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ARTICLE

Development of SSR markers and assessment of genetic diversity in native *Nepenthes* spp. in southern Thailand

Desenvolvimento de marcadores SSR e avaliação da diversidade genética em *Nepenthes* spp. nativas do sul da Tailândia

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Abstract: *Nepenthes* spp. (Nepenthaceae) is one of the popular genera of carnivorous plants, which has a special leaf modification with varying shapes, sizes, and shades of color. These plants exhibit high variation within species and populations, it is difficult to classify them by morphology. As a result, molecular techniques are needed to identify genetic diversity. Therefore, this study aimed to generate simple sequence repeat (SSR) markers and assess the genetic diversity of nine native *Nepenthes* spp. in southern Thailand. In the present study, A total of 20 SSR primer pairs containing dinucleotide or trinucleotide motifs were identified from 2,138 nucleotides and 41 ESTs of Nepenthaceae and 950 nucleotides of carnivorous plants from that database were retrieved from GenBank. 2.0. Only 10 were polymorphic, showing 2-7 alleles and on average 3.7 alleles per locus. Based on SSR markers, the nine *Nepenthes* spp. were clustered into two main groups by SSR scoring data using UPGMA cluster analysis with a cophenetic correlation was 0**.76**. This is the first report that identified *N. mirabilis* var. globosa as distinct from *N. mirabilis* by SSR markers. The results suggested that SSR markers can successfully reveal variability among *Nepenthes* spp. It can be useful to evaluate genetic diversity studies, provide practical information for parental selection and to assist breeding programs and the result is important to the conservation action of endangered species.
Keywords: carnivorous plants, genetic identification, *Nepenthes* spp., pitcher plant, simple sequence repeats.

Resumo: *Nepenthes* spp. (Nepenthaceae) é um dos gêneros populares de plantas carnívoras, que apresenta uma modificação especial da folha, com formas, tamanhos e tonalidades de cor variados. Essas plantas exibem alta variação dentro das espécies e populações, tornando difícil sua classificação apenas por características morfológicas. Como resultado, técnicas moleculares são necessárias para identificar a diversidade genética. Portanto, este estudo teve como objetivo desenvolver marcadores de repetições de sequência simples (SSR) e avaliar a diversidade genética de nove espécies nativas de *Nepenthes* no sul da Tailândia. No presente estudo, foram identificados 20 pares de primers SSR contendo motivos de dinucleotídeos ou trinucleotídeos a partir de 2.138 nucleotídeos e 41 ESTs da família Nepenthaceae, além de 950 nucleotídeos de plantas carnívoras recuperados do banco de dados GenBank. Apenas 10 marcadores foram polimórficos, apresentando de 2 a 7 alelos, com uma média de 3,7 alelos por loco. Com base nos marcadores SSR, as nove espécies de *Nepenthes* foram agrupadas em dois grupos principais por meio da análise de agrupamento UPGMA, com coeficiente de correlação cofenética de 0,76. Este é o primeiro relato que identifica *N. mirabilis* var. globosa como distinta de *N. mirabilis* por meio de marcadores SSR. Os resultados indicam que os marcadores SSR são eficazes na detecção da variabilidade entre as espécies de *Nepenthes*. Eles podem ser úteis em estudos de diversidade genética, fornecer informações práticas para a seleção parental, auxiliar em programas de melhoramento e contribuir para ações de conservação de espécies ameaçadas de extinção.
Palavras-chave: plantas carnívoras, identificação genética, *Nepenthes* spp., planta jarro, repetições de sequência simples.

Introduction

Nepenthes is a genus of carnivorous plants in the monotypic family Nepenthaceae. They are also known as tropical pitcher plants or monkey cups. Its leaf modification known as a pitcher is used to trap prey such as termites, ants, and insects. This adaptation helps them to environmental stress, especially the habitat with a lack of nitrogen and highly acidic soil (Cheek et al., 2020). The pitchers of different species have diverse morphological characteristics, including variations in shape, size, and coloration, which may be linked to different dietary preferences (Ghazalli et al., 2021). Recently, *Nepenthes* were popular as an ornamental plant due to the unique and attractive features of its pitcher. However, wild *Nepenthes* species are frequently extracted from their natural forest habitats for commercial trade, and the processes of hybridization and introgression within the genus contribute to the diversification of pitcher morphological traits (Scharmann et al., 2021).

This genus comprises roughly 192 naturally occurring species found in the tropics (Mcpherson, 2023). They are mostly liana-forming plants and are distributed across South China, Indonesia, Malaysia, Philippines, Madagascar, Australia, India, Sri Lanka and Thailand. In 2021, 80 species of *Nepenthes* were recorded from Indonesia (Mansur et al., 2021). *Nepenthes* spp. is a protected plant and listed for conservation in CITES (Conservation of International Trade in Endangered Species of Wild Fauna and Flora), which regulates the international trade of wild animals and plants that may face extinction. The populations of *Nepenthes* species in the wild are predicted to decline due to various factors such as increasing habitat degradation, climate change, overharvesting, forest fires, illegal logging, conversion of forest land or shrubs into residential areas, plantations, agriculture and mining, and forest reclamation for plantations. (Pistanty, 2023; Mansur et al., 2024) These activities have led to disturbed *Nepenthes* habitats and many wild *Nepenthes* spp. has been lost, which has made them rare in local forests. Therefore, effective conservation measures are needed (Cross et al., 2020).

Currently, 14 species and two variety of *Nepenthes* are recognized in Thailand (Nuanlaong, 2022). Nine species of *Nepenthes* are found in southern Thailand, namely, *N. suratensis*, *N. mirabilis, N. mirabilis* var. globosa, *N. andamana***,** *N. krabiensis, N. rosea, N. gracilis, N. ampullaria* and *N. kongkandana. N. mirabilis* has a high tolerance to various habitat conditions. It was found in a variety of habitats both in shaded areas

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and open areas and has a wide distribution. *N. mirabilis* var. globosa is popularly known as both *Nepenthes globosa* and *Nepenthes* sp. Viking. This variety is immediately recognizable by the oval shape of the pitcher body resembling the prow of a Viking ship, which is usually wholly red. Distribution areas are in sandy soil, on open savannahs and grasslands in Trang and the islands of Phang Nga (Catalano, 2010a). The grower called "Tigers" for a group of *Nepenthes* with longitudinal red streaks on the outer surface and red dots or small patches on the inner side such as *N. thorelii*, *N. andamana*, *N. suratensis*, *N. smilesii* and *N. kampotiana* (Catalano, 2010b, 2010c). Some species were endemic to the southern region of Thailand, such as *N. suratensis* in Surat Thani, *N. krabiensis* in Krabi, and *N. kongkandana* in Songkhla Province and currently found that the wild population was decreasing especially, *N. suratensis* was found only in a protected area. Normally, these plants are characterized by their insect-catching pitchers that exhibit high variation within species and populations because *Nepenthes* spp. is a plant that can hybridize naturally, and morphological characters are easily affected by environmental factors. As a result, identifying a variety of these species by morphological characters is usually difficult. To address this, molecular techniques are essential for accurately identifying the genetic diversity of *Nepenthes*.

Molecular markers, particularly Simple Sequence Repeats (SSRs), have proven to be effective tools in assessing genetic diversity and population structure due to their co-dominant inheritance, high polymorphism, and reproducibility. SSR markers are essential for breeding and crop improvement, conservation, protection, introduction, and reintroduction of endangered and valuable plants such as *Clematis acerifolia*, *Pyrus* spp. and *Saussurea involucrate* (Hussain and Nisar, 2020; Ouni et al., 2020; Tuvesson et al., 2021; Zhao et al., 2023; Hu et al., 2024). Nevertheless, current genetic knowledge is very limited for *Nepenthes* spp., which hinders genetic conservation and improvement of these endangered. Therefore, this study aimed to develop SSR markers and applied to investigate genetic diversity and phylogenetic relationships among native *Nepenthes* populations in southern Thailand. The findings will provide insights into the genetic resources of these unique plants and contribute to their conservation and management.

Materials and Methods

Plant materials and DNA extraction

The *Nepenthes* species analyzed in this study were collected from wild habitats across various geographical regions in southern Thailand. To capture the widest possible genetic diversity, individuals were sampled from locations that maximized interspecies distances, reflecting local variations of these carnivorous plants. *Nepenthes suratensis* was sampled from Amphoe Kanchanadit, Surat Thani Province, while *N. mirabilis* was collected from Amphoe Mueang Surat Thani, Surat Thani Province, and Amphoe Khura Buri, Phangnga Province. The variety *N. mirabilis* var. globosa was obtained from Amphoe Mueang Trang, Trang Province. *N. andamana* was sampled from Amphoe Takua Pa, Phangnga Province, whereas *N. krabiensis* originated from Amphoe Khlong Thom, Krabi Province. *N. gracilis* was collected from Amphoe Thung Wa, Satun Province, and Amphoe Pa Bon, Phatthalung Province. *N. ampullaria* was found in Amphoe Saba Yoi, Songkhla Province, and *N. kongkandana* in Amphoe Khlong Hoi Khong, Songkhla Province. Lastly, *N. rosea* was sampled from Amphoe Mueang Krabi, Krabi Province (Fig. 1. and Fig. 2.). *N. rafflesiana* from Malaysia was included as an outgroup control. This diverse sampling strategy ensures comprehensive genetic representation across multiple habitats, facilitating robust genetic and evolutionary analyses. Fresh young leaves were harvested and immediately stored at -20 °C for subsequent genomic DNA isolation.

Fig. 1. The nine species of *Nepenthes* analyzed in this study: (A) *N. suratensis*, (B) *N. mirabilis*, (C) *N. mirabilis* var. globosa (D) *N. andamana*, (E) *N. rosea*, (F) *N. krabiensis*, (G) *N. kongkandana*, (H) *N. gracilis* and (I) *N. ampullaria,* Bar = 5.0 cm.

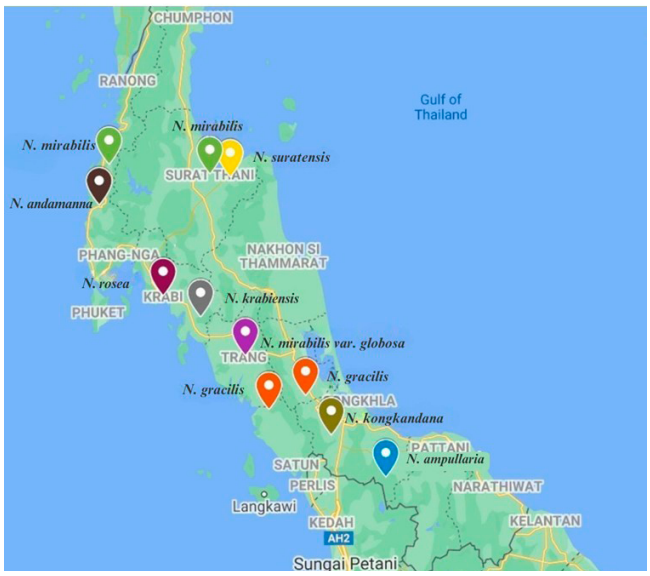

Fig. 2. Map of the collection sites of *Nepenthes* species across various districts in southern Thailand.

The genomic DNA was isolated by using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987) with 2% CTAB to extract the fresh leaves. The integrity and quality of the DNA were evaluated by electrophoresis on 0.7% (w v^{-1}) agarose gel in 1× TBE buffer at 100 volts for 30 min. and photos were taken under UV using a gel documentation system. The purity of DNA was estimated from the ratio of absorbance at 260 nm and 280 nm (A_{260nm}/A_{280nm}), using NanoDrop Lite (Thermo Scientific, USA).

Primer design and screening of primers

The SSR primers were designed from sequences of the 2,138 nucleotides of *Nepenthaceae*, 41 EST sequences of *Nepenthaceae,* and 950 nucleotides of carnivorous plants from the database retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). These sequences were analyzed to identify the perfect SSR loci using Websat software (http://purl.oclc.org/NET/websat) (Martins et al., 2009) using the following criteria: ≥ 5 repeat units for dinucleotides and trinucleotides, ≥ 4 for tetranucleotides and pentanucleotides and ≥ 3 for hexanucleotides. SSR loci with appropriate flanking sequences were selected for primer design using the Primer 3 software (http://primer3plus.com/ web_3.0.0/primer3web_input.htm). The parameters for designing the primers consisted of several parameters: the size of the primers ranging from 19 to 23 bp, amplification product size ranging from 100 – 300 bp, GC content ranging from 40–60%, and optimum annealing temperature (Tm) ranging from 55 – 60 °C. The primers were tested for performance in PCR amplification of *N. mirabilis* samples for their species specificity. Suitable primer combinations were selected that could amplify in all samples having polymorphic bands.

The SSR amplification was performed in a final volume of 10 µL of PCR mixture solution containing 100 ng/µL of DNA template, 3 mM $MgCl_2$, 800 µM dNTPs, 0.5 µM forward primer, 0.5 µM Reverse primer and 1U *Taq* DNA Polymerase (Thermo Fisher scientific, USA). The amplification was performed with the following cycle program: pre-denature at 94 ºC for 3 min., followed by 35 cycles of denaturation at 94 ºC for 45 s, annealing at 55, 58, 60, and 62 ºC for 30 s, and extension at 72 ºC for 1 min. The amplification was finalized by a final extension at 72 ºC for 5 min. PCR products were separated on 1.5% (w v^{-1}) agarose gel by electrophoresis in 1× TBE buffer at 50 volts for 60 min. Photographic imaging under UV light was done with a gel documentation system (Lumi BIS, DNR Bio Imaging Systems).

SSR Amplification

Genomic DNA extraction randomly collected of eight individuals of each *Nepenthes* species were identified. Suitable SSR primers were used in PCR amplification that was carried out in a 15 µL reaction mix containing 1 × Master Mix of Dream Taq PCR master mix (cat. no. K1071, Thermo Fisher Scientific), 0.5 µM of each primer (forward and reverse), and 100 ng μL^{-1} of genomic DNA template. The PCR amplification conditions consisted of 94 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 45 s, annealing at the annealing temperature of each primer pair for 30 s, extension at 72 ºC for 1 min, and the final extension at 72 ºC for 5 min. Negative controls were included for each PCR reaction. Amplification products were separated on 4.5% polyacrylamide gel by electrophoresis in 1× TBE buffer under a 100 W constant power supply for 1 – 3 h. After electrophoresis, the products were visualized by silver staining.

Data analysis

Reproducible and consistent SSR fragments were scored manually in terms of a binary data matrix from the gel electrophoresis photos as present (1) or absent (0) for each of the SSR markers. The cluster analysis was conducted by using NTSYS-pc version 2.20e software (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 2000). The SIMQUAL (Similarity for Qualitative Data) program was used to evaluate simple matching. Similarity matrices were utilized to generate the dendrogram using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) indicative of clustering by similarity matrices calculated using the simple matching (SM) coefficient.

Results and discussion

Primer design and screening of primers

A total of 20 SSR primer pairs were developed, 15 SSR primers were designed from sequences of the 2,138 nucleotides of *Nepenthaceae*, 3 primers from 950 nucleotides of carnivorous plants (*S. purpurea*) and 2 primers from 41 EST sequences of *Nepenthaceae* linked aspartic proteinase production.

Screened using *N. mirabilis* showed that primers have the repeat motifs include dinucleotide sequences (60%) and trinucleotide sequences (40%), which are valuable for detecting genetic polymorphism. 10 SSR markers (50%) from sequences of the 2,138 nucleotides of *Nepenthacea* generated reproducible polymorphic DNA amplification products at an annealing temperature 58 °C (Nep_SSR1, Nep_SSR3, Nep_SSR6, Nep_SSR7, Nep_SSR8, Nep_SSR12, Nep_SSR14 and Nep_SSR18), and 55 °C (Nep_SSR5 and Nep_SSR17) (Table 1), which ensures compatibility in PCR amplification. The product sizes, ranging from 206 bp to 350 bp, are optimal for downstream genotyping, ensuring robust amplification even in cases of degraded DNA (Hu et al., 2024). Such primers have shown high specificity and reliability in consistently amplifying distinct bands, making them indispensable for genetic diversity studies.

Table 1. Ten successful SSR primers that produced a specific band marker for *N. mirabilis*.

primer	primer sequence	Repeat motif	Tm (°C)	Product size (bp)
Nep_SSR1	F 5′-GTTCTAACAAATGGGGTTGACT-3′	$(TA)_5$	58	289
	R 3′-CTATCTTCATTCTCGTCCGATT-5′			
Nep_SSR3	F 5′-ATATTTCTATCGCATTGGCCCT-3′	$(TA)_5$	58	241
	R 3′-TATATGGCGCAACCCTTGTT-5′			
Nep_SSR5	F 5′-CAAAAGAGCGATTGGATTG-3′	$(TTA)_6$	55	216
	R 3′-TAAAAGTAGAGGGTTTCTTGGG-5′			
Nep_SSR6	F 5′-CTACGAGGCATTTCTGTGAGT-3′	$(AT)_5$	58	292
	R 3′-TACCTACAGCTTCTCCCAATTC-5′			
Nep_SSR7	F 5′-CAAAAGAGCGATTGGATTG-3′	$(TTA)_6$	58	209
	R 3′-AGAGGGTTTTTGGGGTTATC-5′			
Nep_SSR8	F 5′-CAAAAGAGCGATTGGATTG-3′	$(TTA)_4$	58	206
	R 3′-GGGTTTCTTGGGTTATCAAA-5′			
Nep_SSR12	F 5′-GCATTCGATTTCGTTGGTA-3′	$(TA)_5$	58	350
	R 3′-TAGATGGGAAAATATGCGGA-5′			
Nep_SSR14	F 5′-TTCTAACAAATGGGGTTGACTG-3′	$(TA)_8$	58	294
	R 3′-CTCTATCTTCATTCTCGTCCGAT-5′			
Nep_SSR17	F 5′-TCAAAAGAGCGATTGGATTG-3′	$(TTA)_6$	55	233
	R 3′-GGACCGGAAACTCAAGTAAAAG-5′			
Nep_SSR18	F 5′-TTCTAACAAATGGGGTTGACTG-3′	$(TA)_8$	58	298
	R 3′-GACTCTATCTTCATTCTCGTCCG-5′			

Note: Tm (°C) = annealing temperature

SSR Analysis

The 10 SSR primer pairs generated a total of 1,095 fragments, with an average of 109.5 fragments per primer pair, ranging from 93 (Nep_SSR3) to 184 (Nep_SSR5) (Fig. 3). The results indicated a moderate level of genetic diversity, with SSR fragments ranging from 2 to 7 alleles per locus, averaging 3.7 alleles. This is comparable to the allelic variation reported in pigeonpea (*Cajanus cajan*) by Manju et al. (2017), although direct comparisons between species should be interpreted with caution due to differences in population structure, sample size, and marker selection. The average alleles per locus can be attributed to genetic diversity in the investigated genotypes. A total of 167 were polymorphic and ranged from 8 to 33 with on average 16.7 polymorphic fragments per primer pair. The percentage of polymorphic bands across the primer pairs varied from 4.34 to 35.48% with an average of 16.82%. Especially, Nep_SSR3, Nep_SSR12, and Nep_SSR1 showed high levels of polymorphism in bands as 33 (35.48%), 28 (29.16%) and 23 (23.95%), respectively (Table 2). The findings indicated that *Nepenthes* populations exhibited greater genetic variation and polymorphism compared to *Clematis acerifolia* and *Zea mays* L. (Shete et al., 2023; Zhao et al., 2023), but lower levels than those reported for *Viburnum* L. and *Lycopersicon esculentum* Mill. (Okumuş and Dağidir, 2021; Hamm et al., 2023). This difference may be due to the limited genomic resources available for *Nepenthes* and the use of primers that were not fully optimized for this genus. However, in the current study, primer Nep_SSR3, Nep_SSR12, and Nep_SSR1 showing highly polymorphism levels. There can serve as powerful tools in future genetic studies of *Nepenthes* species. Their high polymorphism rates suggest they can effectively distinguish between different genotypes, facilitating studies on genetic diversity, population structure, and evolutionary relationships.

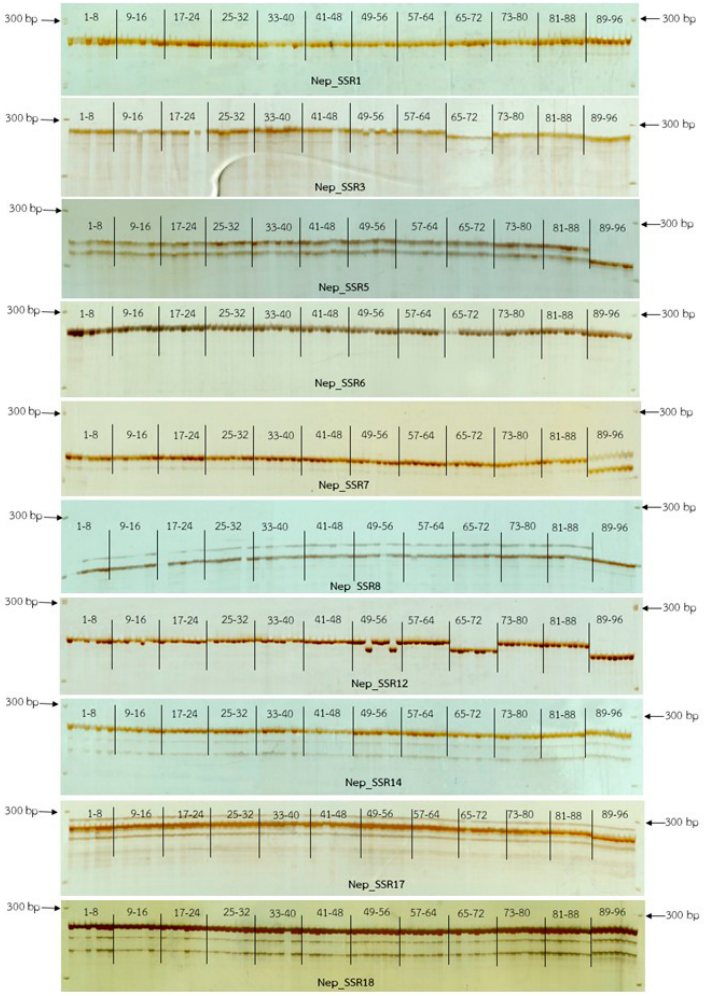

Fig. 3 SSR amplification profiles of nine *Nepenthes* species in southern Thailand using different primers. Lane: 1-*8 N. mirabilis*, Lane: 9-16 *N. mirabilis* "Phangnga", Lane: 17-24 *N. mirabilis* var. globosa, Lane: 25-32 *N. suratensis*, Lane: 33-40 *N. andamana*, Lane: 41-48 *N. gracilis* "Satun", Lane: 49-56 *N. gracilis* 'Phatthalung', Lane: 57-64 *N. kongkandana*, Lane: 65-72 *N. ampularia*, Lane: 73-80 *N.rosea*, Lane: 81-88 *N. krabiensis* and Lane: 89-96 *N. rafflesiana*

Table 2. SSR primer combinations of analyzed *Nepenthes* spp. in southern Thailand.

primer	Total of Number Bands	Number of Polymorphic Bands	Percentage of Polymorphic Bands
Nep_SSR1	96	23	23.95
Nep_SSR3	93	33	35.48
Nep_SSR5	184	8	4.34
Nep_SSR6	96	16	16.66
Nep_SSR7	96	8	8.33
Nep_SSR8	147	8	5.44
Nep_SSR12	96	28	29.16
Nep_SSR14	96	8	8.33
Nep_SSR17	95	8	8.42
Nep_SSR18	96	18	18.75
Total	1,095	167	
Average	109.5	16.7	16.82

A total of 1,095 loci were accounted for to calculate the genetic diversity among the nine *Nepenthes* spp. in southern Thailand. Binary data matrices produced by SSRs were used to estimate the genetic similarity of the genotyped *Nepenthes* spp. samples. A dendrogram was constructed using UPGMA cluster analysis based on the corresponding genetic similarity coefficient among the tested nine *Nepenthes* spp. populations. In this study, the similarity coefficient of 96 samples ranged from 0.34 to 1.00. In this dendrogram, the nine species of *Nepenthes* found in southern Thailand can be distinguished from the foreign *Nepenthes* species (*N. rafflesiana*) used as the control group at the position of 0.34. The nine species of *Nepenthes* can be classified into two groups at a similarity level of 0.76. Group I can be further divided into two subgroups at a similarity level of 0.86. Subgroup I consists of *N. mirabilis* and *N. mirabilis 'Phangnga'*, with similar morphology and geographic locations. Subgroup II can be further divided into two clusters at a similarity level of 0.91. Cluster I includes *N. mirabilis var. globosa*, *N. suratensis*, and *N. andamana*, showing the same morphological characteristics as red dots on the pitcher, while Cluster II consists of *N. gracilis 'Satun'*, *N. kongkandana*, and *N. gracilis 'Phatthalung'*. Group II can also be divided into two subgroups at a similarity level of 0.84. Subgroup I comprises *N. ampullaria*, and Subgroup II consists of *N. rosea* and *N. krabiensis* (Fig. 4).

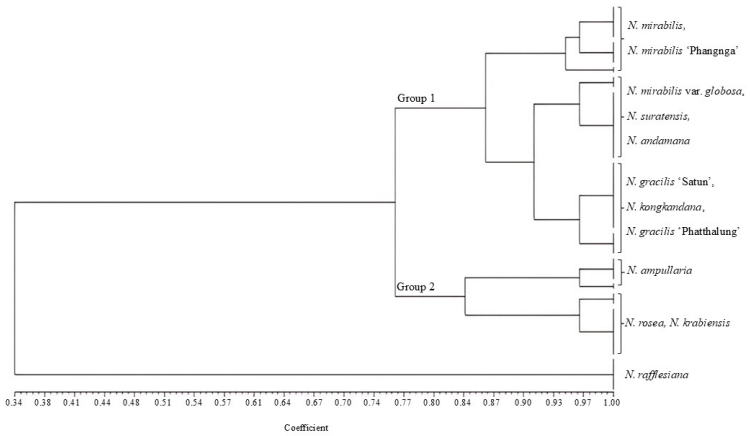

Fig. 4. UPGMA dendrogram of nine species of *Nepenthes* in southern Thailand using 10 SSR markers.

In the set of *Nepenthes* species, only 10 out of the 20 SSR markers tested (50%) were polymorphic, suggesting a relatively low level of genetic polymorphism. However, whether this reflects a genuinely narrow genetic base of *Nepenthes* species or is influenced by primer-source bias (e.g., non-native sequences) requires further investigation. The frequency of SSR markers in *Nepenthes* spp. is comparable to that found in Tunisian local pear (Ouni et al., 2020) but somewhat lower than previously reported for maize (Mathiang et al., 2022). Differences in marker polymorphism across species should be interpreted cautiously, as they may be influenced by population structure, marker selection, and sample size. In present investigation, primers used was little specificity in the material tested. This may be due to most primers having been designed based on nucleotide sequences and ESTs of foreign *Nepenthes* spp. (higher altitude varieties) while this study focused on lowland species. Schwallier *et al*. (2016) also found that genetic divergence varied significantly between high-altitude species and lowland species. These findings accordingly reveal the relatively broad range of SSR densities among plants at the species level.

Nepenthes spp. as bioindicators of anthropogenic environmental degradation and ecosystem disruption. They exhibit highly specialized and sensitive ecological adaptations, predominantly occupying nutrient-deficient habitats where carnivory confers a competitive advantage. Consequently, they are among the first species to experience population decline or local extinction following habitat disturbance, land-use changes, and disruptions to natural ecological processes (Cross et al., 2020). Currently, studies on *Nepenthes* spp. have explored various aspects, including the discovery of new species and the application of molecular markers for species classification and genetic diversity analysis. These efforts aim to support conservation initiatives and promote the sustainable utilization of *Nepenthes* spp. (Bianchi et al., 2020; Cross et al., 2020; Ghazalli et al., 2020; Brearley, 2021; Cross et al., 2022; Dančák et al., 2022). This research assessed the genetic diversity of nine *Nepenthes* spp. populations were assessed using 10 SSR markers designed from the nucleotides of *Nepenthes* spp. The unique microsatellite patterns found in each species allowed for clear discrimination at the species level, despite high similarity coefficients. Results from the SSR banding showed a group of related *N. mirabilis* while *N. mirabilis* var. *globosa* was excluded from this group, which agrees with Saetai et al. (2014). Another group confirmed the relatedness of the "Tiger" group: *N. suratensis* and *N. andamana,* which means that the genetic relationship between them is very close. A third group separated *N. gracilis* from *N. mirabilis*, consistent with previous studies (Chaveerach et al. 2006; Anuniwat et al. 2009; Schwallier et al. 2016) and *N. kongkandana* was distinct from *N. suratensis,* although AFLP assessment were grouped (Suraninpong et al., 2015). The remaining three groups, comprising *N. ampullaria*, *N. rosea, N. krabiensis,* and *N. rafflesiana* differed in genetic code, geographic location, and morphological appearance. The present results demonstrate that SSR markers successfully identified *N. mirabilis* var. *globosa* as distinct from *N. mirabilis* and *N. kongkandana* as distinct from *N. suratensis. Nepenthes* spp. as bioindicators of anthropogenic environmental degradation and ecosystem disruption. They exhibit highly specialized and sensitive ecological adaptations, predominantly occupying nutrient-deficient habitats where carnivory confers a competitive advantage. Consequently, they are among the first species to experience population decline or local extinction following habitat disturbance, land-use changes, and disruptions to natural ecological processes (Cross et al., 2020).

SSR markers (microsatellites) have been successfully used to determine genetic diversity in many plants due to their co-dominant inheritance, high polymorphism, uniform distribution across the genome, and ease of development from genomic libraries and expressed sequence tag (EST) collections. However, molecular marker resources for *Nepenthes* spp. are currently very limited and there is evidence suggesting that certain species within this genus may exhibit polyploidy or aneuploidy. Hybridization in *Nepenthes* species, both natural and artificial, may lead to variations in ploidy levels. While many *Nepenthes* species are diploid with a chromosome number of 2n = 80, instances of polyploidy have been reported. For example, *Nepenthes gracilis* exhibits a decaploid karyotype (2n = 10x = 80), indicating a complex genomic structure resulting from whole-genome duplications (Saul et al., 2023). Natural hybridization is common among *Nepenthes* species, particularly in habitats where multiple species coexist (Scharmann et al., 2021). Such hybridization events can lead to offspring with varying ploidy levels, potentially causing reproductive challenges due to mismatched chromosome numbers. While

specific studies on ploidy variations in *Nepenthes* hybrids are limited, research on related genera, such as *Drosera*, has shown that hybrids can produce tetraploid offspring, suggesting that similar mechanisms may occur in *Nepenthes* (Mohn et al., 2023). Polyploidy significantly impacts SSR marker analysis in *Nepenthes*, affecting allele scoring, genetic diversity assessment, and overall data interpretation. The presence of multiple chromosomes sets in polyploids increases genetic redundancy, potentially leading to an overestimation of genetic similarity among individuals, particularly in closely related *Nepenthes* species or hybrids (Soltis et al., 2015). These challenges complicate population genetic analyses, as many conventional models assume diploidy, necessitating the use of advanced statistical tools and polyploid-aware markers to ensure accurate results (Blischak et al., 2018). This study represents the first report on the development and application of SSR markers for assessing the genetic diversity of *Nepenthes* species. The findings provide a foundational resource for molecular studies in this genus and contribute to the advancement of genetic research in *Nepenthes*. The SSR markers developed in this study will facilitate future investigations into the genetic structure, evolutionary relationships, and conservation strategies for *Nepenthes* species, ultimately improving the efficiency and accuracy of genetic studies in this unique carnivorous plant group.

Conclusions

The results show the high genetic diversity of wild *Nepenthes* populations in southern Thailand. SSR marker profiles gave two clusters at a genetic similarity threshold of 0.76 Our study demonstrated SSR markers as a useful tool that could be applied in future analyses of genetic diversity, population structure, and level of gene flow, as well as in optimizing the breeding conservation program of *Nepenthes* and related species.

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Author Contribution

YS: Conceptualization, Project Administration, Writing – Original Draft. **BS:** Methodology, Data Analysis. **DK:** Writing – Review & Editing. **ST:** Resources, Data Analysis. **PS:** Validation, Visualization.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

All the research data is contained in the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT (OpenAI) in order to translate text from English to Portuguese. After using this tool/service, the author(s) reviewed and edited the content as needed and takes full responsibility for the content of the publication

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