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SEX DETERMINATION IN *NEPENTHES SUMATRANA* (MIQ.) BECK BASED ON MORPHOLOGICAL TRAITS AND MOLECULAR MARKERS

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SUMMARY

Pitcher plants have undergone extensive cultivation as ornamental plants. Breeders made significant efforts on early sex identification, particularly before propagation. The following study may be the first investigation to identify the sex determination based on morphological and molecular traits of Sumatra's endemic pitcher plant. Molecular markers applied related to sex determination comprised the primers DYT1, COX, OPA 15, OPD 05, UBC354, and OPY 7. The pitcher plant samples collected came from South Tapanuli and North Sumatra, Indonesia. Observations based on qualitative and quantitative morphological characters, as described in the descriptor, covered parts of stems, leaves, and pitchers. The results revealed considerable variations among the various species of *N. sumatrana* for stem surface texture, leaf shape, and upper leaf surface texture. Only the male plants exhibited 290 bp of DNA fragments amplified by the primer DYT1. Primer OPA-15 amplification produced 600 bp, OPD05 gave 850 and 650 bp, and UBC354 provided 900 bp of DNA fragments, found only in the female. The primer OPY7 amplified DNA fragments measuring 610, 680, and 750 bp, and they were evident only in the female. The presented research will be a valuable contribution to the development of an early sex determination system.

Keywords: Pitcher plant (*N. sumatrana*), genetic diversity, morphological traits, molecular analysis, sex determination

Key findings: In pitcher plants (*N. sumatrana*), the study based on sex determination was successful through morphological and molecular characterization. The presented research could help in the development of an early sex determination system in dioecious Nepenthes plants and would be greatly beneficial in their cultivation.

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INTRODUCTION

Being an ornamental plant, pitcher plants (*Nepenthes* spp.) belong to rare and intriguing flora with extensive cultivation. The diverse hues and shapes of the bag make the said plant species attractive. The pitcher plants' use as ornamental plants is extremely prevalent abroad. Indonesia seemed the land of nectar, being the home of 65% of the global species population. Overall, the world is hosting around 138 known species (Mcpherson and Robinson, 2012); however, Indonesia has the greatest diversity, followed by Malaysia and the Philippines. The 29 out of 36 species documented only on Sumatra Island are endemic to the island (Mcpherson and Robinson, 2012).

Sex determination is a critical developmental process in plants, bearing economic significance because in commercially important plants, determining the cultivation and breeding practices are effective by their sexual phenotype (Leite *et al*., 2021). Breeders made significant emphasis on the early detection of sex before propagation due to consequential implications for commercial viability, breeding strategies, and the economic worth of genetically modified material. Such type of implementation would substantially decrease the time required to assess the traits, facilitate the rapid evaluation of both plants' sexes through breeding, and substantially reduce the labor and cost.

The efficient establishment of sex-specific collection (female or male) is possible if ascertaining sex can occur during the seedling phase. This is particularly beneficial for populations that differ in sexual reproduction. *Nepenthes* is a dioecious plant, and the distinct individuals produce staminate (male) and pistillate (female) flowers on separate plants. According to Tarigan (2020), the plant's sexual determination is feasible at the reproductive age, which is between five and seven years for seed-propagated plants and one and three years for stem-cutting plants. It also creates a significant challenge in practical application, as in pitcher plants, the sex identification becomes difficult at the vegetative phase, with its detection only being better during the flowering stage.

In pitcher plants, sex determination has never been successful morphologically through roots, stems, leaves, and pitchers. According to Lam and Tan (2020), *N. ampullaria* has a paniculate flower morphology, while in *N. gracilis* and *N. rafflesiana,* male and female flowers have raceme-like shapes. In current research, the focus was on describing the morphology of flowers in each species. In *N. sumatrana*, the detection of sexual maturity involves the utilization of molecular markers. Molecular marker genes, which are generally easily detectable genetic loci, served to identify several characteristics, including the sex and other plant traits.

Molecular markers serve as a valuable technique in the identification of sex in dioecious plants and before the detection of phenotypic variations that occur during the reproductive phase of plants. The current state of molecular sex determination has constraints from the efforts of certain researchers. Scharmann *et al*. (2019) reported the DYT1 gained validity as a male-specific marker with a 290-bp range in multiple Nepenthes populations. Dwiati *et al*. (2021) examined *N. adrianii* with the RAPD marker OPK-16 (GAGCGTCGAA), and they identified a 290-bp band unique to the female genotype. Mokkamul *et al*. (2007) examined *N. gracilis* Korth, *N. mirabilis* Druce, and *N. smilesii* Hemsi with the RAPD marker OPA 15 (TTCCGAACCC) and identified a band consisting of approximately 750 base pairs exclusive to the male genotype.

The pitcher plants species hold significant economic and conservation values. Pitcher plants have an extremely lengthy immature phase in its natural habitat, lasting between one and seven years, and during that time, the sexes are indistinguishable. During development, the early sex identification is necessary to determine the optimal planting ratio. Pitcher plants are native to the

Indonesian Island of Sumatra, and the species *N. sumatrana* have never undergone scrutiny before. This study aimed to identify sex determination based on morphological and molecular characteristics in endemic pitcher plants on the island of Sumatra in Indonesia. Therefore, the presented study may be the first to investigate sex detection in *N. sumatrana* based on morphological and molecular traits. These results could have a considerable contribution to the development of an early sex determination system for dioecious Nepenthes plants. Such a system would enable more precise and timely sex selection, which will greatly assist the cultivation of Nepenthes plants. Moreover, the acceleration in sex selection could lower costs of labor and maintenance regarding its production.

MATERIALS AND METHODS

This research lasted from March 2022 to December 2023. Survey and sampling site selection relied on breeders' information related to the original habitat of pitcher plants on Sumatra Island, Indonesia. The *Nepenthes sumatrana* samples collected came from Malombu, South Tapanuli Regency, North Sumatra Province, Indonesia. Morphological characteristics identification transpired in their habitat. However, the identification of molecular qualities continued at the Laboratory of Plant Genetics and Breeding, University of Gadjah Mada, Yogyakarta, Indonesia. Each *N. sumatrana* sample comprised a male and a female collected from three different individuals.

Morphological parameters

The observations on sample plants proceeded based on qualitative and quantitative morphological characters described in the descriptor, covering parts of stems and leaves (Cheek and Jebb, 2001), pitchers (Wistuba *et al*., 2007), and the morphology of male and female flowers (Lam and Tan, 2020).

Molecular markers parameters

After collecting samples from the leaves, weighing a volume of 0.1 g ensued after cleaning. After weighing the leaves, adding 1500 microliters of CTAB buffer solution to a 2-ml microtube containing the leaves underwent mashing with a mini-bead beater. The employment of the modified CTAB method ran for DNA extraction (Doyle and Doyle, 1990).

The study performed primer screening using the same steps as the previous sex-linked primer screening process, using a PCR machine. The PCR reactions performed had a total volume of 10 µl for each PCR tube and DNA amplification reaction step. The primers used were DYT1-F (5'-AATTCACTGATTCGGATCACG-3´) and DYT1-R (5' CGATCGCGTCGCAAAGTATG-3´), COX-F (5'-GGAGGAGTTGATTTAGC-3´) and COX-R (5'-AAGGCTGGAGGGCTTTGTAC -3´), OPA 15 (5'-TTCCGAACCC -3´), OPD 05 (5'- TGAGCGGACA -3´), UBC354 (5'- CTAGAGGCCG -3´), and OPY7 (5'- AGAGCCGTCA -3´). The PCR reaction optimization had the following amplification conditions: in total, 45 cycles occurred, 45 s of denaturation at 95 °C, 30 s of annealing adjusted to the primer used (Table 3), and 120 s extension at 72 °C. Before starting the first cycle, amplifications continued for 7 min at 95 °C, and a 5-min final extension at 72 °C took place after the final cycle. The amplification mixtures reached investigation on a 1.5% agarose gel.

Data analysis

The morphological data comprised both qualitative and quantitative parameters. Means and standard deviation calculations resulted from analyzing the quantitative data with Microsoft Excel. The information provided appeared through detailed descriptions of both male and female flora. Utilizing the outcome of electrophoresis visualization helped identify particular DNA bands that differentiate the sexes of pitcher plants. Through the comparison of DNA band patterns amplified by male and female pitcher plants, the specific DNA bands in male and female pitcher plants can be clear.

RESULTS AND DISCUSSION

Morphological identification

The data based on qualitative characteristics indicated considerable variations among the *N. sumatrana* species for the stem surface texture, leaf shape, and upper leaf surface texture. The males' stem surface texture was hairy, and their leaves were lanceolate, with hairs on the surface. In contrast, the females have an ovate leaf shape, a smooth surface texture, and a smooth stem surface texture (Table 1, Figure 1). Dzhaparidze (1969) reported morphological variations between the males and females in dioecious plants for the size of leaves, stems, branches, flowers, and canopy. It can only be visible after its reproductive maturity; however, the complete reliability of these morphological features was still unsure. Morphologically, almost all dioecious plants appeared with indeterminate male and female plants in the vegetative stadia. Sex differences present in plant leaves reflect the sex-specific strategies due to variances in reproductive inputs and may produce variations in sex tolerance under unfavorable conditions (Espírito-Santo *et al*., 2003). Several studies reported differences in the growth strategies of male and female plants under varied environmental conditions (Liu *et al.*, 2020). In general, the proportional distribution of reproductive structures and aboveground nutrient growth varies by sex and developmental stage in dioecious plants (Teitel *et al*., 2016).

Table 1. Qualitative characters of male and female plants in *N. sumatrana.*

No.	Plant parts	Characteristics	*N. sumatrana*	
			Male (♂)	Female (♀)
1	Stem	Surface texture	Hairy	Slick
2	Leaves	Leaf shape	Lanceolate	Obovate
3		Upper surface texture	Hairy	Slick

Figure 1. Leaf morphology of female (left) and male (right) plants in *N. sumatrana.*

The study results contrasted with past findings, which state smaller habitus characterized male trees than females, as well as straighter branches and smaller leaves (Marzuki *et al*., 2008). On differences between male and female flowers, the male flowers grow straighter on fruit branches, with a smaller size of flower stalks and flowers and a thinner flower shape than female flowers. Leaf morphology for male trees was generally smaller with an oval shape and tapered leaf base and tip. Despite these tendencies, these attributes cannot yet be applicable as a method for predicting the sex expression phenotype of a nutmeg tree because the character's expression appears when the tree enters the maturity stage. Therefore, these traits proved less useful for early selection.

For male and female sex determination in *N. sumatrana*, the morphological screening methods emerged as successful in distinguishing the differences in the qualitative characteristics (Table 1). The *N. sumatrana* male plants have a taller stem height, a wider digestive zone diameter, and a wider bag lip (peristome) diameter than their female counterparts. In contrast to female plants, males have a longer vine length, greater leaf thickness, and a larger stem diameter. Their upper pouch has a longer length, longer wings, and a broader diameter. The lower pouch exhibits a bigger pitcher length and a wider diameter of pouch cover than the pitcher of female plants (Table 2).

The quantitative characteristics distinguished the male from female plants by their longer tendrils, taller stems, and larger tendril diameters in the *N. sumatrana* species. Compared with female plants, its upper pitcher has a greater diameter of the wax zone and digestive zone, as well as a larger pitcher lid. In comparison with female plants, the waxy zone, digestive zone, lip (peristome), and pitcher lid of the lower pitcher were wider in diameter. The female plants have elongated leaves and a larger peduncle in the flower section than their male counterparts (Table 2). Rakocevic *et al*.'s (2009) studies also demonstrated that male ilex plants have a broader leaf area and photosynthesis intensity than their female counterparts. Furthermore, the research on date palm (*Phoenix dactylifera* L.) disclosed that male seedlings possess numerous lower leaves and a larger trunk circumference than female seedlings, which exhibited a reduced trunk circumference and few lower leaves (Yahaya *et al*., 2022).

Sex determination is a fundamental part of the reproduction system across the entire animal and plant kingdom. However, biological studies have shown the mechanism that controls sex determination considerably varies among different organisms (Herpin and Schartl, 2008). In crop plants, the sex-

Table 2. Quantitative characteristics of male and female plants in *N. sumatrana.*

No.	Plant parts	Characteristics	*N. sumatrana*	
			Male (♂)	Female (♀)
1	Stem	Height	138.33 ±10.41 cm	52.33±2.52 cm
2	Leaves	Length	24.17±5.11 cm	27.33± 7.1 cm
3	Tendrils	Length	4.3±0.52 cm	21±3.6 cm
4		Diameter	4.57±0.12 mm	3.87±0.7 mm
5	Upper pitcher	Pitcher length	18±3.77 cm	16.33±0.6 cm
6		Waxy zone diameter	54.6±3.36 mm	48.17±0.3 mm
7		Pitcher lip diameter	64.93±8.69 mm	52.27±2.4 mm
8		Pitcher lid diameter	71.43±12 mm	64.83±1.7 mm
9	Lower pitcher	Waxy zone diameter	45.47±13.78 mm	36.73±1.6 mm
10		Diameter of digestion zone	55.9±14.86 mm	42.5±2.2 mm
11		Wings length	9.5±2.5 cm	7.27±0.3 cm
12		Pitcher lip diameter	49.83±12.31 mm	31.67±1.5 mm
13		Pitcher lid diameter	59.03±6.56 mm	42.53±0.5 mm
14	Flowers	Peduncle diameter	13.33±3.91 mm	4.8±0.4 mm

Table 3. Annealing temperatures of primers.

No.	Primers	Temperature of annealing (°C)
1.	DYT1	61.0
2.	Combination of COX and DYT1	42.9
3.	OPA 15	34.2
4.	OPD 05	33.8
5.	UBC354	36.3
6.	OPY 7	33.8

Figure 2. Male inflorescence (A), male flowers (B), female flowers (C), seed morphology (D) and (E) in plants of *N. sumatrana.*

determination mechanism is more complex, as regulated by multiple factors, such as sex-determination genes, sex chromosomes, phytohormones, environmental factors, and epigenetic regulation (Cheng *et al*., 2023). These study results were consistent with previous findings in *Silene latifolia* (Delph *et al*., 2010), *Ceratodon purpureus* (McDaniel, 2005), and *R. hastatulus* (Teitel *et al*., 2016).

Flower morphology in *N. sumatrana*

Flowering starts from the base of the plants. Male flowers have a tepal color, and the pedicels' color is gray orange (164 A); peduncle color is yellow green (153 B); and stamens were yellow (5 A) (Figures 2A and B). Female inflorescences have a tepal color, and pedicels' color is gray orange (177 A); peduncle color is yellow green (151 C); capsule color is gray orange (165 C); and the seed color is also gray orange (163 C) (Figures 2C, D, and E). The flower morphology of *N. sumatrana* exhibited paniculate inflorescences. The paniculate inflorescence of *N. sumatrana* was similar to that of *N. ampullaria,* and the flowers generally appeared at the tip of the stem (Lam and Tan, 2020). Male inflorescences typically comprise flower stalks, tepals, stamen columns, and anthers.

In both sexes, the flower development follows an acropetal trajectory, specifically from the base to the apex. Male flowers of a single inflorescence typically open gradually over the course of several weeks, whereas the female flowers typically bloom within a few days. Thus, male inflorescences can continuously produce pollen for several weeks, whereas female inflorescences become susceptible to pollination for one to two weeks. In contrast to perennial flowering, most flowering plants exhibit a seasonally synchronized flowering pattern. In addition to endogenous factors, flower appearance and expression of plant sex phenotypes also have influences from exogenous and environmental factors.

The sex expression of a tree could have influences from genes that regulate sex expression phenotypes located in particular chromosomes. Sex expression phenotypes receive considerable effects from hormone concentration, transport, and sensitivity, while hormonal activity incurs significant modifications from environmental conditions, including light quality, photoperiodicity, nutrition, and temperature (Purwiyanti *et al*., 2018). Das *et al.* (2012) reported the pattern of acquired segregation ratio and the nature of nutmeg sex expression phenotypes may have two genes controlling them, which are fully dominant at both loci.

Molecular markers identification

In the presented study, the primers DYT1, COX, OPA 15, OPD 05, UBC354, and OPY 7 were the samples utilized. The results indicated different banding patterns in male and female Nepenthes. By applying the primers to male and female plants, the use of primer DYT1 alone or in combination with two other primers (COX and DYT1) could produce disparate outcomes. The primer COX amplified 1000-bp DNA fragments in both male and female plants (Figure 3A). However, only male plants exhibited 290-bp DNA fragments amplified by the primer DYT1 (Figure 3B). Past studies mentioned that the dysfunctional tapetum 1 (DYT1) functions as a male-specific marker across multiple Nepenthes populations in Brunei Darussalam (Borneo), Singapore, and Seychelles, with a range of 290 base pairs (Scharmann *et al*., 2019). Furthermore, genes whose expression bears regulations from DYT1 probably contribute to the operation of the tapetum, which generates the necessary material and enzymatic activities for pollen formation. Therefore, DYT1 is a critical component in the regulatory network responsible for tapetum development and function (Wei *et al*., 2006).

A DNA fragment size of 600 bp, as generated by amplification of primer OPA-15, had this fragment exclusively observed in female specimens of *N. sumatrana* (Figure 4A). The findings presented here were inconsistent with those documented in the past study on *N. gymnamphora*, wherein primer OPA15 failed to generate PCR bands that differentiated both sexes (Pertiwi *et al*., 2019). However, an effective generation of a 750-bp DNA fragment occurred by using primer OPA-15 in the male *N. mirabilis* and *N. gracilis* (Mokkamul *et al.,* 2007). This demonstrates that primers designed to differentiate sexes in *N. mirabilis* and *N. gracilis* may not be applicable to other *Nepenthes* species, including *N. adrianii* and *N. gymnamphora*. In the interim, the primer OPA-15-amplified RAPD marker measured 1625 base pairs, as exclusively identified in male *Momordica dioica* (Baratakke and Patil, 2009).

The primer OPY 7 successfully amplified the 610, 680, and 750 bp DNA fragments, which were consistently evident in all female plants but found absent in all examined male plants (Figure 4B). In contrast, the primer OP-$Y7_{900}$ proved highly effective for the discrimination of male *C. papaya* plants. The sequence-characterized amplified region (SCAR) marker generated from the OP-$Y7_{900}$ discriminates male and hermaphrodite plants from the female plants. These results suggested that the location of the SCAR marker is in a region of the Y chromosome found only in male and hermaphrodite plants. Assuming that a Y chromosome is morphologically and functionally distinct, the results suggested that the sequence used to develop the SCAR marker sits only on this chromosome (Modi *et al.,* 2018).

Primer OPD 05 effectively amplified DNA fragments of 850 and 650 bp in *N. sumatrana*; however, these fragments were exclusively visible in female individuals (Figure 5A). Conversely, the primer UBC354 effectively amplified a 900-bp DNA fragment that was exclusive to females of *N. sumatrana* (Figure 5B). In certain instances, however, it was possible to infer that OPD05 primers generate DNA fragments specific to the male sex in *Silene latifolia* Poiret (Mulcahy *et al*., 1992). Previous research reported the primer UBC354 generated DNA fragments specific to the female sex in *Salix viminalis* L. (Alstrom-Rapaport *et al*., 1998).

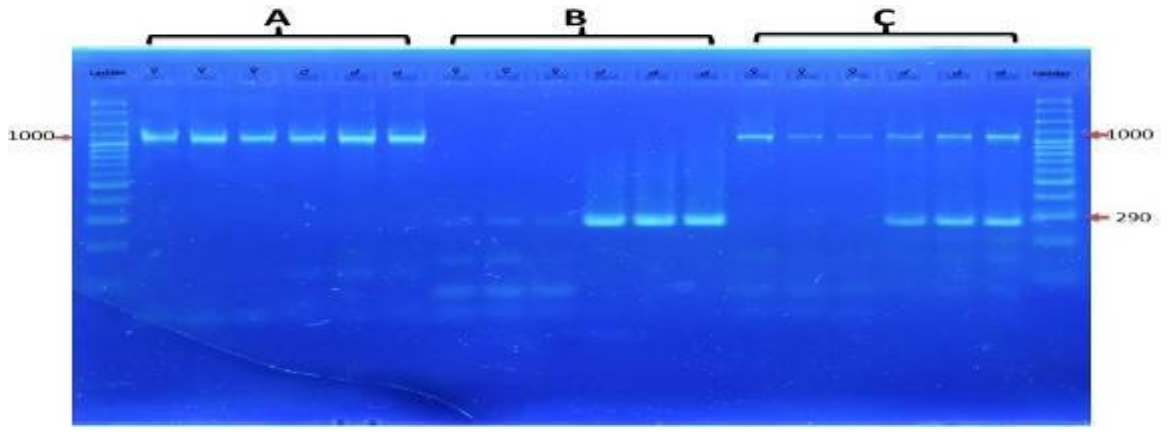

Figure 3. The outcomes of PCR amplification in *N. sumatrana* using primers COX (A), DYT1 (B), and combination of COX and DYT1 (C).

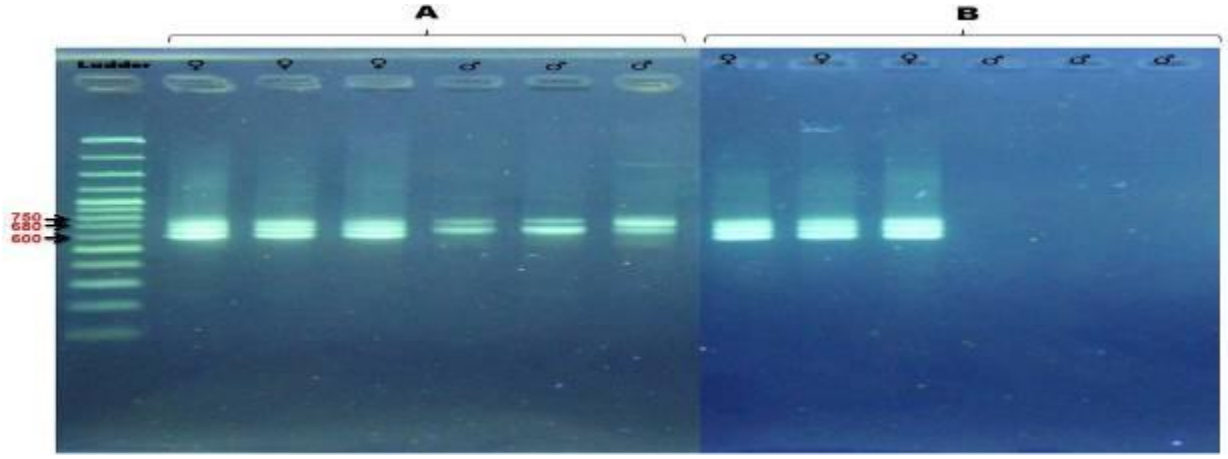

Figure 4. The outcomes of PCR amplification in *N. sumatrana* using primers OPA15 (A) and OPY7 (B).

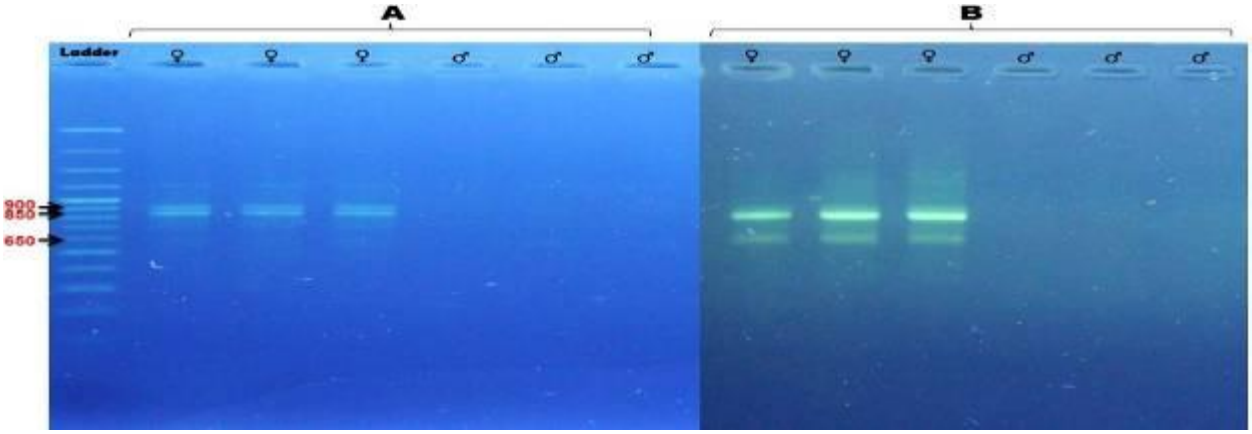

Figure 5. The outcomes of PCR amplification in *N. sumatrana* using primers OPD05 (A) and UBC354 (B).

Table 4. Specific bands of male and female plants in *N. sumatrana.*

No.	Primers	Female (bp)	Male (bp)
1.	DYT1	Absent	290
2.	Combination of COX and DYT1	Absent	290
3.	OPA 15	600	Absent
4.	OPD 05	650, 850	Absent
5.	UBC354	900	Absent
6.	OPY 7	610, 680, 750	Absent

The promising study showed molecular methods for sex determination (male and female) in *Nepenthes sumatrana* succeeded in finding specific bands that appeared on multiple primers (Table 4). Sex identification during the initial stages of development may enable the farming community to selectively transplant female plants while also relocating male plants following the prescribed sex ratio. In dioecious plants, the early identification of sex is crucial for field transfer to save time and reduce expenses, eventually contributing to increased production. Developing early sex detection methods can essentially improve the cultivation of dioecious crops, allowing for the cultivation of a suitable number of productive female trees (depending on market preferences) while minimizing the presence of unproductive male trees.

Utilizing RAPD markers as sex discriminators is easy, cost-effective, and provides rapid analysis. Applying numerous primers enables the search for RAPD markers with only a small quantity of DNA samples being amplified (Dwiati *et al*., 2021). Sequence-characterized amplified region (SCAR) markers may aid in further analyzing the amplification outcomes of particular RAPD markers (Modi *et al*., 2018). The SCAR markers' use verifies the existence of RAPD markers specific to sex (Zhou *et al.,* 2018). Sex-associated RAPD markers reached isolation and refining, with the purification outcomes subsequently ligated into plasmids. Plasmids proceeded subsequent cultivation in cells suitable for cloning. Following the sequencing of the cloned products, the resulting base sequences become useful in the development of SCAR primers. According to Bhagyawant (2016), if the SCAR primers effectively amplify a previously acquired specific marker, then that particular marker also acquires validity.

Anuniwat *et al*. (2009) have implemented the SCAR markers on several *Nepenthes* species, including *N. ampullaria, N. gracilis,* and *N. kampotiana*. As a result of their ability for amplification at elevated annealing temperatures, regions amplified by the SCAR generally emerged more stable than those amplified by alternative PCR methods like RAPD. This stability enables the SCAR to identify the sex of non-flowering plants. The keen interest centered on the sex determination of non-flowering juvenile individuals in numerous commercially valuable crops (Masayuki, 2009). Therefore, we expect that specific bands appearing in some primers can be stronger with SCAR to make them more stable in future applications.

CONCLUSIONS

Based on morphology, qualitative characteristics revealed variations among the *N. sumatrana* species for the stem surface texture, leaf shape, and upper leaf surface texture. On molecular characterization, only the male plants exhibited 290-bp DNA fragments amplified by the primer DYT1. Amplification with the primer OPA-15 produced a 600 bp fragment, OPD05 gave 850 and 650 bp, and UBC354 provided 900 bp DNA fragments solely found in female Nepenthes. The primer OPY7 amplified DNA fragments measuring 610, 680, and 750 bp, which were consistently notable only in female individuals.

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