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The effectivity of *rbcL* marker to identify invasive alien plant species

La eficacia del marcador rbcL para identificar especies de plantas exóticas invasoras

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Abstract

Identifying and managing pathways for the introduction of alien species is one of the Kunming-Montreal Global Biodiversity Framework targets. The alternative approach to identifying the invasive alien plant species (IAPS) can be conducted using the DNA barcoding method. However, not all markers are effective in identifying the specific species. Hence, finding the right and effective marker to identify the specific species for correct identification is critical. This study examined the effectivity of *ribulose 1,5-bisphosphate carboxylase/oxygenase large* subunit (*rbcL*) marker to identify IAPS. Several primer sequences of *rbcL* were retrieved from the literature and then analyzed *in silico* by aligning the primer to the whole chloroplast DNA sequence of the target species. A selected pair of primer sequences was then employed as a marker to identify five IAPS. The sequencing results were aligned to the reference database obtained from NCBI by using BLAST. The results showed that all the species were success to be amplified using *rbcL* gene. The sequence results showed the query cover and per identity 99-100% of the target species. This result suggests that *rbcL* markers could precisely identify the IAPS target. Therefore, further research on protocol development and extensive use of DNA barcoding in IAPS identification.

Keywords: Biodiversity, DNA Barcoding, Invasive Alien Plant Species, Marker, *rbcL*.

Introduction

One of the biggest threats to biodiversity is the emergence of Invasive alien plant species (IAPS). IAPS are plants growing outside of their native area, which manage to survive and spread rapidly, posing a threat to biodiversity and local species (Abywijaya *et al.* 2014). The spread of IAPS occurred unintentionally because of the human population growth which supports the expansion of global relations. It exacerbated by uncontrolled climate change which causes ecosystem changes, thus providing favorable properties for the spread of IAPS (Butkuvienė *et al.* 2022). The term "invasive alien plant species" (IAPS) refers to a variety of creatures that are not native to an ecosystem and might provide dangers to the environment, economy, or human health, such as plants, animals, diseases, and others (Beck *et al.* 2008). Ecotourism destinations have also seen the growth of IAPS (Anderson *et al.* 2015, Aththanayaka *et al.* 2023). The spread of native weeds and exotic plant invasions in protected areas can be exacerbated by increased tourism and human visits, such as animal safaris in national parks because tourism increases the human footprint inside conservation lands and causes anthropogenic disturbances (Anderson *et al.* 2015, Toral-Granda *et al.* 2017, Kays *et al.* 2017).

The emergence of IAPS can be detrimental to the ecosystem, as it creates hybridization, spreads disease, and outcompetes native species, which leading to biodiversity disruption (Langmaier & Lapin 2020, Kumar Rai & Singh 2020). Massive colonization of IAPS dominantly consumes the available nutrient, causing nutrient deficiencies in native plants (Simatupang *et al.* 2023). This circumstance requires prevention control and eradication (Lestari 2021). Therefore, Kunming-Montreal Global Biodiversity Framework aspires to eliminate, minimize, reduce, and mitigate the impacts of invasive alien species on biodiversity and ecosystem services, aiming to restore the damaged or lost biodiversity and also its sustainable use to restore the condition of the earth and nature for the common good (Obura 2023).

Control and prevention of IAPS can begin with an identification process to distinguish it from other plants. Identification activities are usually carried out using morphological characters, although sometimes there are problems with the morphological similarity among different species, often leading to misidentification. Another problem in the identification process based on morphological phenotype is the difficulty in distinguishing between IAPS and non-invasive species, especially during the vegetative phase. Some IAPS have an unstable morphology influenced by age and location where they were found (Damayanti *et al.* 2022). Meanwhile, technological developments in the molecular field have provided faster and more accurate identification methods (Ariati *et al.* 2022).

DNA Barcoding is the advance identification method that uses a short sequence of a specific gene (marker) which has been standardized in each organism's genome and contains variations (Imtiaz *et al.* 2017). The marker must have high interspecies genetic differences but low intraspecies genetic variations, and need to have a few base pairs so it easy to obtain even from degraded tissue (Teklemariam *et al.* 2023). The DNA Barcoding method is potentially useful to identify IAPS that have morphological similarities to non-invasive species or unstable morphology (Hartvig *et al.* 2015). In plants, the commonly used markers are originated from the chloroplast gene region, as chloroplast DNA has a low number and variety of genes (Rohimah *et al.* 2018). Besides, chloroplast DNA has a high level of universality and discriminatory, making it suitable for identification process. The commonly used marker comes from the *rbcL* gene in chloroplast DNA. *rbcL* encodes RuBisCO and has a sequence length of approximately 1400 bp. *rbcL* was chosen as a marker because it has a low mutation rate compared to other genes in chloroplast DNA (Sheikina 2022, Candramila *et al.* 2023, Harnelly *et al.* 2018).

The effectivity of DNA Barcoding methods is sometimes hampered by the use of inappropriate markers. As mentioned previously that *rbcL* marker has a low mutation rate, its application has been approved by the Consortium for the Barcodes of Life (CBOL) as a barcoding locus for plants. However, in several plants, the rbcL marker was not as effective as the other markers such as *megakaryocyte-associated tyrosine kinase* (*matK*), *Internal transcribed spacer* (*ITS*), and *trnH*. Previous study explained that variations in the *matK* gene are much greater than in the *rbcL* gene (Thitikornpong *et al.* 2018). Despite that, several studies reported that the *rbcL* marker is so far effective in certain plants or resulted in exclusivity (Fatima *et al.* 2019, Ho & Nguyen 2020).

This study used *rbcL* primers which had been previously analyzed in-silico using SnapGene software. The primers are obtained from the literature and analyzed in-silico to ensure the attachment location on each IAPS *rbcL* gene sequence. The aim of this study is to differentiate IAPS from native species using *rbcL* markers. The tested IAPS consists of five species based on the quantity found around the SEAMEO BIOTROP area. These species underwent extraction stages, amplification, sequencing, and data analysis using BLAST feature on the NCBI web.

Materials and methods

Plant Materials

Plant material consisting of five species: *Chromolaena odorata* (L.) R.M.King & H.Rob (CO), *Mikania micrantha* Kunth (MM), *Spathodea campanulata* Beauverd (SC), *Rivina humilis* L. (RH), and *Chimonobambusa quadrangularis* (Franceschi) Makino (CQ) were used in this study. These plant species were collected from fields around the SEAMEO BIOTROP, Bogor, Indonesia. Those species are confirmed invasive alien plant species, and *R. humilis* is a quarantine concern in Indonesia (Rahmawati & Rosleine 2023, Zuhri & Mutaqien 2024, Labrada & Medina 2009, Tjitrosoedirdjo *et al.* 2016, Huang *et al.* 2021, Adhikari *et al.* 2023).

DNA Extraction

The collected young leaf samples were crushed using a sterile mortar and pestle by adding 0.02 grams of polyvinyl polypyrrolidone (PVPP) to avoid oxidation. DNA was extracted using the CTAB method. 1 ml of 10% CTAB buffer was put into a 2 ml microtube and then 0.25 grams of leaf powder sample was added. The mixture was vortexed for 2 minutes and incubated at 65ºC for 30 minutes, with a gentle inversion every 10 minutes to homogenize the mixture). The DNA extract solution was purified by adding 750 µl chloroform: isoamyl alcohol (24:1). The mixture was vortexed and centrifuged at 11000 rpm for 10 minutes. The supernatant was transferred into a new 2 ml microtube, and the purification process was carried out again. 1 ml of isopropanol was added to precipitate the DNA and homogenized by inverting the tube until white threads formed. The mixture was incubated at -20°C for 30 minutes. The DNA pellet was centrifuged at 11000 rpm for 10 minutes. The liquid above was discarded and then the DNA pellet was dissolved in the TE buffer (200 µl). 10 µl of RNAse was added to remove RNA. Then the mixture was incubated at 37°C for 1 hour. DNA was precipitated by adding Na-acetate 3M pH 5.2 1/10 x volume and absolute alcohol 2.5 x volume, the mixture was homogenized and incubated at -20°C for 30 minutes. DNA was precipitated again by centrifuging at 11000 rpm for 10 minutes. The liquid was discarded and the DNA pellet was rinsed using 500 µl of 70% alcohol, the DNA pellet was dried and then dissolved using 100 µl of TE buffer. The quality, quantity, and integrity of the extracted DNA were then estimated using a UV-Vis spectrophotometer. The isolated DNA was stored at -20°C.

PCR Amplification and Sequencing

Amplification was achieved in a 50 µL reaction mixture containing 1 µL of diluted template DNA sample, 2 µL of forward primer, 2 µL of reverse primer, 25 µL of MyTaq HS Red Mix, and 20 µL of ddH2O. Thermal cycling was carried out with initial denaturation at 95°C for 1 minute, followed by 25 cycles of denaturation at 95°C for 15 seconds, annealing at 50-60°C for 15 seconds, elongation at 72°C for 20 seconds, ending with a final extension of 10 min at 72°C and followed by a final hold at 4°C. After 25 cycles, the amplified PCR results were resolved in 1% agarose gel. Gel imaging was performed using a UVP gel imaging system (Ho *et al.* 2021, Gonzalez *et al.* 2022).

Table 1. IAPS DNA samples for amplification

Species of IAPS	Family	Sample codes
C. odorata	Asteraceae	CO2
		CO1
M. micrantha	Asteraceae	MM3
		MM1
S. campanulata	Bignoniaceae	SC1
		SC3
R. humilis	Petiveriaceae	RH3
		RH2
C. quadrangularis	Poaceae	CQ2
		CQ1

Table 2. Primers used to amplify the *rbcL* gene region

Primer	Sequence (5'-3')	Reference
rbcL-11F-F	ATGTCACCACAAACAGAGACTAAAGC	(Amandita *et al.* 2019)
rbcL-GTA-R	GTAAAATCAAGTCCACCRCG	(Giovino *et al.* 2020)

Data Analysis

All reference sequences were submitted to GenBank. The NCBI web-based megablast algorithm using default settings was then used to identify the query sequence. Each identification was carried out manually by taking the E-value, maximum identity, number of closely related species represented in the database, and the distribution of the plant in question into consideration. All sequences that yielded an e-value of 0.0 in the BLAST search were then downloaded from GenBank in fasta format to create an expanded reference database for each marker. Sequences longer than 700 bp (plastid markers) were then added to the files and the orientation of the sequences in each file was checked to ensure no back completion was used. The sequences obtained were evaluated by phylogenetic analysis using Mega X (Kumar *et al.* 2018).

Results and discussion

Taxonomy and morphology of IAPS

In this study, we used five IAPS collected from fields around the SEAMEO BIOTROP, Bogor, Indonesia which consists of four different families to present the test results of the *rbcL* gene, for the taxonomy and morphology of IAPS as shown in Table 3 and Figure 1. In this section, we explain the morphological characteristics of each species as the basic information of the species before continuing to molecular analysis.

Table 3. Species of IAPS that used to research (Tjitrosoedirdjo *et al.* 2016).

No.	Species	Classification	
1	*C. odorata*	Division Class Order Family Genus Species	: Tracheophyta : Magnoliopsida : Asterales : Asteraceae : Chromolaena : *C. odorata*
2	*M. micrantha*	Division Class Order Family Genus Species	: Tracheophyta : Magnoliopsida : Asterales : Asteraceae : Mikania : *M. micrantha*
3	*S. campanulata*	Division Class Order Family Genus Species	: Tracheophyta : Magnoliopsida : Lamiales : Bignoniaceae : Spathodea : *S. campanulata*
4	*R. humilis*	Division Class Order Family Genus Species	: Tracheophyta : Magnoliopsida : Caryophyllales : Phytolaccaceae : Rivina : *R. humilis*
5	*C. quadrangularis*	Division Class Order Family Genus Species	: Tracheophyta : Magnoliopsida : Lilianae : Poaceae : Chimonobambusa : *C. quadrangularis*

Figure 1. Morphology of five invasive alien plant species: A. *C. odorata*, B. *M. micrantha*, C. *S. campanulate*, D. *R. humilis* L., E. *C. quadrangularis*.

1. *Chromolaena odorata*

Chromolaena odorata is a shrub habit (Makin *et al.* 2022). This plant has a height of around 1.5 - 2 meters under normal conditions and can grow up to 6 - 7 meters if there are nearby supporting trees. *C. odorata* has ovate leaves, oval where the widest part is at the base of the leaf. The tip of the leaf is acutus or tapered, with the edge of the leaf bi-serratus shaped like a double-edged saw. Leaves are 6 - 10 cm long and 3 - 6 cm wide. *C. odorata* stems contain chlorophyll and are green with a cylindrical shape and many branches. On the surface of the stem, there are fine hairs. The *C. odorata* root system is a taproot system with a fusiform shape and spreading at the base. The flowers of *C. odorata* are located at the ends of the branching stems or are the sitting type of flos terminal flowers with the number of flowers in each bouquet ranging from 20 - 35 flowers. The flowers of this plant are blue when young, and turn brown over time. The flowering cycle begins simultaneously during the dry season with a period of 3 - 4 weeks until the seeds ripen. Ripe seeds will burst and be

carried by the wind until they spread and dominate an area (Zahara 2019).

2. *Mikania micrantha*

Mikania micrantha is an annual plant with a shrub habit and is known for its invasive. *M. micrantha* is a fast-growing herb, growing up to 1 meter per year (Zhang *et al.* 2019). The stems can grow up to 6 meters long. The stem of this plant is characterized by a light green color is covered with fine hairs and has many branches. *M. micrantha* leaves have a triangular or cordate shape with acutus or tapered leaf tips and serrated or serratus leaf edges, the leaves are located facing each other in one segment. *Mikania micrantha* flowers are the sitting type of flos axillaris or are located in the leaf axils, and are small and pink and white. The roots of this plant are tap roots, with primary root growth that continues to enlarge and elongate (de Almeida *et al.* 2017).

3. *Spathodea campanulata*

Spathodea campanulata is a large upright tree with shiny greenish pinnate leaves and beautiful reddish-orange flowers. It can grow up to 80 feet tall in suitable conditions. It has sturdy stems covered with light gray bark. The leaves are usually opposite, 10–15 cm long, elliptical to oval, and clustered at the ends of the branches. In the form of compound, irregular leaves with seven - eight pairs of leaflets, and a petiole 5–6 cm long. It has fruit 5–10 inches long, finger-like, and pointing upwards. Each fruit contains about 500 paper-like seeds (Padhy 2021).

4. *Rivina humilis*

Rivina humilis is a type of herbaceous plant or shrub that grows up to 1 meter high, with spreading branches that are softly hairy when young. The leaves are light green, thin and slender with smooth edges, the leaves are ovate to ovate-elliptical. The first leaf is simple and opposite, with long leaf stalks. The blade is shiny, and round, and the leaf edges are intact. The small white or greenish-to-reddish flowers are arranged in elongated clusters. Flowers appear from October to June. The fruit is round like a berry with one hairy seed per fruit, bright red. The leaves, roots and fruit are poisonous. *R. humilis* has spreading branches. The stems are grooved, dense and soft when young (Sushama *et al.* 2021).

5. *Chimonobambusa quadrangularis*

Chimonobambusa quadrangularis is a plant with leptomorph rhizomes, stolon could reach 4 m in length, and has the characteristic of easily rising to the surface of the ground or rock gaps. The stem of this plant is green, straight, erect, 2–4 m high, 2–3 cm in diameter, 20–25 cm in segments. In contrast to bamboo reeds which are woody, segmented, book-shaped, round, or quadrangular, *C. quadrangularis* has a rectangular reed that is hollow in the middle. *C. quadrangularis* has swollen, prominent stem nodes, with spines vertically to the top of the node and curved downward. A branch complement with three sub-equal branches appears simultaneously. The stem sheath measures 10–22.6 × 4–5 cm, is thinly triangular, and is covered with brown hairs up to 1 mm long, the auricles are absent and glabrous. *C. quadrangularis* has leaves measuring 16–21 × 2 –2.1 cm, linear, adaxially pubescent, very short petioles, and inconspicuous earlobes (Damayanto & Muhaimin 2017).

PCR Amplification

PCR amplification results showed a high success rate of the universal primer *rbcL*, of the 6 IAPS species, 5 species were successfully amplified using the primer pair of *rbcL-11* (Forward) and *rbcL-GTA* (Reverse), with a success percentage of 83.33%. As reported in the previous study, this primer pair was equally effective in obtaining a correct amplicon (Fatima *et al.* 2019, Ho & Nguyen 2020). Therefore, it is selected as the highest success rate in *in-silico* analysis using *Snapgene*. However, there was no *rbcL* gene amplicon on *Praxelis clematidea*.

These findings corroborate previous research on the effectiveness of the *rbcL* gene in plant DNA amplification. The results of the amplification of conifer plants in Vietnam showed a success rate of 76.19% higher using the *rbcL* marker compared to *matK* with a success rate of 66% (Pham *et al.* 2021). In addition, a previous study reported that amplification using *rbcL* provided a success rate of 100% in three species, including *Solanum nigrum*, *Euphorbia helioscopia* & *Euphorbia helioscopia* (Wattoo *et al.* 2016).

Plant DNA isolation techniques play an important role in the success of PCR-based amplification. Isolation of pure DNA with high molecular weight is essential for the successful application of molecular methods. DNA isolation techniques often present challenges in obtaining DNA samples that can be analyzed. DNA is often easily degraded or the plant material contains high amounts of polysaccharides, polyphenols, and other secondary metabolites, such as alkaloids and flavonoids which can affect the results of DNA isolation (Marengo *et al.* 2019). The presence of certain metabolites can interfere with plant DNA isolation procedures and affect PCR amplification, making it possible that DNA sequence bands may not appear (Abdel-Latif & Osman 2017). The results of DNA extraction are expected to have purity values ranging from 1.8 - 2.1 (A260/A280) and 2.0 - 2.2 (A260/A230), below these values, indicate the presence of contaminants as previously explained (Sophian & Syukur 2021, Pineda-Rodriguez *et al.* 2023). The use of temperature in the annealing stage also influences the success of DNA amplification.

Table 4. DNA concentration results using a nanophotometer.

IAPS	Code	Concentration (ng/πl)	Purity	
			A260/A280	A260/A230
C. odorata	CO1	1123.00	1.862	1.267
	CO2	1185.00	1.862	1.279
M. micrantha	MM1	544.50	1.827	1.361
	MM3	680.10	1.865	1.392
S. campanulata	SC1	1326.60	1.967	1.452
	SC3	644.80	1.979	1.443
R. humilis	RH2	822.65	2.178	1.100
	RH3	1105.30	2.080	1.048
C. quadrangularis	CQ1	38.05	1.956	2.447
	CQ2	95.40	1.951	2.060

At the amplification stage using PCR, this research used an annealing temperature gradient, with a range of 50-60oC. Suitable temperature during annealing affects the amplification process. Excessively high temperature (above 60°C) will result in mispriming and inhibit the primer annealing (Khaira *et al.* 2023). Optimization of annealing temperature using the temperature gradient technique is used because of differences in the sequence, length, and composition of the primers attached to the sequence of the target species; it is often difficult to obtain the same melting temperature (Tm) between the two pairs of primers. An annealing temperature mismatch can drastically reduce the results and specificity of PCR and even cause PCR failure (Green *et al.* 2015).

Amplification using primers *rbcL-11-F* and *rbcL-GTA-R* (Table 2) with an annealing temperature of 50°C showed electrophoretic DNA bands in IAPS *S. campanulata* (SC1 and SC3) and *R. humilis* (RH1 and RH2) DNA samples. The failure in amplification of the other four species was possibly caused by annealing temperatures that were not optimal so the primers failed to attach to the sample DNA template (Silalahi *et al.* 2021). For this reason, it is necessary to optimize PCR by varying the annealing temperature so that other samples can be successfully amplified. Amplification was again carried out with varying annealing temperatures (55°C, 54°C, 53°C, 52°C, 51°C, and 50°C) using the same primers, and electrophoresis DNA bands were obtained in the IAPS *C. odorata* (CO2) and *C. quadrangularis* (CQ2) DNA samples. *C. odorata* DNA samples were successfully amplified at annealing temperatures of 50°C and 52°C, while *C. quadrangularis* DNA samples were successfully amplified at 50°C, 51°C, 52°C, 53°C, 54°C, and 55°C. PCR optimization was again carried out to obtain electrophoretic DNA bands for the two remaining species, namely *M. micrantha* and *P. clematidea*. Amplification with varying annealing temperatures (56°C, 57°C, 58°C, 59°C and 60°C) in the two species produced electrophoretic DNA bands only in *M. micrantha* (MM3).

A total of 7 samples were successfully amplified using the *rbcL* marker from 5 IAPS species (Figure 2). Two samples of *P. clematidea* DNA failed to be amplified even after PCR optimization. Therefore, we tried to reanalysis the quality of *P. clematidea* DNA samples using 1% agarose gel electrophoresis and observing them under UV light. The visualization results show that there is no DNA band at all. Previous DNA quality analysis using UV-Vis Spectrophotometer also showed that the purity value of the *P. clematidea* DNA samples was below the proper value (Table 4) which may contain contaminants in the form of phenol and too much solvent used. This is the reason why the amplification of the *rbcL* gene in *P. clematidea* DNA samples failed. Amplification will not produce a PCR product if the DNA sample used has been damaged or lost. This damage can occur due to an incorrect DNA extraction process (Sophian & Syukur 2021).

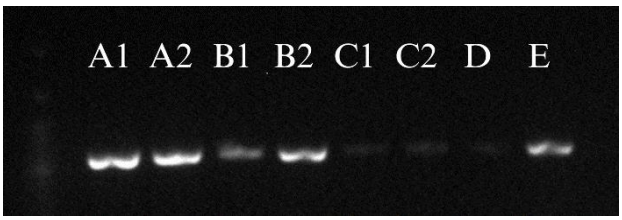

Figure 2. Amplification of the *rbcL* gene sequence was carried out from the five species using the PCR method. The amplification results were visualized by 1% agarose gel electrophoresis under UV light. The five species are *S. campanulata* (A1 and A2), *C. odorata* (B1 and B2), *C. quadrangularis* (C1 and C2), *M. micrantha* (D), and *R. humilis* (E).

The use of the *rbcL* marker has great potential for identifying various plant species. These primers allow amplification across a wide taxonomic range and greatly determine the success rate of PCR, especially in cases of discovery of new or rarely studied species (Thakur *et al.* 2016). In addition, the *rbcL* gene locus has a high success rate for bidirectional sequencing (Basith 2015). Therefore,

the results of PCR amplification in this study will be used for further sequencing and analysis stages

Sequence Analysis

BLAST analysis of each IAPS *rbcL* sequence molecular identification was carried out using a BLAST search on the NCBI database. The results of *rbcL* marker sequencing in 5 IAPS species showed a success rate of 100%. The sequence lengths of CO, SC, RH, MM, and CQ species were 570, 579, 562, and 573 bp respectively. BLAST results for one species showed misidentification. The *rbcL* sequence *C. quadrangularis* was also misidentified with another species called *Phyllostachys purpurata*. It might indicate that the *rbcL* marker was not effective in identifying those species. However, the sequence of *C. quadrangularis* still hit the right species with a coverage of 99.65% (Table 4).

Some studies also show the existence of misidentification in databases. Misidentification still often occurs in mitochondrial DNA subjects (Cheng *et al.* 2023). Around 5% of existing 16S rRNA sequences in public databases are not evaluated and contain substantial errors (Cheng *et al.* 2023). Random and well-studied may be wrong. It is possible that the misidentification was caused by errors in poor DNA isolation techniques, resulting in the amplification of incorrect or chimeric DNA sequences (Stavrou *et al.* 2018).

The sequencing results in this study were successful for 5 IAPS, namely *C. odorata*, *C. quadrangularis*, *R. humilis*, *S. campanulata*, and *M. micrantha*. However, our BLAST result showed the result of *C. quadrangularis* hitting *Phyllostachys purpurata* McClure which is another species but in the same family, Poaceae. There is a need for further investigation of *C. quadrangularis* species. This result also suppots the previous study showed that the *rbcL* gene region has been widely used for DNA barcoding in land plants because it has a high level of universality and is easy to amplify (Nurhasanah *et al.* 2019). This indicates that the *rbcL* gene marker is an effective locus in the DNA sequence (Bell *et al.* 2017).

Table 4. Sequencing results and statistical simulation of BLAST sequence

No.	Code	Sequence length	Targeted Species				
			Species	Query Cover (%)	Per. Identity (%)	E-value	Accession
1	MM	582	*M. micrantha*	99	99,83	0.0	NC_031833.1
2	CO	582	*C. odorata*	98	100	0.0	NC_050055.1
3	RH	566	*R. humilis*	100	100	0.0	NC_041300.1
4	SC	574	*S. campanulata*	99	99,83	0.0	NC_049000.1
5	CQ	579	*P. purpurata*	98	100	0.0	MK982135.1

Sequencing is carried out to obtain DNA sequences from each IAPS and is used for identification at the genus to species level. Sequence variation from reference sequences and phylogenetic reconstruction are the basic principles of species identification in plants (Xu *et al.* 2018). In this study, we amplified and sequenced IAPS types in five different species. These species represent the majority of IAPS samples that are frequently found disturbing local plant species and that have a fairly wide distribution in agricultural areas. There are still many plant genomes that lack sequence information. These findings indicate that the use of the *rbcL* marker in the DNA Barcode method was successful in amplifying, identifying, and grouping IAPS species based on their respective sequences. These results can further help to study the taxonomy, ecology, phylogeny, and morphology of different IAPS species. However, there is still a need to develop new protocols and amplification strategies by combining or modifying markers so that innovations in molecular identification can emerge that describe more detailed genome information from different and broader species (Wattoo *et al.* 2016, Ho *et al.* 2021).

Phylogenetic Analysis

The phylogeny tree was obtained from the *rbcL* IAPS sequence using the Neighbor-Joining method with a bootstrap value of 1000 times. Phylogenetic tree analysis shows the formation of one ingroup cluster/group, namely *S. campanulata*, *C. odorata*, and *R. humilis*, with one outgroup species, namely *C. quadrangularis*. The three ingroup species form a monophyletic group, where *S. campanulata* and *C. odorata* are sister taxa, and *R. humilis* is an outgroup in the monophyletic group. The Asteraceae and Bignoniaceae families are in the same evolutionary line and have a close relationship, therefore in the phylogeny tree, the two species from this family are sister taxa (Sousa-Baena *et al.* 2018). Poaceae is on a different evolutionary line and the most distant common ancestor of the other families, therefore *C. quadrangularis* is an outgroup species on the phylogeny tree (Banerjee *et al.* 2023).

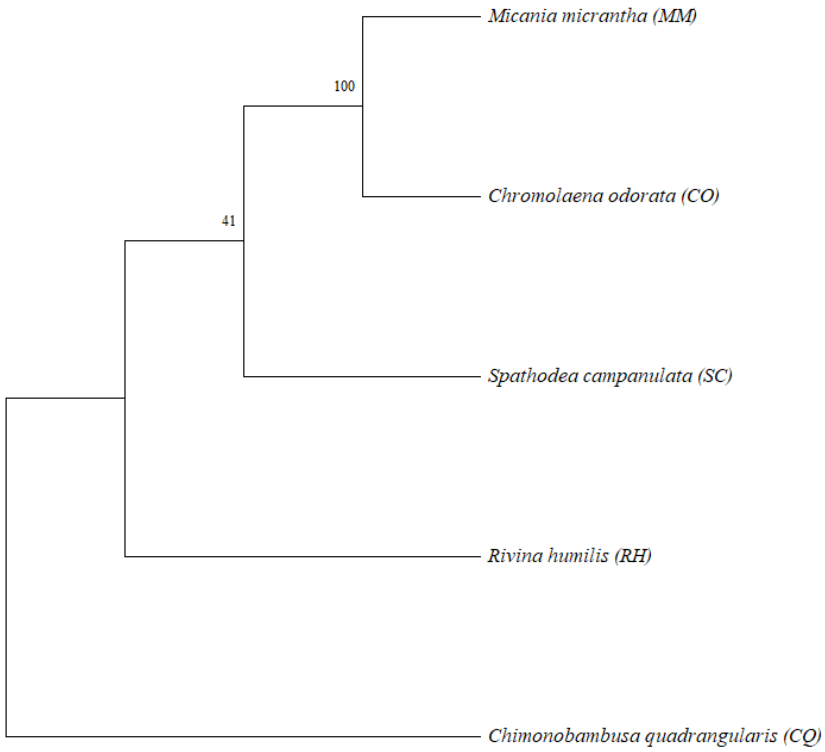

Figure 2. Phylogenetic tree from *rbcL* sequence of 5 IAPS.

Conclusion

In conclusion, these findings demonstrate that the universal primer *rbcL* for DNA barcoding is successful for the amplification, identification, and discrimination of IAPS. The BLAST results showed that the amplified sequence fragments hit the specific species on the database with the query cover and percent identity around 99-100%. The application of the universal primer *rbcL* in DNA barcoding is an effective, fast, and cheap method for IAPS identification. However, it is necessary to compare *rbcL* marker to other markers and utilize more samples. This result shows the potential of *rbcL* marker in identifying IAPS.

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