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Molecular identification of *Scopellaria marginata* from East Java, Indonesia, based on *trn*L-UAA and *trn*L-*trn*F intergenic spacer regions

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Abstract. *Turhadi, Sudarjayanti BN, Solihah FM, Azrianingsih R, Afiyanti M, Arumingtyas EL. 2024. Molecular identification of* Scopellaria marginata *from East Java, Indonesia, based on* trn*L-UAA and* trn*L-*trn*F intergenic spacer regions. Nusantara Bioscience 16: 111-118. Scopellaria marginata* (Blume) W.J.de Wilde & Duyfjes is a wild species in Cucurbitaceae, which was recorded as new expanding their distribution in East Java, Indonesia. The *trn*L-UAA and *trn*L-*trn*F intergenic spacer (IGS) sequences of *Scopellaria* are still limited in publicly accessible databases. This study aimed to evaluate the *trn*L-UAA and *trn*L-*trn*F IGS sequences of *S. marginata* from Malang, East Java. Total DNA of *S. marginata* was used to amplify the *trn*L-UAA and *trn*L-*trn*F IGS region. Then, the PCR products are sequenced using bi-directional Sanger dideoxy sequencing to obtain the DNA sequence of those two regions. The results showed that the partial sequences of *S. marginata* for *trn*L-UAA ranged from 528 to 571 bp, while the sequence for *trn*F-*trn*L IGS ranged from 434 to 445 bp. The *S. marginata* samples are similar to *S. marginata* in the database with similarity levels of 97.14-97.50% and 98.36-98.61%, respectively, based on the *trn*L-UAA and *trn*L-*trn*F IGS. Both *trn*L-UAA and *trn*L-*trn*F IGS showed a Correct Assignment Rate (CAR) of 100% for *S. marginata*. Two-dimensional DNA barcoding with lengths 505 and 417 bp for *trn*L-UAA and *trn*L-*trn*F IGS proposed as specific barcodes for *S. marginata*. These results prove that Malang, East Java was an additional distribution area for *S. marginata* in Indonesia.

Keywords: Cucurbitaceae, DNA barcoding, *Scopellaria marginata*, *trn*L-*trn*F intergenic spacer, *trn*L-UAA intron

INTRODUCTION

Cucurbitaceae is a plant family with 101 genera and about 1000 species with a wide distribution, especially in tropical regions (Simpson 2019; POWO 2024) and a group with diverse economic use. As a member of Cucurbitaceae, *Scopellaria* consists of two species, namely *S. diversiflora* and *S. marginata*. The *S. diversiflora* and *S marginata* are grow wild and not popular in the community. Only *S. marginata* is found in Indonesia because of its wide distribution, while *S. diversifolia* is only found in the central and eastern parts of Borneo (Sabah). According to de Wilde and Duyfjes (2010), *Timun tikus* (*S. marginata*) is divided into two varieties, including *S. marginata* var. *marginata* and var. *penangense* which is differentiated based on the characteristics of their leaf blade, leaf base, and seed size. A previous study showed the presence of *S. marginata* in Malang, East Java, Indonesia, based on the morphological characterization (Arumingtyas et al. 2023).

As a expanding their distribution, *S. marginata* must be characterized molecularly to strengthen evidence that it matches the DNA barcodes in the database. The availability of DNA sequences helps the morphological characterization approach to identify a species. Providing a DNA barcode for *S. marginata* is very important because the morphological characters, especially during the early vegetative stage are similar between several species of the Cucurbitaceae members, such as cucumber, melon, etc. Several characters are very variable and do not correlate with phylogenetic relationships between species of Cucurbitaceae based on chloroplast-based DNA barcode sequences, including petals, fruit characters, and karyotypes (Kocyan et al. 2007). Moreover, morphological characters are also greatly influenced by environmental factors (Kwon et al. 2017; Nadeem et al. 2018), so it takes longer for identification to be carried out until the generative phase is achieved. Therefore, to resolve this problem, a molecular approach such as DNA barcoding can speed up species identification, such as Cucurbitaceae.

DNA barcoding is a molecular biology technique using short standardized sequences that help identify plant species and support conservation and further utilization strategies (Kress 2017). DNA barcodes can be quickly, accurately, and effectively used in species identification, when morphological characteristics difficult to determine the sample (Taberlet et al. 2007; Trivedi et al. 2020). Several types of gene loci are commonly used as plant DNA barcodes, for example, *trn*L-UAA and *trn*L-*trn*F intergenic spacer (IGS). The *trn*L is the chloroplasts genome with a very conservative secondary structure and widely used as a marker for plant phylogenetic analyses (Yulita 2013; Kishor and Sharma 2018). Furthermore, the number of *trn*L-UAA sequences available in databases is already very high, by far the most numerous among non-coding chloroplast DNA sequences (Taberlet et al. 2007). The *trn*L-UAA and *trn*L-*trn*F IGS have been effectively used to identify Cucurbitaceae (Kocyan et al. 2007; Schaefer and Nee 2012), *Taxus* (Taxaceae) (Coughlan et al.

2020), *Prunus* (Rosaceae) (Sevindik et al. 2020), and *Eurycoma longifolia* (Simaroubaceae) (Yulita et al. 2022). Furthermore, the availability of DNA sequences in publicly accessible databases is also crucial in species identification using DNA barcoding (Roslim 2018).

DNA barcode, especially for *Scopellaria* are still very limited in publicly accessible databases. This study aimed to evaluate the *trn*L-UAA and *trn*L-*trn*F IGS sequences from *S. marginata* from Malang, East Java, Indonesia. The availability of those DNA barcodes for *S. marginata* is useful as basic information in conservation strategies. This study is also useful as additional information on new expanding distribution of *S. marginata* in Indonesia, especially in East Java. Based on our findings, it opens up opportunities for further studies, especially exploring the potential of *S. marginata* which has not yet been widely reported.

MATERIALS AND METHODS

Sample collection

All plant material used in this research was wild *S. marginata* collected from Malang, East Java, Indonesia (7°57'7.01420" S; 112°36'41.29880" E) (Figure 1.A). The herbarium specimens (Figure 1.B) were identified by Turhadi and deposited at the Herbarium Universitas Brawijaya (MUBR), Laboratory of Plant Taxonomy, Structure, and Development, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java, Indonesia with collection number SM 08 (Sm_UB1), SM 09 (Sm_UB2), and SM 10 (Sm_UB3).

Procedures

Total DNA extraction

A total of 40 mg of fresh leaf tissues of *S. marginata* were used for total DNA extraction. The total DNA extraction using Wizard® Genomic DNA Purification Kit (Promega, USA) and followed the manufacturer's extraction protocol. The extracted DNA was checked for quality on gel electrophoresis with 0.8% agarose and run using 1X TBE (Tris-Borate EDTA) buffer (Promega, USA) at 100 V for 30 minutes. Subsequently, the extracted DNA was also checked for its concentration and purity level using a NanoPhotometer® NPOS 6.6c (Implen, Inc., USA) at the wavelength (λ) of 260 and 280 nm. The extracted DNA was diluted in TE buffer pH 8.0 (Promega, USA) and stored at -30°C for further analysis.

DNA amplification and sequencing

A Polymerase Chain Reaction (PCR) final volume of 25 µL was used in target region amplification. It consisted of the following components: 12.5 µL GoTaq® Green Master Mixes (Promega, USA), 0.5 µL each forward and reverse primers (10 pmol/µL), 10.5 µL nuclease-free water (Promega, USA), and 1 µL (100 ng/uL) genomic DNA. Amplification of the target region was carried out using the Takara PCR Thermal Cycles Dice Gradient (Takara Bio Inc., USA). The amplification of the target regions using two specific primer pairs which consisted of *trn*L-UAA and *trn*L-*trn*F IGS (Taberlet et al. 1991) (Table 1). The amplification process was carried out in 35 cycles with the following PCR program: pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 10 seconds, and post-extension at 72°C for 10 minutes. The electrophoresis of the PCR product was 80 V for 35 minutes using 1X TBE (Tris-Borate EDTA) buffer and then visualized on a 1% agarose gel and the nucleic acids stained using Diamond™ Nucleic Acid Dye (Promega, USA). Electrophoresis results were documented using a Gel Documentation tool, UV Transilluminator (Major Science Co. Ltd., USA). Subsequently, the PCR products obtained were used for bi-directional Sanger dideoxy sequencing using Genetic Analyzer 3730XL instrument (Thermo Fisher Scientific Inc, USA) at the Macrogen Company, Singapore.

Data analysis

Moreover, the sequence data of multiple individuals in a studied species is very important as it allows comparisons between sequences. The sequencing results obtained were prepared for further analysis using BioEdit Sequence Alignment Editor software ver.7.0.9.0. Then, each sequence was manually edited and verified by examining the sample's placement within the phylogenetic tree (de Vere et al. 2015). The DNA sequences were matched to the database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree construction using the Maximum Parsimony (MP) algorithm with 1000 replicates was carried out in MEGA X version 10.0.5 using the default parameters by comparing the *S. marginata* samples of this study with its relatives in the Cucurbitaceae family (Kumar et al. 2018) (Table 2). Additionally, *Begonia oxyloba* (Begoniaceae) was determined as the outgroup in the phylogenetic tree construction.

Table 1. Primers used in this study

Region	Sequence (5'→ 3')	Amplicon (bp)
*trn*L-UAA	CGAAATCGGTAGACGCTACG GGGGATAGAGGGACTTGAAC	~500
*trn*L-*trn*F IGS	GGTTCAAGTCCCTCTATCCC ATTTGAACTGGTGACACGAG	~400

Figure 1. A. Living *Scopellaria marginata* and B. Herbarium specimen

Table 2. The *trn*L-UAA and *trn*L-*trn*F IGS sequences used for analysis in this study

Region	Species	GenBank Accession Number	Note
*trn*L-UAA	*Coccinia grandiflora* Cogn.	HQ608407	Ingroup
	Citrullus lanatus (Thunb.) Matsum. & Nakai	DQ536761	Ingroup
	Zehneria bodinieri (H.Lév.) W.J.de Wilde & Duyfjes	KY523355	Ingroup
	Zehneria perpusilla (Blume) Bole & M.R.Almeida	KY523367	Ingroup
	Zehneria maysorensis (Wight & Arn.) Arn.	KY523373	Ingroup
	Trochomeriopsis diversifolia Cogn.	DQ536878	Ingroup
	Eureiandra formosa Hook.f.	DQ641905	Ingroup
	Citrullus rehmii De Winter	KP036545	Ingroup
	Citrullus colocynthis (L.) Schrad.	KY613619	Ingroup
	Raphidiocystis phyllocalyx C.Jeffrey & Keraudren	DQ536855	Ingroup
	Peponium caledonicum (Sond.) Engl.	DQ536774	Ingroup
	Blastania cerasiformis (Stocks) A.Meeuse	DQ536803	Ingroup
	Scopellaria marginata (Blume) W.J.de Wilde & Duyfjes	DQ536882	Ingroup
	Sm_UB1	OR703797	*This study*
	Sm_UB2	OR703798	*This study*
	Sm_UB3	OR703799	*This study*
	Begonia oxyloba Welw. ex Hook.f.	AY968563	Outgroup
*trn*L-*trn*F IGS	*Neoachmandra cunninghamii* (F.Muell.) W.J.de Wilde & Duyfjes	KY523360	Ingroup
	Bambekea racemosa Cogn.	DQ536788	Ingroup
	Zehneria guamensis (Merr.) Fosberg	KY523363	Ingroup
	Zehneria polycarpa (Cogn.) Keraudren	KY523381	Ingroup
	Seyrigia humbertii Keraudren	AY973010	Ingroup
	Dieterlea fusiformis (E.J.Lott)	KJ531878	Ingroup
	Ibervillea hypoleuca (Standl.) C.Jeffrey	DQ536829	Ingroup
	Scopellaria marginata (Blume) W.J.de Wilde & Duyfjes	DQ536882	Ingroup
	Ceratosanthes palmata (L.) Urb.	DQ536795	Ingroup
	Neoachmandra japonica (Thunb.) W.J.de Wilde & Duyfjes	DQ536884	Ingroup
	Sm_UB1	OR703800	*This study*
	Sm_UB2	OR703801	*This study*
	Sm_UB3	OR703802	*This study*
	Begonia oxyloba Welw. ex Hook.f.	AY968378	Outgroup

The nucleotide sequences of the candidate barcode markers for *S. marginata* were converted to two-dimensional DNA barcode images using an open-source DNA Barcode and QR code generator (Yu et al. 2016; Khan et al. 2017). Mobile terminals (such as Android and iPhone devices) can read the information as QR code scanners (Ma et al. 2017).

RESULTS AND DISCUSSION

The *trn*L-UAA and *trn*L-*trn*F intergenic spacer characteristics

The Polymerase Chain Reaction (PCR) technique successfully amplified the *trn*L-UAA and *trn*L-*trn*F IGS regions in all *S. marginata* samples with a single band (Figure 2). The PCR products of those two regions were ~500 bp and ~400 bp, respectively. Our results correspond with previous studies that the length of whole chloroplast *trn*L-UAA is ranged from 254 to 767 bp (Taberlet et al. 2007), and *trn*L-*trn*F IGS on various monocots and dicots groups are ranged from 206 to 756 bp (Tsai et al. 2006). DNA sequencing for the *trn*L-UAA and *trn*F-*trn*L IGS in *S. marginata* samples was also successfully carried out using bi-directional Sanger dideoxy sequencing. Furthermore, our results also showed clean calls of chromatograms for those two target regions (Figure 3). The good sequencing chromatogram indicates no overlapping peaks, indicating the potential simultaneous sequencing of two DNA molecules (Aguirre-Dugua et al. 2019). These profiles are also shown in our results.

Contig DNA sequences from pre-processing showed that the sequence length was relatively similar for surveyed samples. The sequence length for the *trn*L-UAA ranged from 528 to 571 bp, while the sequence length for the *trn*F-*trn*L IGS ranged from 434 to 445 bp. Similar results also showed in various studies, for instance the sequence length of *trn*L-UAA ranged from 582 to 602 bp in *Oxytropis* (Fabaceae) (Tekpinar et al. 2016), 525 to 528 bp in *Melothria domingensis* (Cucurbitaceae) and 527 bp in *Cionosicyos excisus* (Cucurbitaceae) (Schaefer and Nee 2012), 555 to 559 bp in *Nepenthes* (Nepenthaceae) (Bunawan et al. 2017), 464 to 465 bp in *Cinnamomum osmophloeum* (Hsu et al. 2019), 519 to 528 in *Laurus nobilis* (Lauraceae) (Sevindik and Okan 2020). While the *trn*L-*trn*F IGS in some previous studies showed 381 to 395 bp in *M. domingensis* (Cucurbitaceae) and 410 bp in *C. excisus* (Cucurbitaceae) (Schaefer and Nee 2012), 372 to 376 bp in *L. nobilis* (Lauraceae) (Sevindik and Okan 2020), 449 bp and 179 bp in *Pisum vera* (Fabaceae) and *Pisum*

sativum (Fabaceae), respectively (Sen et al. 2020), 455 bp in *Gossypium hirsutum* (Malvaceae) (Hocaoglu-Ozyigit et al. 2022), 444 to 473 bp in species of Gramineae (Wang et al. 2022), and 298 to 306 bp in *Salvia miltiorrhiza* (Labiate) (Feng et al. 2022).

The average nucleotide composition of *trn*L-UAA of *S. marginata* was 38.5% A, 28.3% T, 17.5% G, and 15.7% C. The highest A+T content (67.0%) and the lowest G+C content (33.0%) were observed in Sm_UB3, while the lowest A+T content (66.5%) and the highest G+C content (33.5%) were shown in Sm_UB1 (Table 3). While, the *trn*F-*trn*L IGS of *S. marginata* was 30.8% A, 32.3% T, 16.4% G, and 20.5% C. The highest A+T content (63.5%) and the lowest G+C content (36.5%) were observed in Sm_UB1, while the lowest A+T content (62.8%) and the highest G+C content (37.2%) were shown in Sm_UB3 (Table 3). These nucleotide profiles were similar to the previous study in *L. nobilis* (Lauraceae) (Sevindik and Okan 2020). The low proportion of G+C than A+T content in *trn*L-UAA is also found in various taxa, such as *Pistacia vera* (Sarra et al. 2015), *Citrus* (Rutaceae) (Sevindik and Yalçin 2018), and *Dittrichia viscosa* (Asteraceae) (Sevindik et al. 2023). Like *trn*L-UAA, the lower proporsion of G+C than A+T content also showed in *trn*F-*trn*L IGS of *S. marginata*. In *Pennisetum glaucum* (Poaceae) (Almutairi 2022) showed a lower proportion of G+C than A+T content. According to Ismail et al. (2020), higher A+T contents than G+C in a barcode indicate high nucleotide composition variability and higher nucleotide substitution rate in that region.

Identification of *Scopellaria marginata*

The matching results with the GenBank (NCBI) database using BLASTn showed that the samples were identified as *S. marginata* based on the *trn*L-UAA and *trn*F-*trn*L IGS with similarity level of 97.14-97.50% and 98.36-98.61% respectively (Table 4). Furthermore, our samples of *S. marginata* showed similar with *S. marginata* voucher code A. Kocyan AK187 (BKF), which originates from Thailand (Kocyan et al. 2007). A sample is a similar species if the similarity value >97% (Mukhopadhyay et al. 2018). There were 468 and 369 positions in the final dataset for phylogenetic tree construction based on *trn*L-UAA and *trn*L-*trn*F IGS, respectively. Construction of a phylogenetic tree based on both the *trn*L-UAA (Figure 4) and *trn*L-*trn*F IGS (Figure 5) shows that the three samples (Sm_UB1, Sm_UB2, and Sm_UB3) were in the same clade with *S. marginata*. This result indicated that the three specimens identified as *S. marginata*.

Table 3. Nucleotide composition of *trn*L-UAA and *trn*L-*trn*F IGS of *Scopellaria marginata* samples

Region	*Scopellaria marginata*	A (%) Content	T (%) Content	G (%) Content	C (%) Content	A + T (%) Content	G + C (%) Content
*trn*L-UAA	Sm_UB1	38.4	28.1	17.7	15.8	66.5	33.5
	Sm_UB2	38.3	28.6	17.4	15.6	66.9	33.1
	Sm_UB3	38.8	28.2	17.4	15.5	67.0	33.0
	Average	38.5	28.3	17.5	15.7	66.8	33.2
*trn*F-*trn*L IGS	Sm_UB1	31.2	32.3	16.2	20.3	63.5	36.5
	Sm_UB2	30.7	32.3	16.5	20.5	63.0	37.0
	Sm_UB3	30.4	32.4	16.6	20.6	62.8	37.2
	Average	30.8	32.3	16.4	20.5	63.1	36.9

Note: G: Guanin, A: Adenin, C: Cytosin, T: Thymin

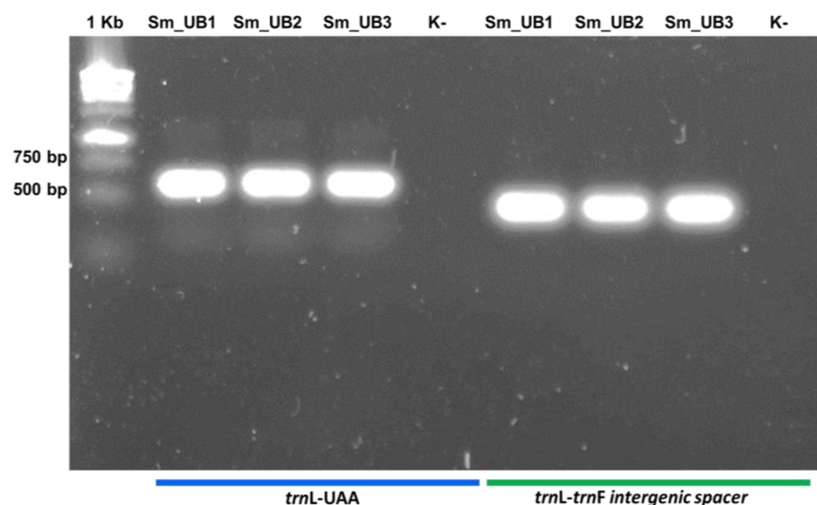

Figure 2. Electrophoregram of PCR results of *Scopellaria marginata* samples using primer *trn*L-UAA and *trn*F-*trn*L. K-: nuclease-free water

Table 4. Results of highest BLASTn pairwise identity (%) for *trn*L-UAA and *trn*L-*trn*F IGS

Region	Sample ID	GenBank/BLAST	Max. ID (%)	Accession No.
*trn*L-UAA	Sm_UB1	*Scopellaria marginata*	97.14	DQ536882
	Sm_UB2	*Scopellaria marginata*	97.50	DQ536882
	Sm_UB3	*Scopellaria marginata*	97.33	DQ536882
*trn*F-*trn*L IGS	Sm_UB1	*Scopellaria marginata*	98.36	DQ536882
	Sm_UB2	*Scopellaria marginata*	98.61	DQ536882
	Sm_UB3	*Scopellaria marginata*	98.39	DQ536882

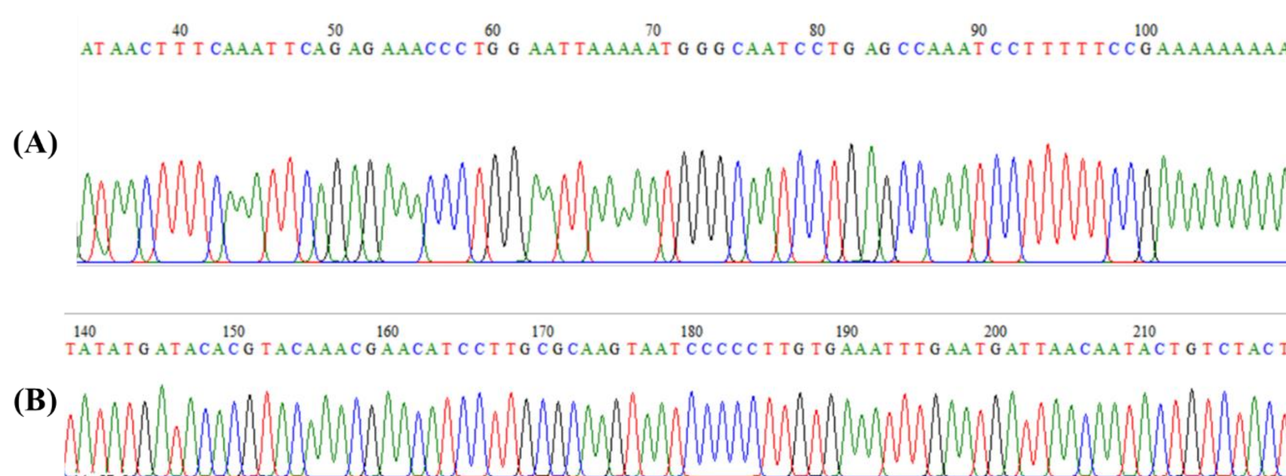

Figure 3. Representative sequencing chromatogram of *Scopellaria marginata* samples using primer *trn*L-UAA (A) and *trn*F-*trn*L (B)

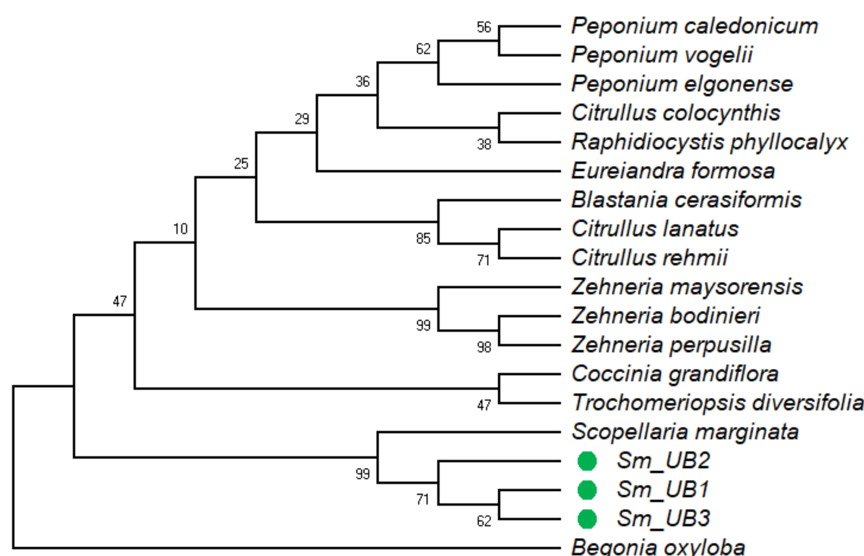

Figure 4. Phylogenetic tree of *Scopellaria marginata* from Malang, East Java, Indonesia (Sm_UB1, Sm_UB2, and Sm_UB3) based on the *trn*L-UAA

The *trn*L-UAA and *trn*L-*trn*F IGS produced in this study were the first sequences obtained from *S. marginata* originating from Indonesia. The *trn*L-*trn*F IGS is a barcode region in the chloroplast genome consisting of two transfer RNA regions, namely *trn*L-UAA and *trn*F-GAA. A non-coding spacer region separates these two regions. The *trn*L region consists of two exons and an intron where the 3'-end joins the exon of the *trn*F gene, which is separated by a spacer, and during the transcription process, these two genes are transcribed simultaneously (Yulita 2013).

Specific DNA barcode of *Scopellaria marginata*

Both *trn*L-UAA and *trn*L-*trn*F IGS showed a correct assignment rate (CAR) of 100% for *S. marginata* samples (Table 5). This result means both *trn*L-UAA and *trn*L-*trn*F IGS are good candidates for DNA barcoding to identify *S.*

marginata. The correct assignment rate at the species level also showed a high result (87.5%) in species of Gramineae, including *Agropyron*, *Bromus*, *Elymus*, *Elytrigia*, *Festuca*, *Leymus*, and *Lolium* (Wang et al. 2022). The good discrimination is also shown by the *trn*L-*trn*F intergenic spacer, which significantly separates the genus *Hedysarum* (Fabaceae) into two sections, *Hedysarum* and *Multicaulia* (Nuzhdina et al. 2018).

This study also successfully generated specific DNA barcodes for *S. marginata* using *trn*L-UAA and *trn*L-*trn*F IGS. DNA sequence with lengths of 505 and 417 bp for *trn*L-UAA and *trn*L-*trn*F IGS proposed as specific barcodes for *S. marginata* (Figure 6.A-B). Two-dimensional DNA barcoding produced in our study is useful for converting the information of *S. marginata* identity. According to Yu et al. (2016), this barcode can be applied to species identification and provide a new clinical safety protection technique. We can apply those barcodes by scanning them using a mobile phone equipped with a barcode scanning device. Subsequently, specific DNA sequence information will be displayed and can be used to identify a species accurately (Khan et al. 2017). Various studies also generated specific barcode markers which transformed into QR codes to benefit the diverse researchers. QR codes were also produced for family level in plants, such as Apocynaceae (Lv et al. 2020), Orchidaceae (Li et al. 2021), Theaceae (Jiang et al. 2022), Apiaceae (Jiang et al. 2023); genera level in plants, such as *Syringa* (Yao et al. 2022), *Clerodendrum* (Gogoi et al. 2020); and species level in plants, such as *Trachelospermum jasminoides* (Yu et al. 2016), *Panax ginseng* (Cai et al. 2016); and also plant-derived product level, such as Shi-Liang tea which made from the processed leaves of *Chimonanthus salicifolius* and *Chimonanthus zhejiangensis* (Ma et al. 2017).

Our results also confirmed that the samples analyzed were *S. marginata* based on morphological data evidence (Arumingtyas et al. 2023) and molecular data obtained from this study. This result also proves that the *S. marginata* found in Malang is a expanding the distribution, especially for the East Java region, and an additional record of the distribution area of *S. marginata* in Indonesia. The existence of *S. marginata* in Indonesia, especially Java, has been reported to be found in West Java (de Wilde and Duyfjes 2006). Based on our study on the digital herbarium collection of Royal Botanic Garden Edinburgh's (RBGE); Rijksherbarium, Leiden (Herb. Ludg. Bat.); and Herbarium Universitas Andalas (ANDA), *S. marginata* was found in Indonesia, including West Java (Purwakarta; Padalarang, Bandung; and Bogor), Southeast Sulawesi (Rantapao Toraja), North Sumatera, West Sumatera (Padang, Payakumbuh, and Bukittinggi). Furthermore, *S. marginata* was also found in several locations in Sumatera, namely Mt. Koeta Boeloer, H.van Tromon, Simeloengoen-Batak landen, Padang, and Sibolangit (Sitorus et al. 2019). Moreover, the distribution of *S. marginata* is based on the description in the Flora Malesiana book, including East Myanmar, China (Yunnan), Thailand, Laos, Cambodia, Vietnam, Philippines, the Malaysian Peninsula, Borneo (Sabah), Sumatera, West Java, and Sulawesi (Schaefer and Renner 2011).

The study concluded that the *Scopellaria* samples found in Malang, East Java, Indonesia, identified as *S. marginata* based on the *trn*L-UAA and *trn*L-*trn*F IGS. The similarity level of *Scopellaria* samples with *S. marginata* in the database showed 97.14-97.50% and 98.36-98.61%, respectively, for the *trn*L-UAA and *trn*L-*trn*F IGS.

Table 5. Correct Assignment Rate (CAR) of species level in *Scopellaria marginata* samples based on *trn*L-UAA and *trn*L-*trn*F IGS

Region	Correct Assignment Rate (CAR) (%)
*trn*L-UAA	100
*trn*F-*trn*L IGS	100

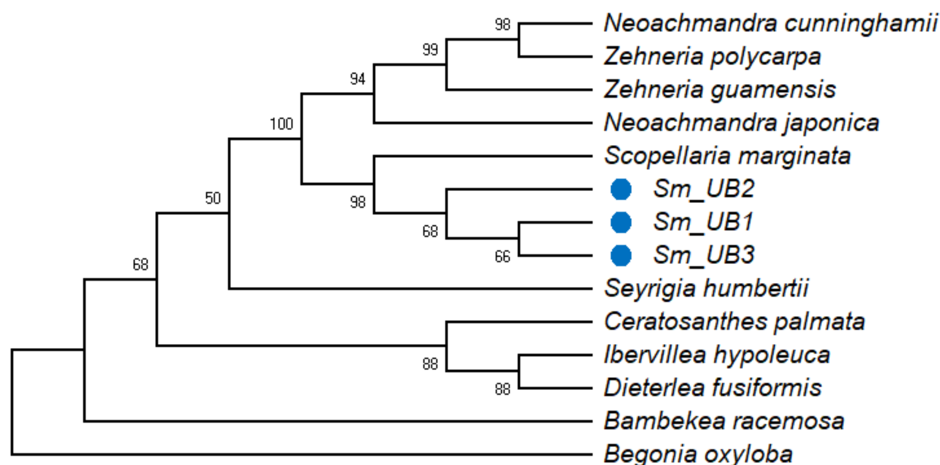

Figure 5. Phylogenetic tree of *Scopellaria marginata* from Malang, East Java, Indonesia (Sm_UB1, Sm_UB2, and Sm_UB3) based on the *trn*F-*trn*L IGS

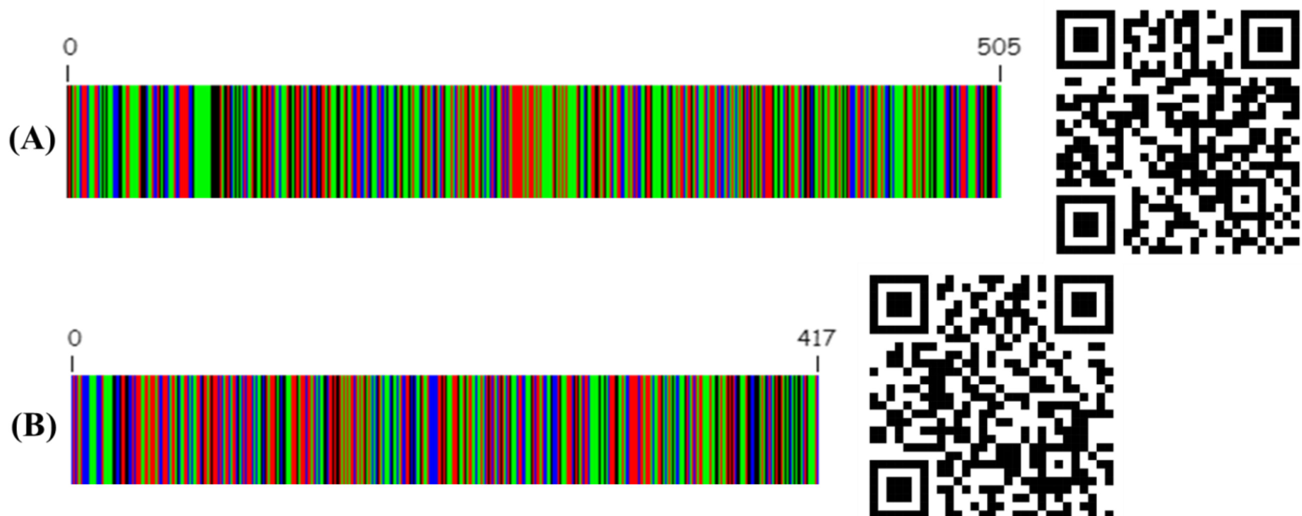

Figure 6. DNA barcodes and two-dimensional DNA barcodes of: A. *trn*L-UAA and B. *trn*L-*trn*F IGS for *Scopellaria marginata* Sm_UB3. Green, red, black, and blue represent base A, T, G, and C, respectively.

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