

Research article

Effectiveness, Sequence Characterization, and DNA QR Code of Internal Transcribed Spacer 1 (ITS1) in Identification of *Scopellaria marginata* (Cucurbitaceae)

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Abstract

Species identification accurately is crucial in plant sciences. DNA barcoding, which is an accurate approach for molecular identification that involves the use of short and standardized regions such as internal transcribed spacer (*ITS1*). To date, the availability of barcode sequences for *Scopellaria marginata* (Cucurbitaceae) is still very limited in public databases including *ITS1*. The purpose of this study was to investigate the effectiveness, to characterize, and to develop the DNA QR code of *ITS1* sequences in the identification of *Scopellaria marginata*. The DNA barcoding was carried out by DNA extraction from three individual *S. marginata*, DNA amplification with one pair barcode primer of *ITS1*, and bi-directional DNA sequencing using the Sanger's method. Nucleotide composition, phylogenetic tree construction, correct assignment rate (CAR), and DNA QR code were evaluated. The results showed that the *ITS1* region in *S. marginata* with the size of ~290 bp comprises nucleotide composition A, T, G, C, G+C, and A+T content of 23.0%, 17.7%, 26.3%, 33.1%, 59.3%, and 40.7%, respectively. The homology analysis using BLAST and phylogenetic tree reconstruction showed that all samples were identified as *S. marginata*. The effectiveness of *ITS1* as a DNA barcode in the identification of *S. marginata* showed 100% as the correct assignment rate value. A region of 218 bp was proposed as a specific DNA barcode for *S. marginata* and also converted as a DNA QR code. Therefore, the *ITS1* was shown as an effective barcode for the identification of *S. marginata*.

Keywords: DNA barcoding; DNA QR code; *ITS1*; *Scopellaria marginata*

1. Introduction

Indonesia is one of the countries in the world that has huge biodiversity. Biodiversity consists of a wealth of living creatures, both animals and plants. Family Cucurbitaceae comprises 120 genera and more than 1000 species with wide distribution in the world, especially in tropical regions (Simpson, 2019; Ho & Nguyen, 2020). One member of the

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genus from the Cucurbitaceae is *Scopellaria*, which consists of two species, namely *S. diversifolia* and *S. marginata* which has two varieties, i.e. var. *marginata* and var. *penangense*. *Scopellaria* are widely distributed in Asia and can be found in Myanmar, China, Thailand, Laos, Cambodia, Vietnam, Peninsular Malaysia, Sumatra, Borneo (Sabah), West Java, Philippines, and Sulawesi (de Wilde & Duyfjes, 2006). However, to date, *Scopellaria marginata* on Java Island is only reported to be found in the West Java province (Padalarang, Bandung; Purwakarta; and Bogor), while its existence in other areas in Java Island has never been reported (de Wilde & Duyfjes, 2006). Recently, the expected species of *S. marginata* has been found in another province in Java Island, Indonesia. Arumingtyas et al. (2023) reported Malang, East Java province as new distribution area for *S. marginata* which was identified based on morphological characters such as stem traits, leaf type, fruit shape, and seed length. Furthermore, molecular evidence using the *trnL* intron and *trnF-trnL* intergenic spacer regions (Turhadi et al., 2024) also supported that finding.

To strengthen the findings on a species based on morphological evidence, molecular data is needed. Molecular identification approaches can be done using the DNA barcoding method as a powerful tool in species identification. As a short and standard region of DNA, the DNA barcode is a powerful molecular-based tool to identify an organism. There are various regions in nuclear and chloroplast genome of plant which are characterized as DNA barcode, such as *ITS*, *rbcl*, *trnH-psbA*, *rpoB*, *rpoC1*, *trnL-trnF*, *psbK-psbI*, dan *atpF-atpH* (Letsiou et al., 2024). However, there is no universal region that can be used as a DNA barcode in plants (Hollingsworth et al., 2011). The advantages of using the DNA barcoding method include high stability and reproducibility, applicability at all stages of plant development, and lower environmental influence than morphological characterization (Trivedi et al., 2020). To date, the *ITS1* barcode DNA sequence has not yet been available in publicly accesible database. Thus, such sequence is provided in this study.

The *ITS1* is one of the nuclear DNA which is widely used in DNA barcoding, especially in species identification. This gene belongs to ribosomal DNA which plays a role in coding the structural components of ribosomes that function in the process of protein synthesis. The *ITS1* is located between the 18S and 5.8 rRNA (Ahmad & Asif, 2023). The *ITS1* has been applied to many plants to support their species identification. Several studies used *ITS1* for species identification on Cucurbitaceae (Hidayat et al., 2020; Turhadi et al., 2025), Orchidaceae (Rohimah et al., 2020), Araceae (Aprilianingsih et al., 2022), Nepentheceae (Saidon et al., 2022), Rubiaceae (Pham et al., 2022), Poaceae (Omelchenko et al., 2022), Balanophoraceae (López-Barrera et al., 2023), and Solanaceae (Dhabal et al., 2024). Furthermore, GenBank has available sequences of molecular markers used to identify *S. marginata* and the data are published by Turhadi et al. (2024). Moreover, de Boer et al. (2015) used *ITS1* sequences in phylogenic analysis of Cucurbitaceae, among them is *S. marginata*.

Hence, the data availability of DNA barcode for *S. marginata* from Malang, East Java is important to characterize and confirm the expected species of *S. marginata*. The aims of this study were to investigate the effectiveness, to characterize, and to develop the DNA QR code of *ITS1* sequences in identification of *S. marginata*. The findings can be valuable to support further utilization of *S. marginata* as a species that is still rarely studied and also as valuable information for species identification in its relatives.

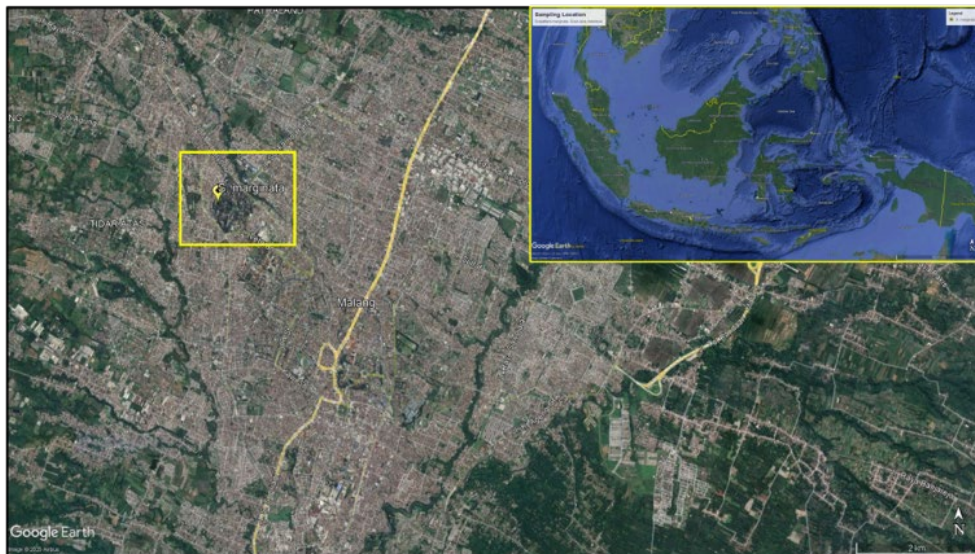
2. Materials and Methods

2.1 Genetic materials

The genetic material used in this research was three individuals of *S. marginata* originated from open area of Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java, Indonesia (S = 7°57'7.01420"; E = 112°36'41.29880") (Figure 1A-B) with elevation of 501 m above sea level.



(A)



(B)

Figure 1. *Scopellaria marginata* sample collected from Malang, East Java, Indonesia (A) and maps showing the sample collection point (B)

2.2 DNA extraction

A 40 mg fresh leaves of *S. marginata* were extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer extraction protocols. The total DNA genome was examined by quantitative and qualitative tests. Quantitative tests were carried out using a NanoDrop® Spectrophotometer ND-1000 (Peglab Ltd., United Kingdom) at wavelengths of 260 and 280 nm to measure the concentration and purity of DNA samples. Qualitative tests were conducted using gel electrophoresis with 0.8% agarose and run with TBE 1x buffer at 50 volts for 50 min.

2.3 DNA amplification and sequencing

DNA samples were amplified using primers (ITS1: TCCGTAGGTGAACCTGCGG; ITS2: GCTGCGTTCTTCATCGATGC) (Innis et al., 1990) with amplicon size ~290 bp. The final PCR mixture volume was 25 µL and consisted of 1 µL DNA template (100 ng/µL), primer forward and reverse (10 pmol) 0.5 µL respectively, 12.5 µL GoTaq® Master mixes (Promega, USA), and 10.5 µL nuclease-free water (Promega, USA). The program PCR was carried out for 35 cycles using the Takara PCR Thermal Cycles Dice Gradient (Takara Bio Inc., USA) with following conditions: pre-denaturation (95°C; 5 min), denaturation (95°C; 30 s), annealing (60°C; 1 min), extension (72°C; 1 min), and post-extension (72 °C; 5 min) (de Boer et al., 2015). Then, the PCR products were electrophoresed on a 1.5% agarose gel at 50 volts for 60 min and visualized using Gel Documentation, UV Transilluminator (Major ScienceCo., Ltd., USA). The PCR products were sent for bi-directional Sanger dideoxy sequencing at Macrogen, Singapore.

2.4 Data analysis

The data analyses were done using MEGA11 software. The chromatograms of the DNA sequencing results were edited to consensus sequences and then analyzed using BLAST (Basic Local Alignment Search Tool) to compare the query sequences with the GenBank sequences (Aprilianingsih et al., 2022; Saudah et al., 2022). The analysis of nucleotide composition to explain the percentage of A, T, G, and C nucleotides from each sample (Prasetya et al., 2020).

The construction of a phylogenetic tree was done using the Neighbor-Joining Tree method with 1000x bootstraps to construct the inferred phylogenetic based on the minimum evolution distance (Li, 2015; Perwitasari et al., 2020). The phylogenetic tree consists of 11 ingroup species i.e. three sequences of *S. marginata* samples, one sequence of *S. marginata* from GenBank, and seven sequences from the Benincaseae tribe, which have the highest similarity with the samples. Then, the outgroup species was *Cucurbita pepo* from the Cucurbitaceae tribe (Table 1).

The value of the correct assignment rate (CAR) defines the accuracy percentage of the DNA barcode to identify species appropriately (Loera-Sanchez et al., 2020). The *ITS1* sequence, recognized as potential barcode marker for *S. marginata*, was transformed into two-dimensional DNA barcode representations utilizing an open-source DNA Barcode tool (QR4Office) in Microsoft Excel 2021 and QR code generator (<https://biorad-ads.com/DNABarcodeWeb/>).

Table 1. The species and its GenBank accession number list of *ITS1* sequences used for phylogenetic analysis

Species	GenBank Accession Number	Note
<i>Benincasa hispida</i>	FJ980302.1	Ingroup
<i>Citrullus naudinianus</i>	KT757533.1	Ingroup
<i>Coccinia longicarpa</i>	HQ608212.1	Ingroup
<i>Dactyliandra welwitschii</i>	KU289684.1	Ingroup
<i>Lagenaria siceraria</i>	KM051438.1	Ingroup
<i>Urceodiscus scabridula</i>	LN896387.2	Ingroup
<i>Zehneria perpusilla</i>	KY523255.1	Ingroup
<i>Scopellaria marginata</i>	LN896367.2	Ingroup
Sm_UB1	PP278013.1	<i>This study</i>
Sm_UB2	PP278014.1	<i>This study</i>
Sm_UB3	PP278017.1	<i>This study</i>
<i>Cucurbita pepo</i>	KT347508.1	Outgroup

3. Results and Discussion

The genomic DNA of *S. marginata* samples were successfully extracted with various concentrations ranging from 497.71-730.97 ng/ μ L with high purity levels, as indicated by the $\text{A}_{260}/\text{A}_{280}$ value of 1.95-2.01. Those genomic DNA also successfully amplified the *ITS1* regions with clearly targeted bands and fragment sizes of ~290 bp (Figure 2A). Based on these results, it was indicated that *ITS1* showed PCR amplification success rate of 100% in the genomic DNA of *S. marginata*. Furthermore, the DNA sequencing also showed good results, as indicated by their chromatogram (data not shown). These results also showed a DNA sequencing success rate of 100% of the *ITS1* region in the genome of *S. marginata*.

Our study showed that amplification of the *ITS1* region was successful achieved in the *S. marginata* (Figure 2A-B) with good quality based on general criteria such as thick band, single band, and correct size (Arslan et al., 2021; Santosa et al., 2020). This result was in accordance with the entire *ITS1* sequence which ranges from 200-300 bp in plants (Ahmad & Asif, 2023). According to Trisyani & Rahayu (2020), smaller difference among the nucleotide bases indicates the smaller genetic variation among the samples. The G+C content of *ITS1* region in *S. marginata* was higher than the A+T content (Figure 2B). Our result was similar to that of Wang et al. (2015), who reported that G+C content in Dicot plants has an average of 56%.

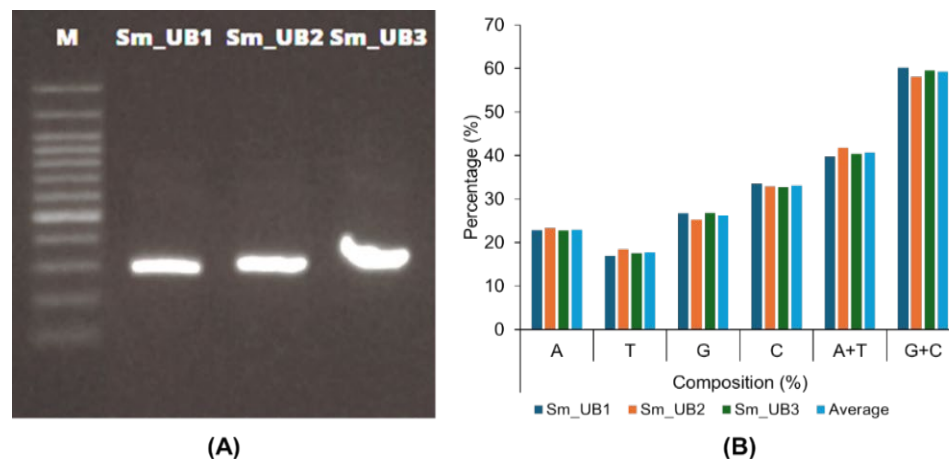


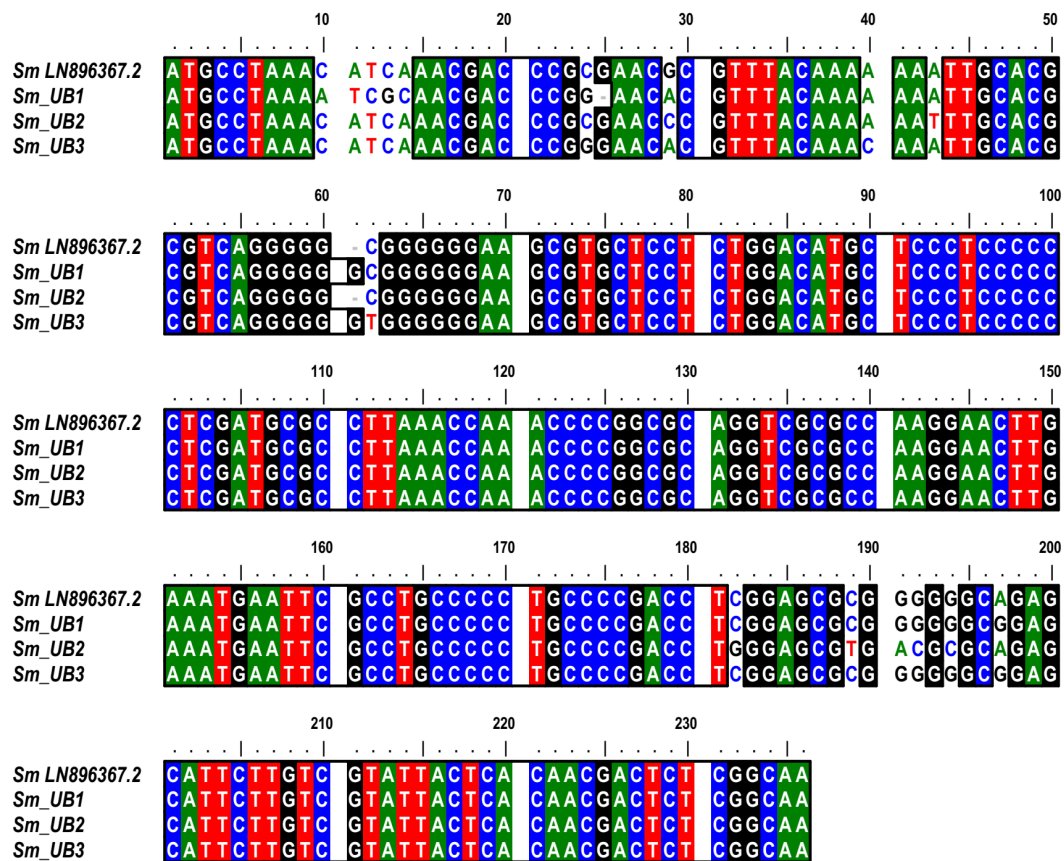
Figure 2. Electrophoregram of PCR products (A) and nucleotide composition (B) of *ITS1* in *S. marginata*. M = 100 bp DNA ladder. T=Thymine; C=Cytosine; A=Adenine; G=Guanine

This study also elucidated the nucleotide composition of the *ITS1* region in *S. marginata*. The average nucleotide composition of A, T, G, and C of *ITS1* region in *S. marginata* was 23.0%, 17.7%, 26.3%, and 33.1%, respectively (Figure 2B). In addition, the G+C content and A+T content of *ITS1* region in *S. marginata* was 59.3% and 40.7%, respectively (Figure 2B). The homology analysis using BLAST NCBI showed that all of our samples exhibited similar percentage identity in the range of 97.06-97.35% compared to *S. marginata* (Accession no. LN896367.2) originating from Thailand (Table 2). In our bioinformatics analysis using NCBI-BLAST homology analysis, the query cover of all our samples was 100% (Table 2) and the multiple sequence alignment result (Figure 3) further confirmed the high level of homology value between our samples and the sequences in the GenBank NCBI database, *S. marginata* originating from Thailand. The high degree of homology between *S. marginata* from East Java, Indonesia and Thailand was likely due to the similar ecological conditions of those two areas. Moreover, the high level of homology might be due to the limited distribution area of *S. marginata* (de Wilde & Duyfjes, 2006). Our result, also supported by the phylogenetic tree, showed all the samples in the same clade with *S. marginata* LN896367.2 with a high confidence level of 99% (Figure 4A) and showed monophyletic with other Benincaseae tribe members. The lower E-values (Table 2) also indicated that our samples were more identical to the *S. marginata* sequence in GenBank NCBI database (Mukhopadhyay et al., 2018, Ilham et al., 2022). A high bootstrap value reflects a high level of confidence in the branching of a phylogenetic tree (Stevanus & Pharmawati, 2021) and this result is shown in our study with bootstrap value of 99%. High bootstrap values were also shown by phylogenetic trees based on the *ITS* region to identify *Eugenia* spp. (Chika & Zahro 2024). Overall, our results indicated that the *ITS1* barcode was able to determine the sample correctly and group the same species in the same clade.

In addition, the *ITS1* demonstrated a correct assignment rate value of 100% to identify species, genus, and family of *S. marginata* (Figure 4B). Furthermore, The *ITS1* region with length of 218 bp is proposed as specific DNA barcode for *S. marginata* (Figure 4C) and this specific DNA barcode was subsequently converted into a DNA QR code (Figure 4D).

Table 2. BLAST analysis of *S. marginata*

Sample	GenBank / BLAST	Accession Number	Query Cover (%)	E-value	% Identity
Sm_UB1	<i>S. marginata</i>	LN896367.2	100	4e-106	97.06
Sm_UB2	<i>S. marginata</i>	LN896367.2	100	3e-113	97.35
Sm_UB3	<i>S. marginata</i>	LN896367.2	100	3e-113	97.20

**Figure 3.** Multiple sequence alignment between *ITS1* region in *S. marginata* LN896367.2 (as reference sequence) and *S. marginata* (Sm_UB1, Sm_UB2, Sm_UB3) originating from East Java, Indonesia

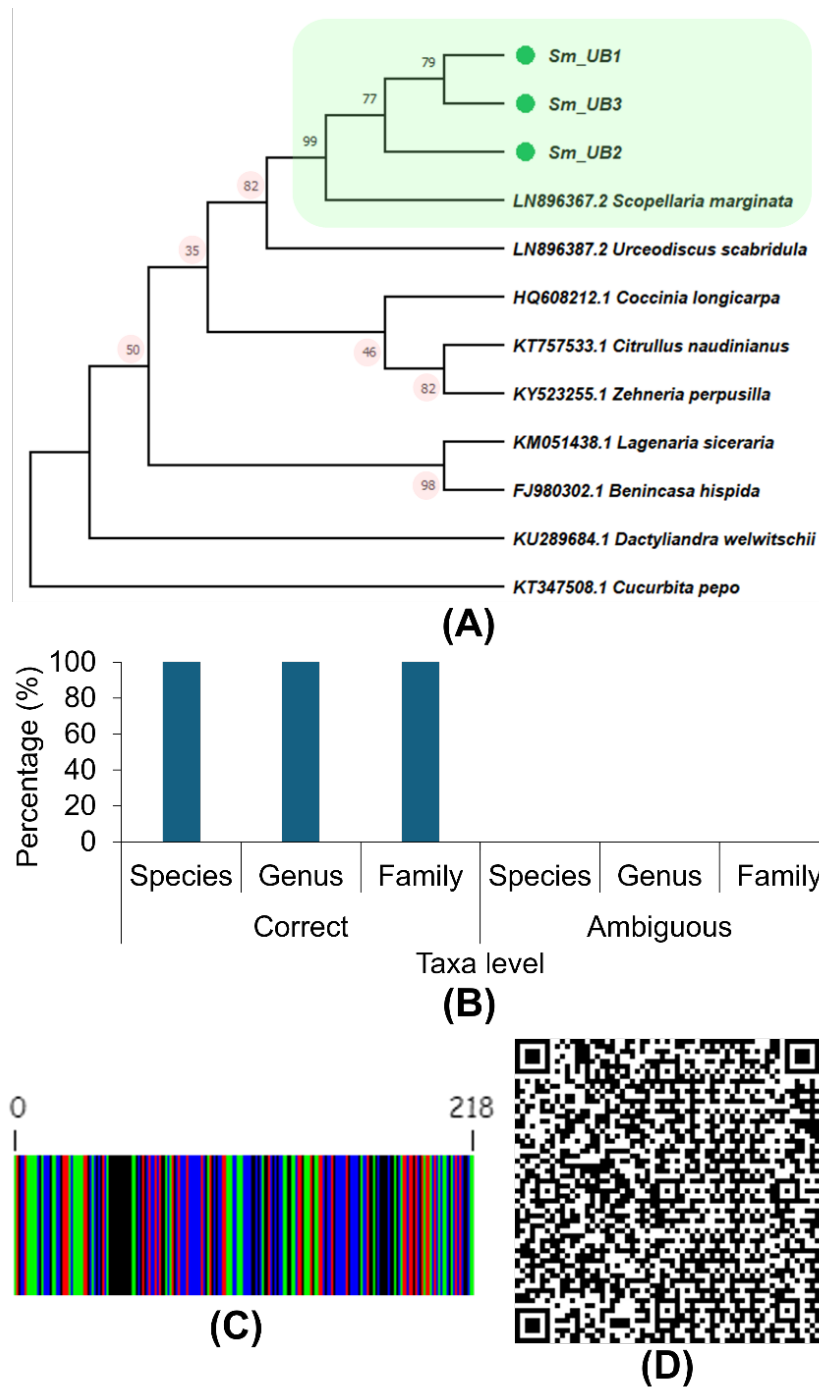


Figure 4. Phylogenetic tree (A), correct assignment rate (CAR) value (B), two-dimensional specific DNA barcode (C), and DNA QR code (D) of *S. marginata*. Green, red, black, and blue represent the A, T, G, and C bases, respectively.

Identification accuracy is a crucial parameter in DNA barcoding. In this study, the identification accuracy is shown by correct assignment rate (CAR) to the species, genus, and family level (Figure 4B). The CAR represents the percentage accuracy of a DNA barcode in correctly identifying a sample. This value is obtained from the ratio of the number of samples correctly identified per the number of samples analyzed. Therefore, a high CAR value illustrates that the DNA barcode can identify species accurately (Loera-Sanchez et al., 2020). Our results based on CAR value were in line with those of previous studies. According to Wang et al. (2022), *ITS1* showed 100% of CAR value in the identity of the Gramineae family, which consisted of the genera *Agropyron*, *Bromus*, *Elymus*, *Elytrigia*, *Festuca*, *Leymus*, and *Lolium*. In addition, *ITS1* also showed a high CAR value in identifying the genus of *Cycas* and *Macrozamia* (Li et al., 2015).

In order to make specific DNA barcode easy to access by public, a DNA QR code was produced in this study. The DNA QR code can be scanned by using a mobile phone equipped with a scanner QR code application. As an easy, effective, and accurate method, this DNA QR code also was proposed as an authentication tool and genetic label for *S. marginata*. In addition, the DNA QR code from our recent study was the general identity for *S. marginata*. This method was also previously used for plant species identification and also plant-based product authentication (Lv et al. 2020; Li et al. 2021; Jiang et al. 2022; Yao et al. 2022; Ma et al. 2017).

4. Conclusions

The *ITS1* showed an effective region for the identification of *S. marginata*. The *ITS1* region in *S. marginata* with size of ~290 bp showed a nucleotide composition of A, T, G, C, G+C, and A+T content, which were 23.0%, 17.7%, 26.3%, 33.1%, 59.3%, and 40.7%, respectively. The homology analysis using BLAST, phylogenetic tree reconstruction, and correct assignment rate on species, genus, and family level indicated that all of our samples confirmed as *S. marginata*. Furthermore, a specific DNA barcode for *S. marginata* based on *ITS1* region with length of 218 bp already converted into DNA QR code.

5. Acknowledgements

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6. Authors' Contributions

Briliyan Natalina Sudarjayanti: performed the experiment, data analysis and interpretation, writing - original draft; Brian Rahardi: Project administration, review and editing; Estri Laras Arumingtyas: Project administration, supervision, review and editing; Turhadi Turhadi: Conceptualization, principal investigator, supervision, data interpretation and visualization, and writing - review and editing.

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7. Conflicts of Interest

The authors declare that there are no conflicts of interest related to this article

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