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New specific primer matK and rbcL region for DNA barcode pitcher plant *Nepenthes spathulata*

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Abstract. *Utama MN, Etikawati N, Sugiyarto, Susilowati A. 2024. New specific primer matK and rbcL region for DNA barcode pitcher plant* Nepenthes spathulata. *Biodiversitas 25: 2515-2523.* The matK and rbcL genes were proposed as the preferred plant barcoding loci by The Consortium for the Barcode of Life (CBOL). DNA barcoding efficiently identifies samples at the species level using short standard DNA sequences. The identification disclosure needs is an important basis given the confusion in systematics in *Nepenthes spathulata*. Therefore, this study aims to design matK and rbcL region-specific primers for DNA barcoding pitcher plants. The primary design uses Primer3Plus with a blueprint from the GenBank KDQ007081.1 for matK and MH346374.1 for rbcL. Following testing primary candidates with Oligo Analyzer and Primer-BLAST, the primers MkNs1, and RbNs4 were selected as the optimal criteria based on the values of ΔG and primer specificity. Optimization of annealing temperature with PCR gradient shows that the temperature range 52-57°C produces a good band for both regions and corresponds to the matK and rbcL region with product sizes of 800-900 bp from seven samples, which could be observed from the gel electrophoresis. Sequence similarity using blast-N, the matK sequence has similarity to the *N. spathulata* matK gene of 99.64% and 99.63% for the rbcL gene of *N. ventricosa* x *N. alata*. This MkNs1 and RbNs4 primer can be used to discover the identity of *N. spathulata* and the *Nepenthes* genus.

Keywords: MatK, *Nepenthes spathulata*, primer, rbcL

INTRODUCTION

Nepenthes spathulata Danser is a pitcher carnivorous plant belonging to the Nepenthaceae family. The distribution of pitcher plants is commonly found on the islands of Sumatra and Java (Hernawati et al. 2022). According to the IUCN Red List, conservation status is the least concerning (Clarke 2018). However, it is a protected species under the Indonesian Ministry of Environment and Forestry laws. These pitcher plants can usually be found epiphytes or attached to tree branches or can also be found terrestrially. On Sumatra Island, *N. spathulata* is commonly found at an altitude of 900-2,850 meters above sea level/masl (Clarke and Moran 2001; Normasiwi et al. 2015). Therefore, optimal growth requires high humidity and low temperatures (Mansur 2013).

This unique carnivorous plant is characterized by pouch-shaped sacs at the tips of leaf tendrils (Labonte et al. 2021), which hang with smooth inner walls as insect traps (Zhang et al. 2021; Lenz and Bauer 2022). Having a green-to-red color attracts insects to approach, which will ultimately be trapped and become a plant food source (Jürgens et al. 2015; Perkovich et al. 2022). This process aims to provide nutrition for *Nepenthes*'s life. The pitcher on this plant can reach a diameter of 5 cm to 10 cm, with fine feather decoration around it in a parallel formation. *N. spatulata* has thick leaves with a smooth surface and shapes like a spoon or spatula (Jebb and Cheek 1997), which is the basis for the name of this species.

Moreover, the clarity in naming this species is crucial; Wartono and Batoro (2017) stated that Javanese endemic *Nepenthes* are a species called *N. adrianii*. However, in the Indonesian carnivorous plant community, *N. adrianii* and *N. spathulata* are the same, just synonyms, because morphologically, they are similar. Morphologically, there have been no specific studies comparing these two species, but many carnivorous plant enthusiasts consider these two species to be the same. This obscurity in the *Nepenthes* species name must be studied further based on morphology and genetic data as the basis of modern taxonomy (Rouhan and Gaudeul 2021).

Molecular methods are urgently crucial to support taxonomic data. Molecular systematics is a method of confirming the kinship of a species using molecular data, observable data, and DNA or GenBank data (Li et al. 2021). Gene markers are specific genes that distinguish one species from another (Ismail et al. 2020). Amplification maturase K gene marker (matK) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (rbcL) of chloroplasts as a specific marker that distinguishes species commonly called DNA barcoding (Poovitha et al. 2015). The matK gene marker has a total sequence length of 1500 bp, and rbcL has approximately 1400 bp, which has high accuracy for DNA barcoding. DNA barcoding using matK and rbcL gene markers has been very popular because it is highly conserved in plant systematics to distinguish at species levels; rbcL is superior in low levels of mutation, and both of these genes can differentiate and have specific base sequences that can differentiate between one species

and another in plant systematically (Ho et al. 2021; Ahmed 2022; Chac and Thinh 2023). The matK and rbcL gene amplification requires a primer in the PCR (Polymerase Chain Reaction). The primer is a single oligonucleotide with a length of 19-30 bp as the initiation of the reaction in DNA amplification (Garafutdinov et al. 2020). The primer becomes an essential component during the amplification process, which consists of forward and reverse primers. The primer we are referring to must be particular because there is obscurity over the naming of *N. spathulata*, so it is necessary to design a new, specific primer to prove the naming of this type of carnivorous plant because universal primers sometimes fail to provide good discrimination or polymorphism at the species level (von Cräutlein et al. 2011; Chandrasekara et al. 2021). This research proposes a new primer using the matK and rbcL gene markers for DNA barcoding *N. spatulata*.

MATERIALS AND METHODS

Study area

Nepenthes spathulata samples were taken from Mount Kunyit, Jambi Province, Indonesia, with latitude 2.276972°S and longitude 101.485474°E at an altitude of 1,970 m asl. (Figure 1).

Sample collection

Samples collected from wildlife will first be identified to ensure whether the sample taken is *N. spathulata*. A total of 7 samples are categorized and sorted based on the height of the place where they were found. The higher the number, the higher the altitude found. If identification has been done, leaves will be taken from *N. spathulata*. The leaves are stored by spraying alcohol first, then wrapped using aluminum foil and plastic clips. During laboratory analysis, the leaves are stored in the refrigerator freezer.

Primer design

DNA markers in this research select the matK and rbcL as potential gene markers for the DNA barcoding plant carnivore *N. spathulata*. The primer design uses the Primer3Plus website, presenting several primer pair candidates (forward and reverse) (Untergasser et al. 2012; Siswanto et al. 2022). The candidate search process requires *N. spathulata* matK and rbcL sequence as a blueprint in the primer candidate search on the Primer3Plus website. Complete matK gene data of *N. spathulata* in the GenBank with accession number KDQ007081.1 used as the blueprint. The rbcL gene blueprint *N. mirabilis* data with accession number MH346374.1 were used because *N. spathulata* rbcL gene data is unavailable in GenBank. Our primer design specification determines for product size range 800-1,000 bp, primer size 19-25 bp, melting temperature primer 57-63°C, primer GC% 45-65%, max Tm difference 5, and in advanced setting GC clamp 1 (Kumar and Chordia 2015).

The primer candidate recommended by Primer3Plus will be tested for quality to the OligoAnalyzer Tools (https://sg.idtdna.com) to reveal the secondary structure of all recommended primer candidates. Secondary structure is the possibility of malfunctions of primer ability in attachment and elongation during the amplification (Owczarzy et al. 2008). The secondary structure observed is the recommended primer candidate's hairpin, self-dimer, and hetero-dimer. From the quality test process with OlygoAnalyzer Tools, the best primer will be selected based on the ΔG value with criteria close to 0. It is balanced, and the energy needed to break the secondary structure decreases. Furthermore, a primer specificity test (in-silico) was performed in PrimerBLAST to look for similarities and sticks to regions in the GeneBank so that the selected primer pairs' specificity level could be observed.

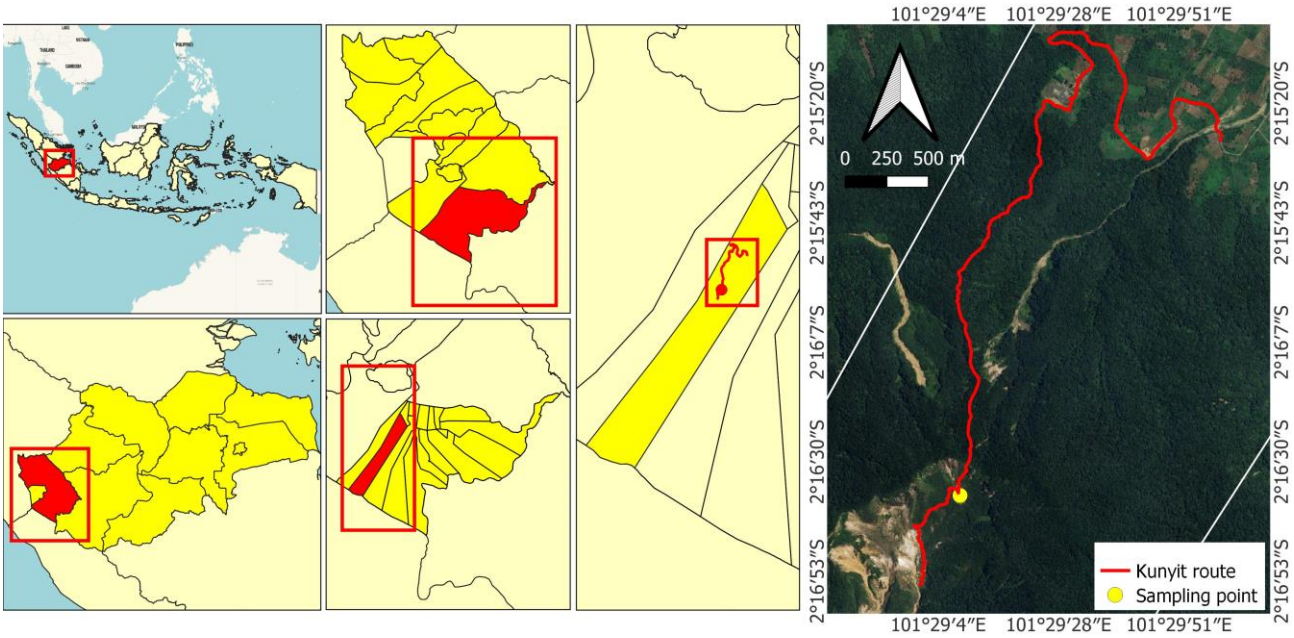


Figure 1. Sample information of *Nepenthes spathulata* in Mt. Kunyit, Jambi Province, Indonesia

DNA extraction

DNA extraction using the Geneaid Genomic DNA mini kit (Plant) with protocols per provider recommendations. This experiment used leaf samples from *N. spathulata* that we collected directly from Mount Kunyit, Jambi, Sumatra, for DNA extraction. DNA extraction utilizes liquid nitrogen when grinding the sample to a fine powder because liquid nitrogen is essential in obtaining high-quality DNA (Aboul-Maaty and Oraby 2019). Based on the extraction protocol from Geneaid, the standard protocol uses Buffer GP1 to lysis the most common plant species. Alternatively, Buffer GPX1 is provided with the kit to ensure efficient cell lysis of plant species with high polysaccharide content. GPX1 buffer was used in this extraction because *N. spathulata* leaves have a high polysaccharide content.

Gene amplification (PCR)

Primer MkNs1 and RbNs4 were synthesized by Integrated DNA Technology (https://sg.idtdna.com/) synthesized primers MkNs1 and RbNs4. The selected primers test directly while optimizing the annealing temperature using PCR gradient using Veriti™ 96-Well Fast Thermal Cycler, Applied Biosystems™ with a temperature range of 52°C to 57°C. PCR mix reaction Bioline MyTaq HS Redmix 25 µL, forward primer 2 µL, reverse primer 2 µL, DNA template 5 µL and ddH_20 up to 50 µL. PCR products were visualized via gel electrophoresis with 1% TAE agarose. The PCR protocol consisted of an initial 2 min predenaturation at 94°C, 35 cycles of 30 s at 94°C (denaturation), 30 s at gradient temperature (annealing), and 1 min at 72°C (extension), and a final 5 min extension at 72 °C.

Sequencing

PCR products are sent to PT Genetica Science Indonesia for single-pass DNA sequencing with ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA. The basic sequencing chemistry used is BigDye® Terminator v3.1 Cycle Sequencing Kit.

Data analysis

The analysis uses BLAST-N software (https://blast.ncbi.nlm.nih.gov/) to determine sequence similarity by matching the sequence data obtained with data in GenBank.

RESULTS AND DISCUSSION

Primer candidates

The study uses the Primer3Plus website to search for primer candidates, and the blueprint uses the KDQ007081.1 gene for the matK sequence from *N. spathulata* and the MH346374.1 gene for rbcL sequence from *N. mirabilis*. Primer3plus recommends primer candidates with a total of 10 pairs. This can be seen in Table 1 and Table 2.

All candidates issued by Primer3Plus are very likely primer candidates. This primer candidate was chosen based on variables such as melting temperature gaps and the presence of secondary structures that can affect primer performance during the amplification process.

Primer candidate quality test

The OlygoAnalyzer (https://sg.idtdna.com) is used to test the quality of the primer candidates recommended by Primer3Plus. This test was performed to determine the presence of secondary structures such as hairpins, self-dimers, and hetero-dimers as a factor in selecting the optimal primer pairing. During the amplification process, the secondary structure produces additional structures from one or both primers, such as the formation of a loop structure, attachment of comparable primers, and attachment between the two primers (forward and reverse). Table 3 and Table 4 shows the quality test of the primer candidate.

Table 1. Design primer products based on sequence KDQ007081.1

Code	O	Sequence	Tm (ºC)	GC (%)	Product size (bp)	Length (bp)
MkNs1	F	GATACCCCATCCTGTCCATCTG	59.7	54.5	885	22
	R	TTGTGTTTCCGAGCCAAAGTTC	59.9	45.5		22
MkNs2	F	GCTACTGGACGAAAGATGCTTC	59.4	50	832	22
	R	CGAGCCAAAGTTCTAGCACAAG	59.8	50		22
MkNs3	F	TTCGCTACTGGACGAAAGATGC	61	50	840	22
	R	GTTTCCGAGCCAAAGTTCTAGC	59.8	50		22
MkNs4	F	AGATAGATCTTGGCAACGTGAC	58.2	45.5	982	22
	R	TCGCACACTTGAAAGAAAACCC	59.8	45.5		22
MkNs5	F	GAGGGATTTGCATTCATTGTGG	58.2	45.5	887	22
	R	ACCGGCTTACTAATGGGATGTC	59.9	50		22
MkNs6	F	TCATTTTTCTCCGCAACCAACC	60	45.5	800	22
	R	ACGTCCTCTATAGAACCTCTGTG	58.8	47.8		23
MkNs7	F	GGAAAATGCCGGTTATGAGAAG	57.7	45.5	901	22
	R	TACCACGGAAGGATTTAGTCGC	60.2	50		22
MkNs8	F	ATACTGATACCCCATCCTGTCC	58.5	50	893	22
	R	CTTTTGTGTTTCCGAGCCAAAG	58.9	45.5		22

Code: Name of primer candidates, O: Oligo, F: Forward primer, R: Reverse primer, Length: Length of primer, GC%: Guanin-Cytosine chain percentage, and Tm: Melting temperature (°C)

Table 2. Design primer products based on sequence MH346374.1

Code	O	Sequence	Tm (°C)	GC (%)	Product size (bp)	Length (bp)
RbNs1	F	GTATTTGGGTTCAAAGCCCTGC	60.4	50	869	22
	R	TTTCCCCAAGGGTGTCCTAAAG	59.9	50		22
RbNs 2	F	TATCCGCTAAGAACTACGGTCG	59.5	50	819	22
	R	TACTCCATTTGCTAGCCTCACG	60.2	50		22
RbNs 3	F	TGCGAATCCCTCCTGCTTATTC	60.5	50	816	22
	R	GGTGTCCTAAAGTTCCTCCACC	60.3	54.5		22
RbNs 4	F	CTGATATCTTGGCAGCATTCCG	59.5	50	864	22
	R	CAGACAGACGTAACGCTTTAGC	59.7	50		22
RbNs 5	F	TAGCTGCCGAATCTTCTACTGG	59.6	50	803	22
	R	ATCTCCACCAGACAGACGTAAC	59.6	50		22
RbNs 6	F	AGTGTTGGATTCAAAGCTGGTG	59.4	45.5	858	22
	R	ACGGTGGATGTGAAGAAGTAGG	59.8	50		23
RbNs 7	F	TATGAATGTCTTCGGGGTGGAC	59.8	50	806	22
	R	GCAGCAGCTAGTTCAGTACTCC	60.7	54.5		22
RbNs 8	F	TGCTCTACGCTTGGAGGATTTG	60.7	50	832	22
	R	TCCTAAAGTTCCTCCACCGAAC	59.7	50		22

Code: Name of primer candidates, O: Oligo, F: Forward primer, R: Reverse primer, Length: Length of primer, GC%: Guanine-Cytosine chain percentage, and Tm: Melting temperature

Table 3. matK primer candidates quality test with OligoAnalyzer

Code	O	Sequence	Product size	Tm (°C)	GC%	Hairpin (ΔG kcal/mole)	Selfdimer (ΔG kcal/mole)	Hetero dimer (ΔG kcal/mole)
MkNs1	F	GATACCCCATCCTGTCCATCTG	885	56.7	54.5	1.37	-3.05	-1.95
	R	TTGTGTTTCCGAGCCAAAGTTC		56.1	45.5	2.25	-3.9	
MkNs2	F	GCTACTGGACGAAAGATGCTTC	832	55.8	50	1.34	-3.61	-6.69
	R	CGAGCCAAAGTTCTAGCACAAG		56.2	50	-0.32	-4.16	
MkNs3	F	TTCGCTACTGGACGAAAGATGC	840	57.3	50	-1.15	-7.13	-5.7
	R	GTTTCCGAGCCAAAGTTCTAGC		56.2	50	1.46	-4.16	
MkNs4	F	AGATAGATCTTGGCAACGTGAC	982	54.6	45.5	0.77	-7.82	-5.12
	R	TCGCACACTTGAAAGAAAACCC		56.1	45.5	-0.61	-3.61	
MkNs5	F	GAGGGATTTGCATTCATTGTGG	887	54.6	45.5	1.15	-7.05	-5.37
	R	ACCGGCTTACTAATGGGATGTC		56.6	50	0.67	-9.75	
MkNs6	F	TCATTTTTCTCCGCAACCAACC	800	56.3	45.4	3.18	-3.61	-5.12
	R	ACGTCCTCTATAGAACCTCTGTG		55.3	47.8	0.65	-9.75	
MkNs7	F	GGAAAATGCCGGTTATGAGAAG	901	54.1	45.5	2.12	-9.75	-6.68
	R	TACCACGGAAGGATTTAGTCGC		56.7	50	-0.78	-3.61	
MkNs8	F	ATACTGATACCCCATCCTGTCC	893	55.6	50	0.57	-3.05	-1.95
	R	CTTTTGTGTTTCCGAGCCAAAG		55	45.5	1	-5.84	

Hm-n: Name of primer candidates, O: Oligo, F: Forward primer, R: Reverse primer, GC%: Guanine-Cytosine chain percentage, and Tm: Melting temperature (°C)

Table 4. rbcL primer candidates quality test with OligoAnalyzer

Code	O	Sequence	Product size	Tm (°C)	GC%	Hairpin (ΔG kcal/mole)	Selfdimer (ΔG kcal/mole)	Hetero dimer (ΔG kcal/mole)
RbNs1	F	GTATTTGGGTTCAAAGCCCTGC	869	56.9	50	-2.48	-6.14	-1.95
	R	TTTCCCCAAGGGTGTCCTAAAG		56.9	50	-1.03	-6.14	
RbNs2	F	TATCCGCTAAGAACTACGGTCG	819	55.9	50	-1.74	-6.68	-6.69
	R	TACTCCATTTGCTAGCCTCACG		56.7	50	1.28	-10.44	
RbNs3	F	TGCGAATCCCTCCTGCTTATTC	816	57.1	50	0.35	-4.99	-5.7
	R	GGTGTCCTAAAGTTCCTCCACC		57.2	54.5	-0.78	-6.37	
RbNs4	F	CTGATATCTTGGCAGCATTCCG	864	56	50	1.43	-3.61	-5.12
	R	CAGACAGACGTAACGCTTTAGC		55.8	50	-0.35	-4.95	
RbNs5	F	TAGCTGCCGAATCTTCTACTGG	803	56.3	50	1.21	-6.34	-5.37
	R	ATCTCCACCAGACAGACGTAAC		56.1	50	1.19	-6.3	
RbNs6	F	AGTGTTGGATTCAAAGCTGGTG	858	55.8	45.5	0.69	-6.34	-5.12
	R	ACGGTGGATGTGAAGAAGTAGG		56.5	50	1.57	-3.61	
RbNs7	F	TATGAATGTCTTCGGGGTGGAC	806	56.6	50	0.62	-3.61	-6.68
	R	GCAGCAGCTAGTTCAGTACTCC		57.4	54.5	-1.05	-6.84	
RbNs8	F	TGCTCTACGCTTGGAGGATTTG	832	57.2	50	-0.88	-3.61	-1.95
	R	TCCTAAAGTTCCTCCACCGAAC		56.4	50	-0.5	-4.86	

Hm-n: Name of primer candidates, O: Oligo, F: Forward primer, R: Reverse primer, GC%: Guanine-Cytosine chain percentage, and Tm: Melting temperature (°C)

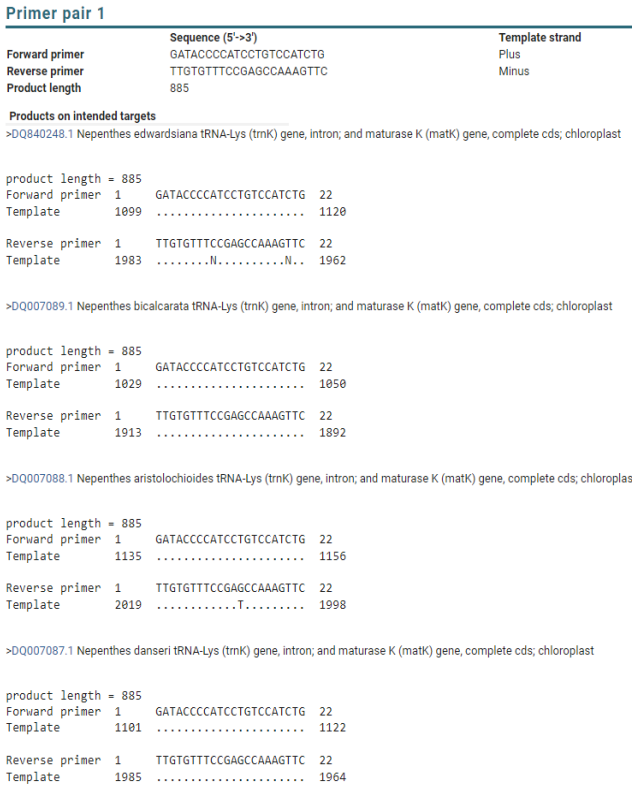
The analysis from the quality test is the primary key to choosing the best primer pair. A good primer criterion is a forward and reverse primer pair. The melting temperature difference does not exceed 5°C because if a pair of primers have a melting temperature difference that is too large, then if used, there must be one primer that is less than optimal and does not even work (Panjkovich and Melo 2005; Green et al. 2015). Other criteria of the best primer seen from the possibility of secondary structure are hairpin, self-dimer, and hetero-dimer (Rodríguez et al. 2015; Bustin and Huggett 2017; Liber et al. 2018). The formed secondary structure will interfere with primer performance; by analyzing gibs, free energy units or ΔG can be seen as the value of the secondary structure. ΔG values close to 0 are the most equilibrium and are best for the performance of a primer.

The best candidate for the quality test of all primer candidates is the MkNs1 and RbNs4 primer pair. This primer pair has the lowest secondary structure value of all primer candidates. MkNs1 has a hairpin value that is the probability of looping of F: 1.37 (ΔG kcal/mole) R: 2.25 (ΔG kcal/mole), a self-dimer value that is the probability of attachment of a similar primer F: -3.05 (ΔG kcal/mole) R: -3.9 (ΔG kcal/mole), and a hetero-dimer value that is a value of the probability of self-attachment of a forward and reverse primer of -1.95 (ΔG kcal/mole). MkNs1 primer pairs have a melting temperature difference of 0.6°C. RbNs4 primer with 22 bp each primer forward and reverse has melting temperature difference of 0.2°C, with low secondary structure hairpin value F: 1.43 (ΔG kcal/mole) R: -0.35 (ΔG kcal/mole), self-dimer value F: -3.61 (ΔG kcal/mole) R: -4.95 and hetero-dimer value is -3.61 (ΔG kcal/mole). Based on all analyses we selected the MkNs1 and RbNs4 primers as the best primer out of all candidates. Therefore, it will proceed to the in-silico specificity test.

Best primer specificity test

Primer-BLAST performed the primer specificity test. Technically, the primer pairs will attach automatically according to their match or degree of similarity to all data nucleotides in the GenBank (Sayers et al. 2021). Figure 2 presents the primer specificity level test MkNs1, and Figures 2 and 3 present RbNs4.

The specificity test shows that from the overall primer gene bank data, selected MkNs1 and RbNs4 showed specific to the genus *Nepenthes*. Although the top target does not specify the species *N. spathulata*, the target referring to the genus *Nepenthes* is considered specific because the matK gene is minimal in GenBank. The specificity shows similarities for hybrid pitcher plants, making it possible to distinguish species. Next, candidate primers are ordered via IDT, which will continue with the annealing temperature optimization on the PCR process to determine the optimum annealing temperature.



Primer pair 1

	Sequence (5'->3')	Template strand
Forward primer	GATACCCCATCCTGTCCATCTG	Plus
Reverse primer	TTGTGTTTCCGAGCCAAAGTTC	Minus
Product length	885	

Products on intended targets

>DQ840248.1 Nepenthes edwardsiana tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene, complete cds; chloroplast

```
product length = 885
Forward primer  1     GATACCCCATCCTGTCCATCTG  22
Template        1099  ......................  1120

Reverse primer  1     TTGTGTTTCCGAGCCAAAGTTC  22
Template        1983  ........N.........N..  1962
```

>DQ007089.1 Nepenthes bicalcarata tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene, complete cds; chloroplast

```
product length = 885
Forward primer  1     GATACCCCATCCTGTCCATCTG  22
Template        1029  ......................  1050

Reverse primer  1     TTGTGTTTCCGAGCCAAAGTTC  22
Template        1913  ......................  1892
```

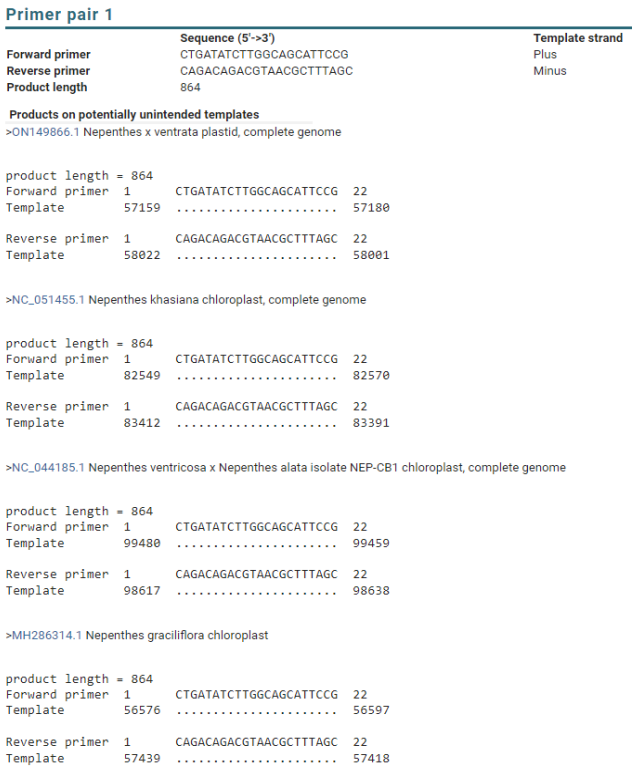
>DQ007088.1 Nepenthes aristolochioides tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene, complete cds; chloroplas

```
product length = 885
Forward primer  1     GATACCCCATCCTGTCCATCTG  22
Template        1135  ......................  1156

Reverse primer  1     TTGTGTTTCCGAGCCAAAGTTC  22
Template        2019  ............T.........  1998
```

>DQ007087.1 Nepenthes danseri tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene, complete cds; chloroplast

```
product length = 885
Forward primer  1     GATACCCCATCCTGTCCATCTG  22
Template        1101  ......................  1122

Reverse primer  1     TTGTGTTTCCGAGCCAAAGTTC  22
Template        1985  ......................  1964
```

Figure 2. Test the level of specificity of selected matK primers in Primer-BLAST NCBI



Primer pair 1

	Sequence (5'->3')	Template strand
Forward primer	CTGATATCTTGGCAGCATTCCG	Plus
Reverse primer	CAGACAGACGTAACGCTTTAGC	Minus
Product length	864	

Products on potentially unintended templates

>ON149866.1 Nepenthes x ventrata plastid, complete genome

```
product length = 864
Forward primer  1      CTGATATCTTGGCAGCATTCCG  22
Template        57159  ......................  57180

Reverse primer  1      CAGACAGACGTAACGCTTTAGC  22
Template        58022  ......................  58001
```

>NC_051455.1 Nepenthes khasiana chloroplast, complete genome

```
product length = 864
Forward primer  1      CTGATATCTTGGCAGCATTCCG  22
Template        82549  ......................  82570

Reverse primer  1      CAGACAGACGTAACGCTTTAGC  22
Template        83412  ......................  83391
```

>NC_044185.1 Nepenthes ventricosa x Nepenthes alata isolate NEP-CB1 chloroplast, complete genome

```
product length = 864
Forward primer  1      CTGATATCTTGGCAGCATTCCG  22
Template        99480  ......................  99459

Reverse primer  1      CAGACAGACGTAACGCTTTAGC  22
Template        98617  ......................  98638
```

>MH286314.1 Nepenthes graciliflora chloroplast

```
product length = 864
Forward primer  1      CTGATATCTTGGCAGCATTCCG  22
Template        56576  ......................  56597

Reverse primer  1      CAGACAGACGTAACGCTTTAGC  22
Template        57439  ......................  57418
```

Figure 3. Test the level of specificity of selected rbcL primers in Primer-BLAST NCBI

Primer annealing optimization

Optimization of annealing temperature PCR gradient with 52°C, 53°C, 54°C, 55°C, 56°C, and 57°C temperatures because the best annealing temperature range is 52°C-57°C. PCR gradients are visualized with gel electrophoresis in 1% TAE agarose (Figure 4).

Optimization of annealing temperature obtained that DNA bands stretched throughout the temperature range 52°C, 53°C, 54°C, 55°C, 56°C, and 57°C. The PCR gradient product explains that the optimal annealing temperature can be used in the temperature range 52°C-57°C with a thick band, no smear, no double band, no faint, and following the target band of 885 bp MkNs1 primer and 864 BNs4 primer.

Primer performance test

MkNs1 and RbNs4 are the best primers after in-silico tests constructed by IDT (Integrated DNA Technology) and tested directly for amplification with DNA template from *N. spathulata*. Gel electrophoresis of DNA amplicon MkNs1 primer is present in Figure 5, and RbNs4 primer in Figure 6.

The gel electrophoresis shows excellent primer performance of the MkNs1 and RbNs4. Seven samples of original *N. spathulata* obtained a thick band with high concentration and product size according to a plan of 800-900 bp, revealing that primers can amplify the matK gene and rbcL of *N. spathulata*.

Sequence similarity

Sequence similarity was tested in BLAST-Nucleotide (https://blast.ncbi.nlm.nih.gov/) to identify sequences obtained with data available in GenBank, as shown in Tables 5 and 6.

Sequence similarity shows that the sequence amplified using the MkNs1 primer with *N. spathulata* in GenBank has a similarity of 99.64%, indicating very high similarity with the sequence amplified with the MkNs1 primer. The MkNs1 primer is similar to other specific species in the *Nepenthes* genus, which directly targets the matK gene. Meanwhile, samples amplified with the RbNs4 primer showed on-target similarity to the rbcL gene. The highest similarity is in *N. ventricosa* x *N. alata* and *N. graciliflora*, with a similarity percentage of 99.63%. No *N. spathulata* is similar to this rbcL sequence because *N. spathulata* data is unavailable in GenBank.

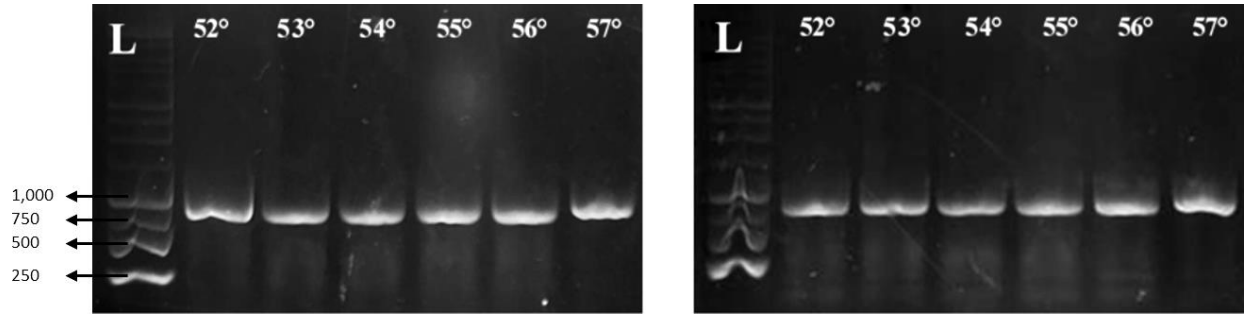


Figure 4. Gel electrophoresis of annealing temperature-optimization PCR gradient. A. MkNs1 primer; B. RbNs4 primer

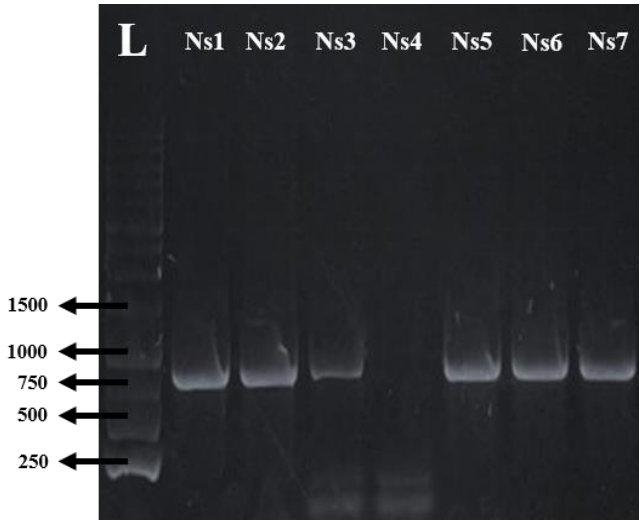


Figure 5. The electrophoresis gel from the matK gene *Nepenthes spathulata* amplicon showed the sample's DNA measurement band in the 800-900 bp range in the 1Kb ladder.

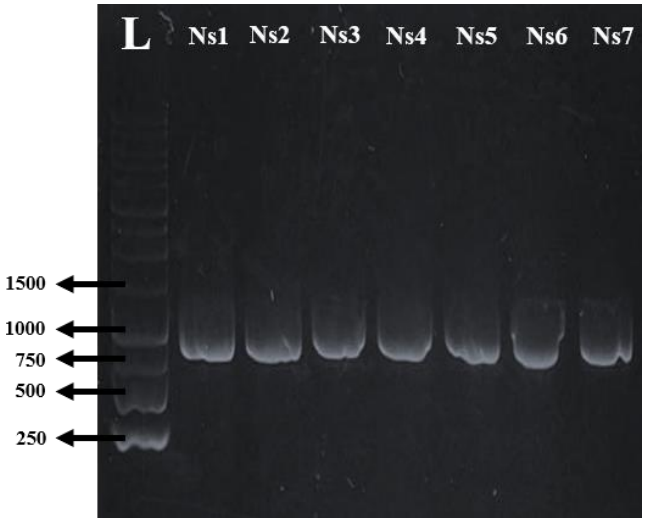


Figure 6. Electrophoresis gel from amplicon of rbcL gene *Nepenthes spathulata*, showed DNA measurement band of the sample in the 800-900 bp range in 1Kb ladder

Table 5. Sequence similarity based on sequence primer MkNs1

Description	Scientific name	Max score	Total score	Query cover	E value	Per. Ident	Acc. Len	Accession
Nepenthes spathulata tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene, complete cds; chloroplast	*Nepenthes spathulata*	1519	1519	99%	0.0	99.64%	2315	DQ007081.1
Nepenthes spathulata translocated tRNA-Lys (trnK) pseudogene, partial sequence	*Nepenthes spathulata*	1365	1365	99%	0.0	96.62%	2887	DQ007145.1

Table 6. Sequence similarity based on sequence primer RbNs4

Description	Scientific name	Max score	Total score	Query cover	E value	Per. Ident	Acc. Len	Accession
Nepenthes ventricosa x *Nepenthes alata* isolate NEP-C81 chloroplast, complete genome	*Nepenthes x ventricosa*	1495	1495	93%	0.0	99.63%	156637	NC_044185.1
Nepenthes graciliflora chloroplast	*Nepenthes graciliflora*	1495	1495	93%	0.0	99.63%	156176	MH286314.1
Nepenthes mirabilis plastid, complete genome	*Nepenthes mirabilis*	1495	1495	93%	0.0	99.63%	156381	MK397881.1
Nepenthes x ventrata plastid, complete genome	*Nepenthes x ventrata*	1495	1495	93%	0.0	99.63%	156637	ON149866.1

Discussion

DNA barcoding is based on the premise that a short standardized sequence can distinguish individuals of a species because genetic variation between species exceeds that within species (Hebert et al. 2003; Kolter and Gemeinholzer 2020; Antil et al. 2023). This method is needed in the current era with many taxonomic confusion or misconceptions phenomena. Modern taxonomy using genetic data will further increase accuracy in the specified grouping species (Goldstein and DeSalle 2010). Genetic data can support morphological, anatomical, and habitat data for grouping species (Padial and De la Riva 2021; Rouhan and Gaudeul 2021). The selection of gene markers is crucial when researchers, especially taxonomists, want to identify species based on DNA sequence (Abbas et al. 2020). Gene markers are specific DNA fragments that can be used as species-specific markers compared to other species; it is helpful if species have similar morphology, anatomy, or habitat. Therefore, the availability of the DNA sequence data is more appropriate when there is no way out of the taxonomical problem. The Consortium for the Barcode of Life (CBOL) Plant Working Group proposed the chloroplast gene rbcL and matK as the core barcodes of plant species. matK is a conserved gene and one of the most rapidly evolving plastid coding regions, and it consistently showed high levels of discrimination among angiosperm species (CBOL Plant Working Group 2009). Therefore, the same species will have matK sequences that tend to be similar (Mathew and Ramesh 2020). If the matK gene sequence differs significantly, it can be ascertained that the two species are different. The matK has also been widely used in DNA barcode research in plants due to its high accuracy in species grouping (Ismail et al. 2020; Mathew and Ramesh 2020; Li et al. 2021). At the same time, rbcL genes have been widely used for phylogenetic analysis within families and subclasss of angiosperm and even among the different groups of seed plants. For example, Jamdade et al. (2021) compared ~1,180 rbcL sequences (with each more than 1,000 bp) collected from GenBank by a distance method and found that rbcL did recognize 89.79% of plant species distinguished plants within the same genus.

Disclosure of identity using molecular data is essential if taxonomists need clarification in naming. In this case, the big theme is finding the true identity of *N. adrianii*, which is still considered a synonym for *N. spathulata* based on discussions at the Indonesian Carnivorous Plant Community and the lack of more in-depth publications about *N. adrianii*. Therefore, the primer construction of this matK and rbcL region will be the basis for continuing to reveal the original identity of this carnivore plant. Genetic data is important in modern taxonomy to strengthen further morphological, physiological, and ecological data for claims to the establishment of a species (Cornwell et al. 2019).

After a quality test, it has been proven to amplify the matK and rbcL target gene of *N. spathulata* with MkNs1 and RbNs4. MkNs1 primer with a primer length of 22 bp each, a melting temperature difference of 0.6, with low secondary structure detail namely hairpin value F: 1.37 (ΔG kcal/mole) R: 2.25 (ΔG kcal/mole), self-dimer value is the possibility of attachment of similar primers F: -3.05 (ΔG kcal/mole) R: -3.9 (ΔG kcal/mole) and hetero-dimer values are the value of the probability of self-attachment of primer forward and reverse -1.95. (ΔG kcal/mole). RbNs4 primer with 22 bp each primer forward and reverse has melting temperature difference of 0.2, with low secondary structure hairpin value F: 1.43 (ΔG kcal/mole) R: -0.35 (ΔG kcal/mole), self-dimer value F: -3.61 (ΔG kcal/mole) R: -4.95 and hetero-dimer value is -3.61 (ΔG kcal/mole). A

small ΔG value means that the possibility of secondary structure occurs minimal, and the number 0 is the Gibbs free energy equilibrium number (ΔG), which affects primer performance in the concept of thermodynamic law. Furthermore, the MkNs1 and RbNs4 primers do not have more than 5 mass repeats, for example, GGGGGGA, because with too high base repetition, there will be the potential for secondary structure in the primer when used for target gene amplification. Our primer is considered adequate with its characteristics, with a GC content is 50% and a GC clamp at the end of the sequence. G and C bases have stronger hydrogen bonds and help primer stability in the attachment process. However, GC content should not be too high because of the potential for dimer formation in the amplification process (Kumar et al. 2017). Bases G and C also affect Tm, G, and C, resulting in higher temperatures than A and T (Delghandi et al. 2022).

These MkNs1 and RbNs4 primers are designed specifically for the species *N. spathulata*. However, based on primer data, this BLAST primer and BLAST nucleotide can also be used for other species in the genus *Nepenthes*. This specific primer will be helpful for other research related to the genus *Nepenthes*. It can be used for identity disclosure or population genetics purposes. It may also be related to its preservation in the wild because this species is protected according to Indonesian law.

In conclusion, MkNs1 with forward sequence GATACCCCATCCTGTCCATCTG and reverse sequence TTGTGTTTCCGAGCCAAAGTTC also rbcL with forward sequence CTGATATCTTGGCAGCATTCCG and reverse sequence CAGACAGACGTAACGCTTTAGC were successful at amplifying chloroplast DNA in the matK region and rbcL region from *N. spathulata*. Due to primer optimization, the annealing temperature for PCR is 52-57°C with 800-900 bp product. This primer can amplify the matK and rbcL region, which can be used for DNA barcoding specifically for the *Nepenthes* genus, as proven by sequence similarity based on BLAST-Nucleotide.

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