

Phylogenetic analysis of pitcher plant *Nepenthes* spp. from South Sumatra Based on ITS rDNA sequence

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ABSTRACT. *Nepenthes* spp. are carnivorous plants that have special leaf modifications with various shapes, sizes, and color patterns. Knowledge of *Nepenthes* spp. species in South Sumatra is still limited because there has been no comprehensive research. This study aims to identify and analyze the phylogenetics of *Nepenthes* spp. from South Sumatra based on Internal Transcribed Spacer (ITS) rDNA sequences. Sampling was carried out in the forests of Tekorejo Village, Air Itam Village and cultivation sites in Palembang, South Sumatra, Indonesia. Molecular identification was carried out by DNA isolation, qualitative and quantitative tests, amplification of the ITS rDNA region using primer pairs ITS1 and ITS4, identification of ITS rDNA sequences, and phylogenetic analysis using the NJ (Neighbor Joining) tree method in MEGA 6 software. The identification results showed that there are 9 variants of *Nepenthes* spp. included in the species *N. mirabilis*, *N. gracilis*, and *N. sumatrana*. The phylogenetic tree from molecular analysis shows a monophyletic line between *N. mirabilis*, *N. gracilis*, and *N. sumatrana* with a genetic closeness between *N. mirabilis* and *N. gracilis* of 0.009 while the relationship between *N. mirabilis* and *N. sumatrana* is relatively distant, with a genetic distance of 0.066. The results of this study provide new genetic baseline data that serve as an important source of information for taxonomic purposes and conservation efforts of *Nepenthes* spp. in South Sumatra for sustainable development and utilization of local plants.

Keywords: molecular identification; *N. mirabilis*; *N. gracilis*; *N. sumatrana*; neighbor-joining

Article History: Received 5 August 2025; Received in revised form 8 August 2025; Accepted 28 January 2026; Available online 30 June 2026.

How to Cite This Article: Lestari W, Jumari. 2026 Phylogenetic analysis of pitcher plant *Nepenthes* spp. from South Sumatra Based on ITS rDNA sequence. Biogenesis: Jurnal Ilmiah Biologi, vol 14(1): 93–101. doi: <https://doi.org/10.24252/bio.v14i1.60544>.

INTRODUCTION

South Sumatra Province is located in the southern lowlands of Sumatra Island. Decree of the Minister of Forestry of the Republic of Indonesia Number SK.866/Menhut-II/2014 dated September 29, 2014, states that South Sumatra Province has a forest area of approximately 3,466,901 hectares (Keputusan Menteri Kehutanan, 2014). The forest area consists of conservation forests, protected forests, and production forests (Badan Planologi Kehutanan, 2002). This forest area consists of a variety of plants, ranging from tall trees, shrubs, to undergrowth, all of which play an important role in maintaining environmental balance and biodiversity. One of the plants found in this forest is the pitcher plant (*Nepenthes* spp.) (Hernawati, 2004; Lestari *et al.*, 2018).

Nepenthes spp. are typical plants of Southeast Asia, particularly Indonesia. *Nepenthes* spp. have modified leaves called pitcher plants with various shapes, sizes, and colors (green, patterned, and reddish) (Buch *et al.*, 2015; Mardianto *et al.*, 2016; Dančák *et al.*, 2022). *Nepenthes* produces lower pitchers near the base of the plant, and upper pitches form whorls on tendrils, which are used to coil around surrounding plants (Fitmawati *et al.*, 2023). These pitcher plants function as insect traps and contain the enzyme aspartate protease to digest prey trapped inside. Trapped insects are digested and used to meet nitrogen needs (Buch *et al.*, 2015). Therefore, pitcher plants can grow in nitrogen-deficient soil (Fitmawati *et al.*, 2023).

Nepenthes spp. have many phenotypic variations. Phenotypic variations can occur because *Nepenthes* spp. are plants that can hybridize naturally. In 2001, the number of *Nepenthes* spp. in the world was known to be 87 species (Clarke, 2001). This number continues to increase along with the

discovery of new species. From 2004 to 2012, several new species were discovered in Indonesia, including *N. rigidifolia*, *N. glandulifera*, *N. adrianii*, *N. jamban*, *N. flava*, *N. naga*, *N. pitopangii*, *N. epiphytica*, *N. nigra*, and *N. undulatifolia*. Currently, the number of *Nepenthes* spp. in the world is 139 species and the majority live in Indonesia, namely 85 species with 59 endemic species (Cheek & Jebb, 2001; Bunawan *et al.*, 2017). *Nepenthes* spp. which live in Indonesia are spread across various islands such as Sumatra (34 species), Kalimantan (22 species), Java (3 species), Sulawesi (11 species), Maluku (3 species) and Papua (11 species) (Mansur, 2013).

Sumatra Island is part of the largest distribution of *Nepenthes* spp. in Indonesia. A review of the presence of *Nepenthes* spp. on Sumatra Island was conducted by Hernawati (2004) with the species found in South Sumatra being *N. gracilis* and *N. mirabilis*. The most recent information regarding the number of species found on Sumatra Island was conducted by Mansur (2013) with the discovery of 34 species, but did not mention the specific location. A review of the discovery of *Nepenthes* spp. from three regions in South Sumatra by Lestari *et al.* (2018) as many as 3 species based on morphological characteristics. However, the study of *Nepenthes* spp. species in South Sumatra still needs to be updated at this time.

Based on a literature review, it is known that *Nepenthes* spp. exhibit high morphological diversity, especially in stems, leaves, and pitcher plant color variations (Handayani, 1999; Tamizi *et al.*, 2023). Research on *Nepenthes* spp. in Sumatra by Hernawati (2004), Mansur (2013), and Lestari *et al.* (2018) was conducted based on morphological characters. To date, there has been no publication that comprehensively documents the diversity of *Nepenthes* spp. in South Sumatra Province, nor has there been a review of phylogenetic relationships using a molecular approach.

The use of DNA markers is a molecular characterization method based on genomic variation (Hidayat *et al.*, 2005). DNA markers can provide polymorphic DNA banding patterns in greater numbers, consistently, and are not influenced by the environment or plant developmental stage (Lukmanasari *et al.*, 2024). DNA contains information about the evolutionary process of a group of organisms to reveal the level and distribution of variation in plant species diversity (Hailu & Asfere, 2020). DNA sequences also provide a wealth of characters due to large differences in the rate of nucleotide base changes at different loci. DNA sequences have been shown to produce more natural phylogenetic relationships (Chase *et al.* 1993; Jose *et al.*, 2023). The use of DNA markers is expected to resolve issues related to species diversity and phylogenetic relationships of *Nepenthes* spp. to the intraspecific level, thus revealing classifications below species, given the high variability of *Nepenthes* spp. One widely used molecular analysis approach is the use of internal transcribed spacer (ITS) sequences.

Molecular analysis of the ITS region, consisting of ITS1, 5.8S, and ITS2. ITS1 and ITS2 are highly variable regions, while the 5.8S gene is highly conserved (Hilis & Dixon, 1991; Harnelly *et al.*, 2022). Sequencing the ITS region has significant potential as a source of nuclear DNA characters for phylogenetic reconstruction in plants (Baldwin, 1992; Su'udi *et al.*, 2023). This is due to its small size, high conservation, high sequence variation, high mutation rate, and rapid evolution (Hidayat *et al.*, 2005).

Based on the background explained, related to the ability of *Nepenthes* spp. to hybridize naturally, its population continues to decline in its natural habitat (IUCN, 2013), and the status of *Nepenthes* spp. in South Sumatra, a study is needed with a more comprehensive identification approach, such as the use of molecular markers. This study will be able to determine the species and phylogenetic relationships of *Nepenthes* spp. and strengthen its conservation in South Sumatra.

MATERIALS AND METHODS

Materials. Sampling was carried out at the location based on the survey results. A total of 9 samples of *Nepenthes* spp. were collected from three different locations in South Sumatra, namely Tekorejo Village (104°31'10" E -4°7'-31" S), Air Itam Village (104°45'6" E -2°57'-40" S), and the *Nepenthes* spp. cultivation location in Palembang city (104°42'58" E -2°56'-2" S) (Fig. 1).

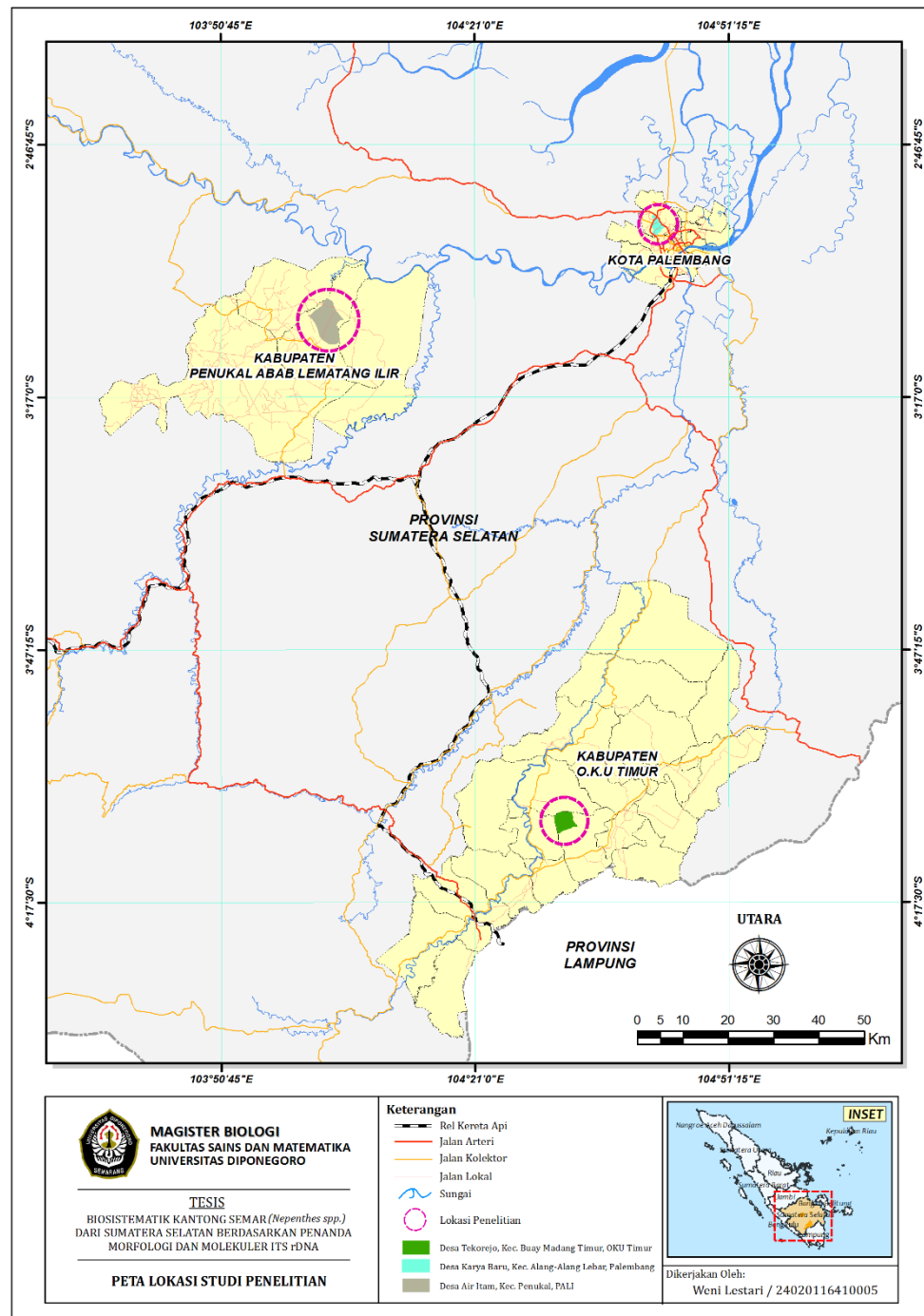


Fig. 1. Map of research sampling locations

DNA Isolation. DNA isolation using young leaves of *Nepenthes* spp. The leaves were cleaned using running water, 70% ethanol, and stored at -20°C until the DNA extraction process (Anuniwat *et al.*, 2009). Genomic DNA was extracted using the Doyle & Doyle (1990) method with a modification of the addition of 2% 2-ME and 1% PVP.

Qualitative and Quantitative Tests. Qualitative and quantitative DNA tests were performed using a Thermo scientific nanodrop 2000 spectrophotometer at 260 and 280 nm wavelengths. DNA concentration was determined based on the absorbance value A260. Purity was determined based on A260/A280 (Desjardins & Conklin, 2010).

Regional Amplification of ITS rDNA. DNA amplification using an ITS1 primer (5'-TCC GTA GGT GAA CCT GCG G-3 ') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). Amplification was performed using 25 µl reactions consisting of 12.5 µl Go Taq Green Master Mix Kit (Promega), 1.5 µl forward primer (ITS1), 1.5 µl reverse primer (ITS4), 2.5 µl DNA template, and 7 µl nuclease free water. PCR reactions were carried out in 30 cycles consisting of initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, and final elongation at 72°C for 5 minutes (White *et al.*, 1990). PCR products were separated on 2% agarose gel, stained with red gel, and visualized under UV light. Informative DNA tape was chosen for further analysis.

Identification of the ITS rDNA Sequence. Identification of ITS rDNA sequences using sequenced DNA base sequence data from 1st Base Malaysia Laboratory services. DNA base sequences in the form of chromatograms from forward and reverse primers are combined using the ClustalW sequence alignment method in Molecular Evolutionary Genetics Analysis (MEGA) 6 software (Tamura *et al.*, 2013). The sequence is stored in FASTA format and their sequence similarity is identified with gene bank data using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) site (Hamdan *et al.*, 2013).

Phylogenetic Analysis. Phylogenetic analysis starts with sequence alignment using the MEGA 6 program. *Silene acutifolia* was designated as an outgroup in the phylogenetic analysis because it belongs to the same order as the Nepenthaceae family. Therefore, it served as a reference point for determining the evolutionary relationships of the ingroup by forming the basis of the phylogenetic tree and identifying shared ancestral traits (Post *et al.*, 2023). Phylogenetic tree construction uses the NJ (Neighbor Joining) tree method by using the total number of nucleotide differences as a distance (Saito & Nei, 1987) in the MEGA program 6. Phylogenetic tree reliability test using bootstrap analysis with 1000 replications (Tamura *et al.*, 2013).

RESULTS AND DISCUSSION

The results of the study showed that the regions amplified by the primers used were the 18S fragment, the complete sequences of the ITS1, 5.8S, and ITS2 regions, and the 28S fragment (Table 1). These regions are commonly targeted in molecular phylogenetic studies due to their variability (in ITS regions) and conservation (in 18S and 28S), which together allow for accurate species identification and analysis of evolutionary relationships (Somasundaram & Yu, 2025).

Table 1. Length of DNA bases in the ITS region of *Nepenthes* spp.

No	Sample	18S (bp)	ITS1* (bp)	5,8S (bp)*	ITS2* (bp)	28S (bp)	Total (bp)
1.	TR1	30	233	165	224	51	703
2.	TR2	30	233	165	224	51	703
3.	TR3	30	233	165	224	51	703
4.	BP1	30	233	165	224	51	703
5.	BP2	30	240	165	224	51	710
6.	BP3	29	257	165	232	49	732
7.	AI1	30	240	165	225	51	710
8.	AI2	30	240	165	225	51	710
9.	AI3	30	240	165	225	51	710

Note: The * sign indicates the ITS area (ITS1, 5,8S, and ITS2)

The length of the sequenced DNA from the ITS region ranged from 703-732 bp consisting of the 18S fragment, the complete sequences of the ITS1, 5.8S, and ITS2 regions, and the 28S fragment (Table 1). The size of the ITS region fragments (ITS1, 5.8S, and ITS2) in this study ranged from 622-654 bp. Baldwin *et al.* (1995) and Alvarez & Wendel (2023) stated that in angiosperms plants, the ITS sequence has a length of between 500-700 bp. Furthermore, Yao *et al.* (2010) stated that for plants, the lengths of ITS2 sequences from dicotyledons were distributed between 100 and 700 bp.

ITS is commonly used for DNA barcoding, molecular phylogenetics, and biodiversity studies (Cheng *et al.*, 2016).

The DNA sequences from each sample varied in fragment size in the ITS1 and ITS2 regions, while the 5.8S gene fragment was identical in length and base sequence across all samples, at 165 bp. Hilis and Dixon (1991) stated that the ITS1 and ITS2 regions exhibit variable base sequences, making them useful for tracking diversity and assigning strains to species. The 5.8S gene is a conservative gene useful for constructing universal phylogenetic trees because it undergoes relatively slow changes and reflects evolutionary chronology. ITS is a plant DNA fragment that is often used in systematic molecular research down to the species level because of its great potential in terms of interspecific and intraspecific relationships (Staggemeier *et al.*, 2015; Yuan *et al.*, 2014).

DNA base sequence analysis to determine the species of each sample was performed by comparing the sample's genetic information with known *Nepenthes* spp. species in the GenBank database. The DNA bases from each sample were analyzed using Basic Local Alignment Search Tool (BLAST) from the NCBI website (Table 2).

Table 2. BLAST analysis of ITS regions of *Nepenthes* spp. samples

No	Sample	Species	Total score	Query cover (%)	Identity value (%)	Accession
1.	TR1	<i>Nepenthes mirabilis</i> voucher RMS049	1313	97%	100%	KP978795.1
2.	TR2	<i>Nepenthes mirabilis</i> voucher RMS049	1313	97%	100%	KP978795.1
3.	TR3	<i>Nepenthes mirabilis</i> voucher RMS049	1313	97%	100%	KP978795.1
4.	BP1	<i>Nepenthes mirabilis</i> voucher RMS049	1313	97%	100%	KP978795.1
5.	BP2	<i>Nepenthes gracilis</i>	1336	100%	99%	AB675882.1
6.	BP3	<i>Nepenthes sumatrana</i>	1294	100%	99%	AB675904.1
7.	AI1	<i>Nepenthes gracilis</i>	1354	100%	99%	AB675882.1
8.	AI2	<i>Nepenthes gracilis</i>	1354	100%	99%	AB675882.1
9.	AI3	<i>Nepenthes gracilis</i>	1354	100%	99%	AB675882.1

In this study, BLAST analysis of ITS sequences from *Nepenthes* spp. samples showed significant matches with sequences published in the NCBI GenBank database, with identity scores ranging from 99% to 100%. Identity value is the highest value of the percentage of identity or match between the query sequence and the aligned database sequence. An identity value of 99–100% indicates a near-perfect match that is identical in the alignment. This means that almost all DNA sequences in the aligned region are identical. These results confirm the usefulness of ITS markers for accurate species delimitation in *Nepenthes* spp. species. Based on BLAST results, it is known that the *Nepenthes* spp. found in South Sumatra consist of *N. mirabilis*, *N. gracilis*, and *N. sumatrana*. Wu *et al.* (2013) stated that the ITS rDNA region is a very important genetic locus in the study of angiosperm systematics, both at the intraspecific and interspecific levels. The hypervariable nature of the ITS nucleotide sequence, located between the 18S and 28S rDNA genes, makes it effective for distinguishing and identifying various plant species.

The closeness of the kinship relationship between samples is indicated by genetic distance (Table 3). Genetic distance indicates the degree of difference or similarity in genetic material between two or more individuals, populations, or species (Toha *et al.* 2016). The closest and furthest genetic distance values are 0.000 and 0.321, respectively. Dogan & Dogan (2016) state that genetic distance is the degree of genomic difference between species or populations as measured by several numerical methods. Genetic distance measurement has been established as a tool for analyzing data on interspecies gene differentiation. The smaller the genetic distance, the closer the samples are to each other. This indicates that the closer the genetic distance, the higher the level of similarity (Lukmanasari *et al.*, 2023). Meanwhile, BP3 (*Nepenthes sumatrana*) has a large genetic distance

(0.063–0.068) compared to other species in samples AI1, AI2, AI3, BP2, TR1, TR2, TR3, and BP1, this indicates a lower level of similarity.

Table 3. Genetic distance of *Nepenthes* spp. samples based on the ITS rDNA region

Sampel	AI1	AI2	AI3	BP2	TR1	TR2	TR3	BP1	BP3	<i>S. acutifolia</i>
AI1	-									
AI2	0.000	-								
AI3	0.000	0.000	-							
BP2	0.004	0.004	0.004	-						
TR1	0.009	0.009	0.009	0.013	-					
TR2	0.009	0.009	0.009	0.013	0.000	-				
TR3	0.009	0.009	0.009	0.013	0.000	0.263	-			
BP1	0.009	0.009	0.009	0.013	0.000	0.210	0.000	-		
BP3	0.063	0.063	0.063	0.068	0.066	0.263	0.066	0.066	-	
<i>S. acutifolia</i>	0.315	0.315	0.315	0.313	0.318	0.318	0.318	0.318	0.321	-

The clustering patterns and relationships of *Nepenthes* spp. and *Silene acutifolia* as an outgroup based on genetic distance were analyzed using MEGA 6 to reconstruct the phylogenetic tree (Fig. 2).

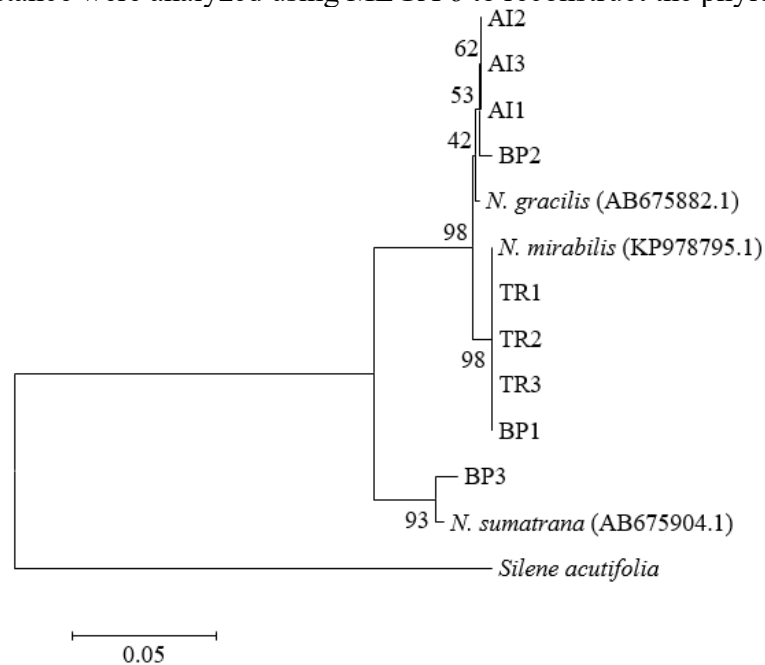


Fig. 2. Phylogenetic tree of *Nepenthes* spp. from South Sumatra

The phylogenetic tree shows that *N. mirabilis*, *N. gracilis*, and *N. sumatrana* are descended from a common ancestor (monophyletic) because they are in the same branch (clade) and share many similar characteristics. A group of organisms within a single group or clade will form a monophyletic group, where they share many similar characteristics or traits, are considered closely related, share similar genetic and biochemical patterns, and are thought to be descended from a common ancestor (Hidayat & Pancoro, 2008). Taxa contained within a monophyletic group are considered more closely related to each other than to any taxon classified outside that group (Slobodian & Pastana, 2020).

Bootstrapping is a non-parametric statistical method commonly used in phylogenetic analysis to assess the reliability of branches of an evolutionary tree (Baltzis *et al.*, 2025). The bootstrap values for the main clades are 98 and 93%. According to Nei & Kumar (2000), bootstrap is a value that describes the level of confidence at a branch point. Branching with a bootstrap value $\geq$ of 95% is considered to reflect a true level of confidence, but a bootstrap value $\geq$ of 70% provides sufficient and realistic confidence. Holmes (2003) also stated that bootstrap values of 98% and 93% reflect the stability of the clade structure against small variations in the data.

The research results indicate that *N. mirabilis* is more closely related to *N. gracilis* than to *N. sumatrana*, with a genetic distance of 0.009. The relationship between *N. mirabilis* and *N. sumatrana* is relatively distant, with a genetic distance of 0.066. This close relationship can be seen from the differences in nucleotide bases in the ITS region of each species. These base differences result in genetic variation. Genetic variation can occur due to changes in the nucleotide composition of DNA. This can occur due to mutations and recombination during sexual reproduction. Salgotra *et al.* (2023) stated that genetic diversity is the amount of genetic variability that exists among individuals of a variety or population within a species. Genetic diversity is the result of recombination of genetic material (DNA) during mutation and sexual reproduction. Genetic diversity can change over time due to mutation, selection, genetic drift, and gene flow. Karmana (2008) states that nucleotide sequence-based evolutionary change is a component of molecular evolution associated with mutations, insertions, deletions, and substitutions, which can cause changes at the gene (nucleotide) level. These changes are agents of evolution at the molecular level (microevolution).

CONCLUSION

Based on the identification of the ITS rDNA sequence it can be concluded that *Nepenthes* spp. from South Sumatra consisting of *N. mirabilis*, *N. gracilis*, and *N. sumatrana*. Phylogenetic analysis of *N. mirabilis*, *N. gracilis*, and *N. sumatrana* shows monophyletic lines. The closest genetic relationship in *N. mirabilis*, and *N. gracilis*.

ACKNOWLEDGEMENTS

The author would like to thank the Integrated Laboratory of Diponegoro University for providing extraordinary facilities so that this research can be carried out.

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