the *S. samarangense* cultivar Citra from several villages in Demak Regency, namely Betokan Village, Jungpasir Village and Wonosari Village stated that there was no differences about qualitative characters such as the surface, color, texture of fruit, leaves, flowers, or stems. They differed in quantitative characters such as length, width, and weight but it was insignificant. Furthermore, the environmental parameters from Betokan Village, Jungpasir Village, and Wonosari Village are known to have no significant differences. In line with Rachmah, (2023), who made observations in Boyolali Village, Demak Regency shown that three cultivars of *S. samarangense* were found, including the *S. samarangense* cultivars such as Citra, Delima, and Madu Deli Hijau. There is morphological diversity in three *S. samarangense* cultivars. Morphological characterization of plants was carried out to determine the phenotypic characteristics displayed. This research was conducted at the same location so it has homogeneous environmental characteristics. The diversity obtained is influenced by the existence of cultivar differences. The differentiation of genetic composition cause appearance variations expressed in the phenotypes of each cultivar so that when their grown in the same environment, these cultivars will produce different phenotypic appearances (Sinay *et al.*, 2016).

Table 5. Genetic distance *S. samarangense* cultivars based on sequence of ITS2

No.	Species	1	2	3	4	5	6	7
1.	*S. samarangense* cultivar Citra Botorejo							
2.	*S. samarangense* cultivar Citra Tempuran	0.0000						
3.	*S. samarangense* cultivar Citra Wonosari	0.0028	0.0028					
4.	*S. samarangense* cultivar cultivar DelimaWonosari	0.0057	0.0057	0.0028				
5.	*S. samarangense* cultivar Nongke	0.0057	0.0057	0.0086	0.0115			
6.	*S. samarangense* cultivar Yinnidaye	0.0086	0.0086	0.0115	0.0014	0.0028		
7.	*S. samarangense* cultivar Yeshengzhong	0.0028	0.0028	0.0057	0.0086	0.0028	0.0057	
8.	*S. aqueum*	0.0086	0.0086	0.0057	0.0028	0.0086	0.0115	0.0057

The samples of *S. samarangense* cultivar Citra revealed low genetic distance and indicated that their samples were closely related to each other (Table 5.). Meanwhile, the *S. samarangense* cultivar Delima Wonosari had a higher genetic distance value toward other Citra cultivars because it was different cultivar. The differences of *S. samarangense* cultivars influenced by the genetic distance value and differentiation of cultivated location. Therefore, *S. samarangense* cultivar will be classified based on cultivar type.

CONCLUSION

There is a low intraspecies variation that is marked with few nucleotide variations between *S. samarangense* in the ITS2 area. *S. samarangense* cultivar Citra Wonosari and cultivar Delima Wonosari had the identical secondary structure of ITS2. However, this was slightly different between *S. samarangense* cultivar Citra Botorejo which consisted of only two loops in helix I and cultivar Citra Tempuran second loop in helix I which bigger than all samples. According to the phylogenetic tree, *S. samarangense* Delima and Citra cultivars separated by cultivar type. The genetic relationship of *S. samarangense* are more influenced by cultivar type rather than cultivated location. Therefore, DNA Barcode ITS2 can be utilized for genetic variation analysis of *S. samarangense* cultivars.

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