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Research Article

DNA Barcoding, Prey Spectrum Analysis, and Vegetative Propagation of *Nepenthes mirabilis* × *rafflesiana*, A Rarely Sighted Pitcher Plant Hybrid from Peninsular Malaysia

Amin Asyraf Tamizi[1]*, Salasiah Mohamad[2]*, Aqilah Awg Abdul Rahman[2], Siti Noratikah Mustafa[3], Muhammad Shafiq Hamdin[2], Mohammad Zulhusni Zakaria[2]

1)Agri-Omics and Bioinformatics Programme, Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia
2)Centre of Research for Sustainable Uses of Natural Resources, Faculty of Applied Science and Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), Pagoh Education Hub KM 1, 84600 Pagoh, Johor, Malaysia
3)Johor State Forestry Department, Bangunan Dato' Mohamad Ibrahim Munsyi, Kota Iskandar, 79660 Nusajaya, Johor
* Corresponding author, email: salasiah@uthm.edu.my, aminasyraf@mardi.gov.my

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ABSTRACT

A solitary female *Nepenthes* hybrid (*Nepenthes mirabilis* × *rafflesiana*), bearing 20-cm tall reddish pitchers, was discovered in the last remaining peat swamp forest in Johor, Peninsular Malaysia. The nothospecies (natural hybrid) is considered a very rare occurrence and has been designated as a taxon of high ecological importance, underscoring the urgent need for in-depth study and targeted conservation initiatives. To confirm its genetic identity, DNA sequencing was conducted, and the phylogenetic analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) indicated a strong genetic relationship between the hybrid (accession no. OR741796) and *N. rafflesiana*. Consistent with this, the ITS1 secondary structure of the hybrid exhibited a conserved folding structure similar to that of *N. rafflesiana*. It was recorded that the nothospecies primarily preys on hymenopterans from the family Formicidae, including at least seven genera. Furthermore, mosquito larvae from the genus *Toxorhynchites* were observed in both upper and lower pitchers, highlighting the pitcher plant's importance as a crucial breeding site for the predatory elephant mosquito. Given that only one living specimen of the nothospecies was discovered, we optimised a shoot-based vegetative propagation method that allowed several clones to be produced, which will be vital for the success of future reintroduction efforts. This is the first report to simultaneously address the molecular, ecological, and horticultural aspects of a *Nepenthes* natural hybrid in Peninsular Malaysia.

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INTRODUCTION

Plant taxonomists have primarily directed their attention towards true species, while *Nepenthes* nothospecies (natural hybrids) and intergrades are underexploered and occasionally regarded as biological outliers. Furthermore, these hybrids are challenging to resolve—through both traditional taxonomy and modern phylogenetics (Scharmann et al. 2021). *Nepenthes* nothospecies are generally fertile, capable of recurrent hybridisation, a form of adaptive radiation, and may possess unique attributes (in both ornamental and scientific aspects) (Rosli et al. 2021; Scharmann et al. 2021). According to Blanckaert and Bank (2018), hybridisation may also result in the displacement or even the creation of entirely new species, a concern that is particularly relevant in the animal kingdom. However, we believe that the occurrence of the *Nepenthes* hybrids in a particular ecosystem reflects an evolutionary advantage driven by a combination of natural and anthropogenic factors. It is imperative to thoroughly document this occurrence for a comprehensive understanding of its dynamics.

The ecological study of *Nepenthes* natural hybrids from Peninsular Malaysia remains a relatively unexplored topic. In 2021, a peculiar reddish *N. mirabilis* × *rafflesiana*, which is the focal subject of our study, was discovered in the Ayer Hitam Utara Forest Reserve (AHUFR) administered by the Johor State Forestry Department (Figure 1). Its taxonomic designation as a nothospecies was predetermined based on its clear intermediate features between those of its parental species, *N. mirabilis* and *N. rafflesiana* (Ghazalli et al. 2021). This taxon is considered a rarity despite the widespread occurrence of its two progenitors in the lowland and hilly habitats of Peninsular Malaysia (Tamizi et al. 2023b). Even within the AHUFR, only one living specimen *N. mirabilis* × *rafflesiana* was discovered. Clarke (2001) briefly mentioned *N. mirabilis* × *rafflesiana* in his monograph '*Nepenthes* of Sumatra and Peninsular Malaysia'; however, details regarding its characteristics and specific locality were not mentioned. We estimate that this population is likely located in the northern part of Selangor State. Unfortunately, this particular population is currently threatened by land conversion and development, as it is not situated within any forest reserve. Mansur (2007) performed diversity research on *Nepenthes* in Kalimantan, Indonesia, and included *N. mirabilis* × *rafflesiana* in the listing, albeit no taxonomy information was provided. Here, we did not focus on the formal description or taxonomical treatment of Johor's *N. mirabilis* × *rafflesiana*. Instead, we analysed its DNA barcode and prey preferences, along with the rooting potential of shoot cuttings. This integrated approach is crucial for gaining a comprehensive understanding of the biological and ecological aspects of the hybrid, thereby supporting conservation efforts such as the reintroduction programme. Throughout this article, we refer to this specific hybrid by the taxonomic label '*N. mirabilis* × *rafflesiana* AHUFR'. With the treatment of the Johor's type as a taxon of conservation priority, our findings are projected to deepen our understanding of this nothospecies, forming a critical basis for the formulation of targeted future conservation strategies.

MATERIALS AND METHODS

Sample collection from the study site

A single shoot about 25 cm long with three petiolated laminas (leaves) was excised from the sole *N. mirabilis* × *rafflesiana* AHUFR plant and kept in an air-tight bag. The bag was filled with some water to ensure freshness of the material and prevent wilting during transportation. This shoot sample would be subsequently used for the DNA study and vegetative propagation. A soil sample was taken from the plant's base, and its pH was measured using a portable pH probe (HI 8424) from Hanna Instruments (Woonsocket, RI, USA). The precise locations (coordinates) of the studied *Nepenthes* in the for-

est reserve were recorded but are not disclosed in this article for conservation purposes. The map of the study site was sourced from the NASA/NGA Shuttle Radar Topography Mission (SRTM) datasets provided by the U.S. Geological Survey's EROS Data Center (https://www.usgs.gov/centers/eros) and underwent additional editing using PhotoScape v3.7 (http://www.photoscape.org/).

DNA purification and ITS isolation from the studied specimen

Genomic DNA was extracted from *N. mirabilis* × *rafflesiana* AHUFR leaves using the DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. The barcode region, ITS1-5.8S-ITS2 (~600bp), was then PCR-amplified from the DNA using universal ITS1-2 primers (ITS_F: 5'-AGGAGAAGTCGTAACAAGGTT; ITS_R: 5'-GATGCAACCTTGGCCTT) and Q5 High-Fidelity DNA polymerase (New England Biolabs, UK). Thermocycler settings included an initial denaturation at 98 °C for 30 seconds, followed by denaturation at 98 °C for 10 seconds, annealing at 61 °C for 15 seconds, extension at 72 °C for 20 seconds, and a final extension at 72 °C for 2 minutes. Subsequent to gel purification using the QIAquick Gel Extraction Kit (Qiagen, Germany), each PCR product was subjected to Sanger sequencing in two reactions using the aforementioned primers. The resulting reads were manually edited and aligned using BioEdit Sequence Alignment Editor (Raleigh, NC, USA). The sequenced DNA barcode was further analysed using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ascertain its identity. Ultimately, the sequences were submitted to the GenBank® database (https://www.ncbi.nlm.nih.gov/genbank/).

ITS acquisition, sequence analysis, and ITS1 secondary structure prediction

The ITS of *Nepenthes* native to Peninsular Malaysia were downloaded from the GenBank database, and information on the specimen provenance and voucher data are provided in Table 1. These ITS sequences (including an outgroup taxon) were aligned using ClustalW multiple alignment tool and manually trimmed using Molecular Evolutionary Genetics Analysis (MEGA11) software (Pennsylvania State University, PA, USA). A phylogenetic analysis was then computed with 1000 bootstrap replications in the same software using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura & Nei 1993). Following this, the ITS sequences of *N. mirabilis* × *rafflesiana* AHUFR and its parental species (*N. mirabilis* and *N. rafflesiana*) were multiple-aligned and exported as a FASTA file for custom visualisation using BioEdit software.

The boundaries of ITS1, 5.8S, and ITS2 from *N. mirabilis* × *rafflesiana* AHUFR and its parental species were determined by comparing them with the ITS sequences of *Nepenthes* characterised by Alejandro et al. (2007, 2008). Subsequently, the first internal transcribed spacer (ITS1) foldings were queried for minimum free energy (MFE) secondary structures using the RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) (Gruber et al. 2008; Tieng et al. 2023). The resulting models were downloaded in JavaScript Object Notation (JSON) format and custom-visualised using the forna (force-directed RNA) tool (http://rna.tbi.univie.ac.at/forna/) for this publication (Kerpedjiev et al. 2015).

Analysis of pitcher fluids and captured prey

The sampling of the pitchers' digestive fluids, conducted in February 2023, followed the methodology outlined by Ristiawan and Hikmat (2022) and Mo-

Table 1. Specimen provenance of the taxa used for the phylogenetic analysis with *Ancistrocladus robertsoniorum* serves as an outgroup (Meimberg et al. 2010). The *Nepenthes* samples are originating from Peninsular Malaysia (PM) or greenhouse-grown specimens cultivated at the Botanical Garden of the University of California-Berkeley (UCBG), Bogor Botanical Garden (BBG), and Komunitas Tanaman Karnivora Indonesia (KTKI).

No.	Taxon	Specimen Voucher / Material	Origin	GenBank Accession No.	Source
1.	*Nepenthes albomarginata*	TR10	UCBG	HM204892	Renner & Specht 2011
2.	*Nepenthes ampullaria*	KRB27100901	BBG	AB675914	Alamsyah & Ito 2013
3.	*Nepenthes benstonei*	INBIOSIS-N BEN-2011	Bkt. Bakar, Kelantan, PM	JX042560	Bunawan et al. 2017
4.	*Nepenthes domei*	MD1 12423	Terengganu, PM	OR722473	This study; Ghazalli et al. 2020
5.	*Nepenthes gracilis*	KRB27100902	BBG	AB675882	Alamsyah & Ito 2013
6.	*Nepenthes malayensis*	MDIAATNMAL001 / MDI 12242	Terengganu, PM	MN347033	Tamizi et al. 2020a
7.	*Nepenthes mirabilis*	INBIOSIS-N MIR-2011	Rawang, Selangor, PM	JX042556	Bunawan et al. 2017
8.	*Nepenthes rafflesiana*	INBIOSIS-N RAF-N-2011	Mersing, Johor, PM	JX042557	Bunawan et al. 2017
9	*Nepenthes rafflesiana*	TR25	UCBG	HM204904	Renner & Specht 2011
10.	*Nepenthes sanguinea*	BL4110906	KTKI	AB675898	Alamsyah & Ito 2013
11.	*Nepenthes sanguinea*	MDIBN1 Np05A	Fraser's Hill, Pahang, PM	OR722474	This study
12.	*Nepenthes sanguinea*	MDIBN1 Np05B	Cameron Highlands, Pahang, PM	OR722475	This study
13.	*Nepenthes ×intermedia*	TR20	UCBG	HM204899	Renner & Specht 2011
14.	*Nepenthes mirabilis* × *rafflesiana* AHUFR	MDIBN1 Np0902A	AHUFR, Johor, PM	OR741796	This study
15.	*Ancistrocladus robertsoniorum*	GB-8	East Africa	GQ443551	Meimberg et al. 2010

ran (1999), with some modifications. Digestive fluids from several lower and upper pitchers of the studied *Nepenthes* plants at AHUFR were decanted into sterile 50 mL conical centrifuge tubes. The pitchers were then refilled with sterile water without exceeding the hip level of the pitchers to compensate for the fluid lost. The pH of the digestive fluid from opened pitchers was measured using a pH probe from Eutech Instruments (model pH700). The fluid was then filtered through a filter paper and the contents were retained and preserved in 70 % ethanol for prey analysis. At the laboratory, the samples were analysed on a grid-marked Petri dish and classified with the aid of a dissecting microscope. The identification of prey taxa (ants and other terrestrial invertebrates) was carried out by the insect taxonomist and according to Bolton (1994) and Maryati (1999). We identified all captured prey up to the Order level. Insects from the Order Hymenoptera and larvae from the Order Diptera could be identified down to the genus level because most of the specimens were intact. Nonetheless, for some taxa, classification below the family level was not possible due to the digestion or dismemberment of the prey invertebrates by the fauna residing and feeding inside the pitchers.

Vegetative propagation of shoot cuttings

The primary shoot cutting previously acquired from the site was first washed with an ample volume of tap water followed by sterile water. The leaves (except for the young apical leaves) were partly removed and the stem was further cut using a sharp sterile blade leaving only three petioles (Figure 5A). A sterile, moistened sphagnum mix (three parts dry sphagnum to one part perlite) was prepared and placed in an orchid pot (ø 7 cm). The shoot cutting was firmly inserted into the sphagnum mix and encased within a clean, transparent bag. This setup was then placed in an area with 50 % shade in the greenhouse, and the bag was kept sealed for one month to maintain 100 % moisture, which promotes root growth. Ensuring the cleanliness and sterility of the materials and equipment used was essential to prevent excessive fungal contamination and, consequently, enhance the survival rate of the new plant.

Once the cutting had successfully rooted and produced a new leaf blade (usually about after one month), the bag was gradually punctured with ten holes (ø 1 cm). This step was taken to acclimatise the young plant to the ambient humidity. After the plant had acclimatised (within two weeks), the rooted shoot was then transplanted into a larger pot, with more sphagnum-perlite mix added. To provide stability to the newly rooted shoot, a support pole was installed, and the plant continued to be grown under 50 % sunlight shade for approximately a year. Watering was carried out once every two days to prevent the sphagnum from drying out. When the plant reached one year of age, a 25 cm shoot was excised, and the entire rooting protocol was repeated to obtain new clones.

RESULTS

Ecological notes

The Ayer Hitam Utara Forest Reserve (AHUFR) (elevation *ca.* 40 m above sea level) is the largest and last remaining peat swamp forest in Johor (Muhammad Jais et al. 2022). A very small portion of the forest was mined in the past and now a total of 3795.84 hectares has been gazetted as a permanent forest reserve (Figure 1) (Muazam et al. 2022). It is home to many Peninsular Malaysian lowland *Nepenthes*, with at least four species and five nothospecies have been recorded within the study site (Ghazalli et al. 2021). The site experienced a slight human disturbance; hence, the phenology of these *Nepenthes* was unpredictable and sporadic. The sole specimen of *N. mirabilis* × *rafflesiana* AHUFR was determined to be a female plant based on the presence of an unpollinated infructescence, and its fecundity has not been determined. *Nepenthes mirabilis* × *rafflesiana* AHUFR (Figure 2) was discovered growing as a terrestrial climbing plant in clay-like laterite soil (pH = 5.2) close to a body of blackwater swamp, and occasional flooding has been observed at this site. A few taxa of *Nepenthes*, including *N. ampullaria*, *N. gracilis*, *N. mirabilis*, *N. rafflesiana*, *N.* ×*hookeriana*, and *N.* ×*trichocarpa*, were observed growing in close proximity to the nothospecies. These *Nepenthes* plants thrived well in open areas within the forest reserve as well as at the forest margin.

Phylogenetics inference of *N. mirabilis* × *rafflesiana* AHUFR and ITS1 secondary structure characterisation

The ITS region (ITS1-5.8S-ITS2) was sequenced and deposited to the GenBank® database (accession no. OR741796). Nucleotide search of the ITS using Nucleotide Basic Local Alignment Search Tool (BLASTn, https://blast.ncbi.nlm.nih.gov/Blast.cgi) validated the isolated DNA sequence belonged to the genus *Nepenthes* and the top five taxon hits sharing the highest ITS identity similarity were *N.* ×*intermedia*, *N. rafflesiana*, *N. gracilis*, and *N. mirabilis* (Table 2). The ITS was used to compute the phylogenetic position of *N. mirabilis* × *rafflesiana* AHUFR among 13 Peninsular Malaysian *Nepenthes*

Figure 1. Location of the Ayer Hitam Utara Forest Reserve (AHUFR) in the state of Johor, Peninsular Malaysia. The forest reserve is an isolated and protected peat swamp forest, gazetted as a state park by the government of Johor. An old mining site within the forest reserve is visible from the satellite, an area where various *Nepenthes* taxa could be spotted.

Figure 2. The sole living specimen of *Nepenthes mirabilis* × *rafflesiana* at Ayer Hitam Utara Forest Reserve (AHUFR) discovered in 2021. **A.** Lower pitchers exhibiting the gross shape of *N. mirabilis* but with the lid and colouration of *N. rafflesiana*; **B.** The mature plant can produce lower pitchers exceeding 20 cm tall; **C.** An upper pitcher (20 cm tall); **D.** The inner margin of the peristome is serrated with fine teeth, a trait inherited from *N. rafflesiana*; **E.** Habit of *Nepenthes mirabilis* × *rafflesiana* (AHUFR) showing basal and upper stems with petiolated leaves; **F.** Unpollinated female inflorescence of *N. mirabilis* × *rafflesiana* AHUFR. Image credits: Amin Asyraf Tamizi, Salasiah Mohamad, and Siti Noratikah Mustafa.

accessions. This analysis revealed the formation of two well-supported *Nepenthes* clades (bootstrap value > 90 %). *Nepenthes sanguinea*, *N. malayensis*, *N. domei*, *N. benstonei*, and *N. albomarginata* formed the highland (HL) and intermediate-highland (I-HL) clade, while *N. ampullaria*, *N. gracilis*, *N. mirabilis*, and *N. rafflesiana* formed the lowland (LL) clade. *Nepenthes mirabilis* × *rafflesiana* AHUFR was clustered together with *N.* ×*intermedia* (a hybrid of *N. rafflesiana* of an ambiguous lineage) under the same branch within the LL clade, indicating these two are closely related (Figure 3A). Despite being an interspecific hybrid, *N. mirabilis* × *rafflesiana* AHUFR (along with *N.* ×*intermedia*) emerged as a sister branch to *N. rafflesiana*, rather than *N. mirabilis*. Furthermore, the ITS sequence of *N. mirabilis* × *rafflesiana* AHUFR showed a much higher sequence similarity with that of *N. rafflesiana* with only two nucleotide mismatches found for the entire 516 bp length (Figure 3B). Whereas, there were four nucleotide mismatches and an indel on the ITS between the nothospecies and *N. mirabilis*.

The ITS1 secondary structures of *N. mirabilis* × *rafflesiana* AHUFR and its parental species were further studied. This was accomplished by transcribing the respective ITS1 nucleotide sequence into an RNA molecule, and we utilised several bioinformatics tools to predict and compute its folding structure. The ITS1 secondary structures of *N. mirabilis* × *rafflesiana* AHUFR and its parental species, generated with optimal minimum free energy (MFE), are illustrated in Figure 4. All of the ITS1 secondary structures consisted of two long and two short helices radiating from a large central loop. Conserved helix structures were observed between the ITS1 of *N. mirabilis* × *rafflesiana* AHUFR and that of *N. rafflesiana*. In contrast, the structure of ITS1 in *N. mirabilis* exhibited a different conformation on both Helix 1 and Helix III. Overall, the generated ITS1 structures displayed a common core.

Characteristics of pitcher fluids and prey composition

In this segment of the study, we conducted an analysis of the prey composition of *N. mirabilis* × *rafflesiana* AHUFR and related taxa. A total of 15 samples were examined, comprising upper and lower pitchers of the nothospecies, *N. mirabilis*, and *N. rafflesiana* (Table 3). It is noteworthy that, in all the studied *Nepenthes*, the pitcher fluids were strong to slightly acidic with levels ranging from pH 1.74 to 6.17 (Table 3). It is intriguing to note that several mature pitchers, including *N. mirabilis* × *rafflesiana* AHUFR upper pitcher 2, exhibited a diminished acidic pH, reaching 6.17.

In terms of prey composition, our result is in common with findings from previous studies reported by Moran (1999), Chin et al. (2014), and Marina et al. (2018). The pitchers trapped a wide range of arthropod prey. Overall, a total of 278 individuals of trapped fauna from six orders of arthropods were

Table 2. Top Peninsular Malaysian species search hits for ITS of *N. mirabilis* × *rafflesiana* AHUFR (query length = 512 bp) in Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sampling locality of *Nepenthes* from Peninsular Malaysia (PM) is indicated next to the taxon name; otherwise, isolate numbers are provided.

	Scientific Name (Isolate / Origin)	Total / Max Score	Query Cover (%)	Identity Similarity (%)	GenBank Accession No.
1.	*Nepenthes* ×*intermedia* (TR20)	933	100	99.61	HM204899
2.	*Nepenthes rafflesiana* (Mersing, PM)	928	100	99.41	JX042557
3.	*Nepenthes rafflesiana* (TR25)	922	100	99.22	HM204904
4.	*Nepenthes gracilis* (KRB27100902; Sabah; UKM Bangi, PM)	911	100	98.83	KP978789; AB675882; JX042555
5.	*Nepenthes mirabilis* (Rawang, PM)	902	100	97.46	JX042556

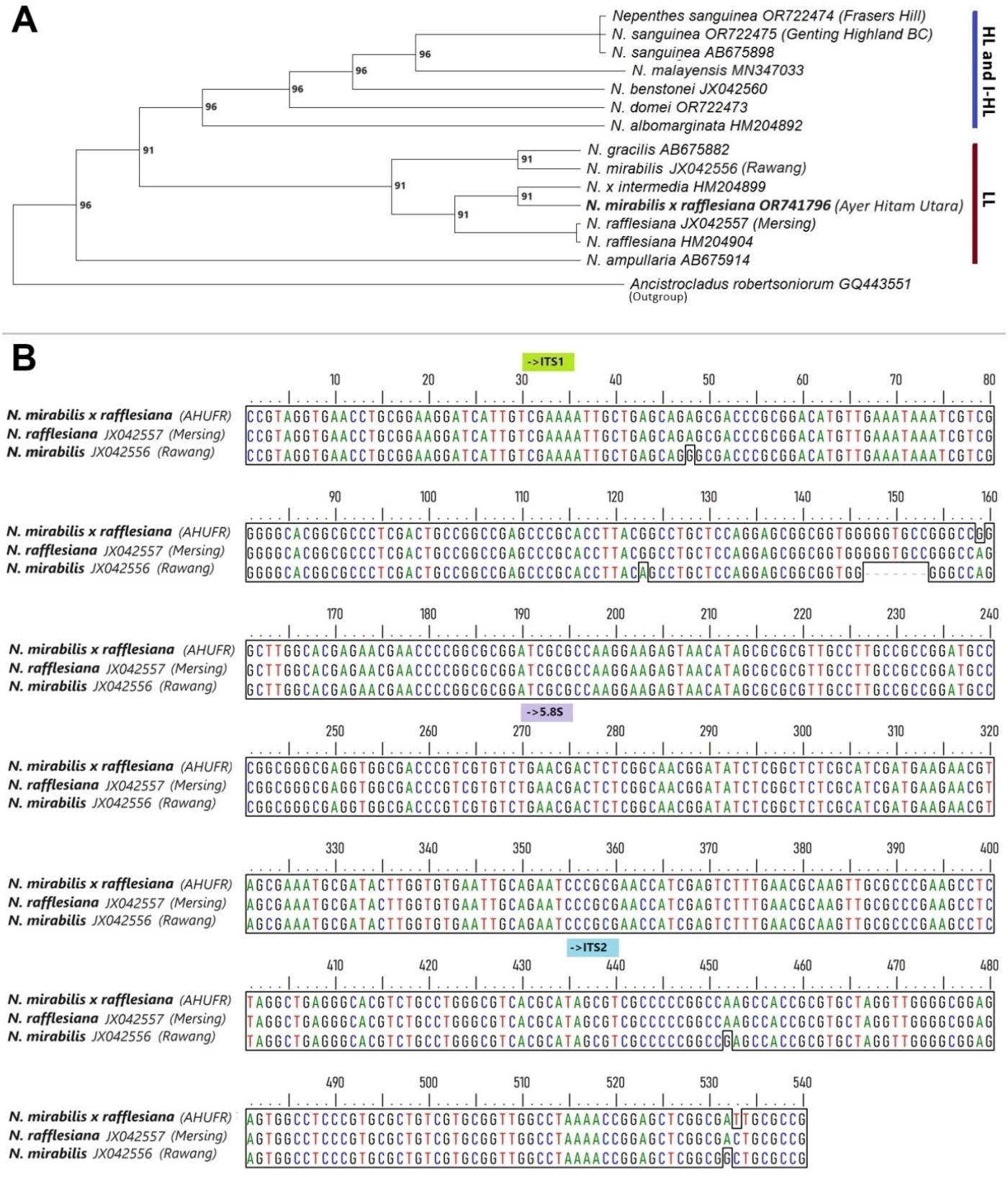

Figure 3. Phylogenetic analysis and alignment of ITS sequences. **A.** The tree displays the phylogenetic relationships of 14 *Nepenthes* taxa and accessions, including the studied taxon, and was inferred using Maximum Likelihood (ML) in MEGA11. Bootstrap values (%), based on 1000 replicates, are shown close to the nodes; **B.** Comparative ITS sequence alignment of *N. mirabilis* × *rafflesiana* AHUFR (OR741796) with its parental species, *N. mirabilis* (JX042556) and *N. rafflesiana* (JX042557). Non-consensus nucleotides are indicated by the outline, and the ITS1, 5.8S, and ITS2 boundaries are highlighted.

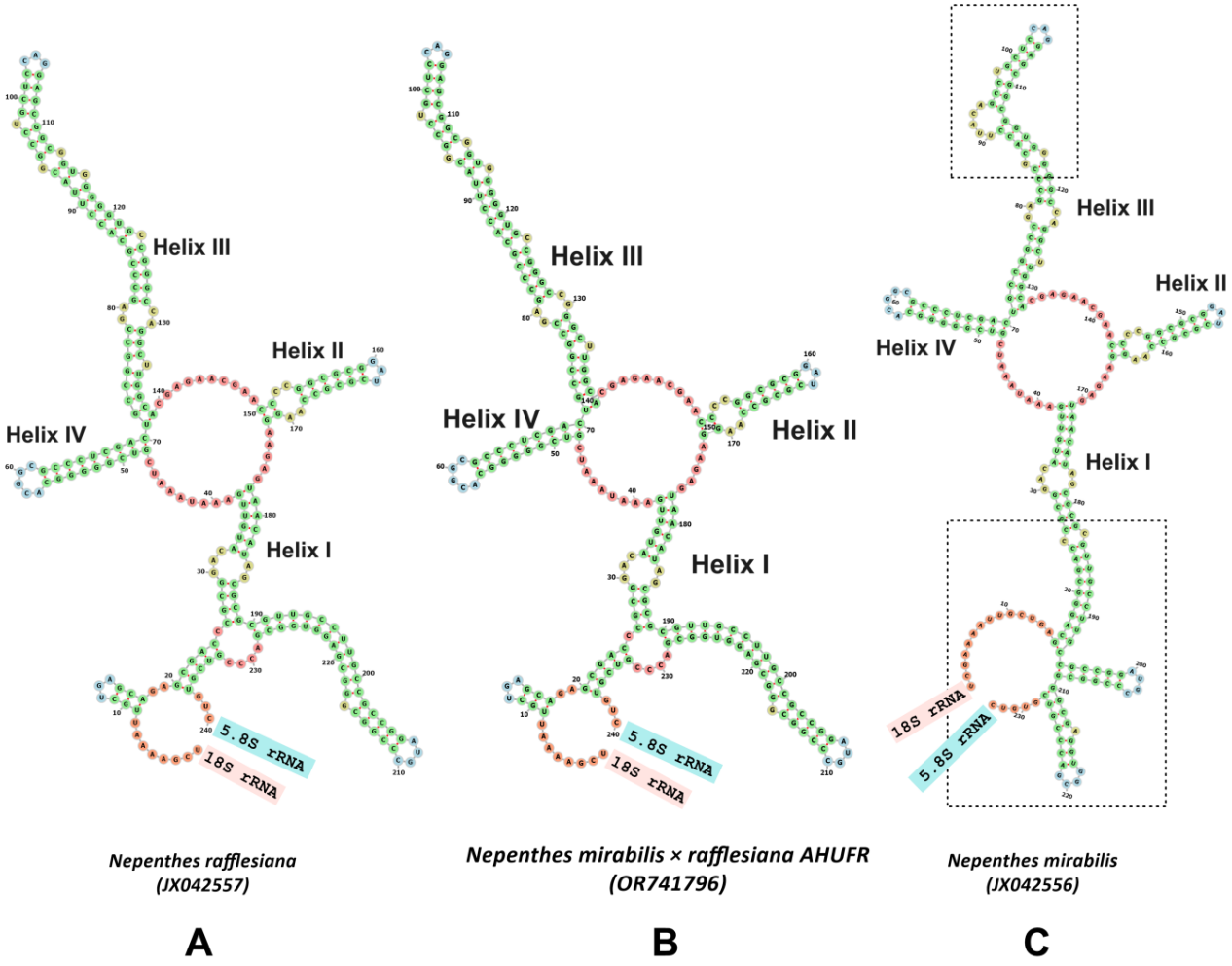

Figure 4. Inferred secondary structure models of the first internal transcribed spacer (ITS1) in 18S-5.8S pre-rRNA molecule for **A.** *Nepenthes rafflesiana* (JX042577), **B.** *N. mirabilis* × *rafflesiana* AHUFR (OR741796), and **C.** *N. mirabilis* (JX042556). The models were computed by an energy-minimisation approach using the RNAfold web server and final adjustments of folded structures were performed with forna tool. The dotted-line boxes indicate non-conserved fold structures of the ITS1 from *N. mirabilis* when compared with those of ITS1 from *N. mirabilis* × *rafflesiana* (AHUFR) and *N. rafflesiana.*

found in the traps of three taxa. Most of the arthropods are from the class Insecta. Table 3 shows the pitcher fluid pH values and Table 4 enlists the trapped prey of the three *Nepenthes* taxa. Insects from the order Hymenoptera recorded the highest number of individuals in all *Nepenthes* taxa. Several genera of ants identified in *N. mirabilis* × *rafflesiana* AHUFR were *Cerapachys* (raider ant), *Paraparatrechina*, *Polyrachis* (spiny ant), and *Crematogaster* (Saint Valentine ant). Living dipteran mosquito larvae from the genera *Tripteroides*, *Aedes* (tiger mosquito), *Malaya*, and *Toxorhynchites* (elephant mosquito) were also found in the pitchers of the studied nothospecies. Interestingly, the elephant mosquito larvae can be found in both *N. mirabilis* × *rafflesiana* AHUFR and *N. rafflesiana.* Meanwhile, mosquito larvae from the genus *Tripteroides* can be found in both *N. mirabilis* × *rafflesiana* AHUFR and *N. mirabilis.* Drowned spiders (Araneae) were only found in *N. mirabilis* × *rafflesiana* AHUFR. Most of the invertebrates found in pitcher plants are considered as prey except for the infauna from the order Diptera. The dipteran larvae were observed to be thriving and using the pitchers to obtain nutrients and as their habitat to complete the life cycle. Table 4 enlists the prey/infauna composition of the nothospecies, *N. mirabilis* × *rafflesiana* AHUFR, and its parental species, *N. mirabilis* and *N. rafflesiana*, from the study site.

Table 3. The pH values of pitcher fluid of the three *Nepenthes* taxa in AHUFR.

Taxa	Types of pitchers	Condition of the pitchers	pH values	pH average
N. mirabilis × *rafflesiana*	Upper pitcher 1	Opened	2.41	4.84 (acidic)
	Upper pitcher 2	Opened	6.17	
	Lower pitcher 1	Opened	5.73	
	Lower pitcher 2	Opened	5.92	
	Lower pitcher 3	Opened	3.95	
N. mirabilis	Upper pitcher 1	Opened	5.32	3.86 (acidic)
	Upper pitcher 2	Opened	4.25	
	Upper pitcher 3	Opened	1.74	
N. rafflesiana	Upper pitcher 1	Opened	2.32	3.98 (acidic)
	Upper pitcher 2	Opened	4.16	
	Upper pitcher 3	Opened	5.44	
	Upper pitcher 4	Unopened	3.40	
	Lower pitcher 1	Opened	4.19	
	Lower pitcher 2	Opened	4.79	
	Lower pitcher 3	Opened	3.55	

Table 4. Prey/infauna composition of the studied *Nepenthes* in AHUFR. *N. m*×*r* = *Nepenthes mirabilis* × *rafflesiana* AHUFR; *N. m* = *Nepenthes mirabilis*; *N. r* = *N. rafflesiana*.

Orders	Families	Genera	*N. m*×*r* upper pitchers	*N. m*×*r* lower pitchers	*N. m* upper pitchers	*N. r* upper pitchers
Hymenoptera	Formicidae	*Componotus*	-	-	-	✓
		Meranoplus	-	-	-	✓
		Cerapachys	✓	✓	-	-
		Crematogaster	✓	✓	✓	-
		Dolichoderus	✓	✓	-	-
		Myrmecaria	✓	✓	-	-
		Paratrechina	✓	✓	✓	-
		Paraparatrechina	✓	✓	-	-
		Polyrachis	✓	✓	-	-
Diptera	Culicidae	*Tripteroides*	✓	✓	✓	-
		Aedes	✓	✓	-	-
		Malaya	✓	✓	-	-
		Toxorhynchites	✓	✓	-	✓
	Corethrellidae	unidentified	-	-	✓	-
	Ceratopogonidae	*Dasyhelea*	-	-	✓	-
	unidentified	unidentified	-	✓	-	-
Araneae	unidentified	unidentified	-	✓	-	-
Orthoptera	unidentified	unidentified	-	✓	-	✓
Coleoptera	unidentified	unidentified	-	✓	✓	-
Hemiptera	Membracidae	unidentified	-	✓	-	-

Regenerative and rooting potential of cut shoots

In this part of the study, we successfully rooted and grew the primary shoot cutting excised from the original *in situ* plant of *N. mirabilis* × *rafflesiana* AHUFR into a mature plant bearing both lower and upper pitchers (Figure 5). Rooting was achieved within one month, with the first pitcher appearing approximately a month later. After a year, a second shoot cutting was excised from the primary clone, resulting in the successful production of a second clone. Subsequently, more clones were created. As of the writing of this article, 10 clones have been successfully produced using the vegetative propagation method, all with a 100 % recovery rate. Since these clones originated from a single upper stem, specifically the apical shoot, the initially produced traps were aerial (or upper) pitchers and had a consistent colouration similar

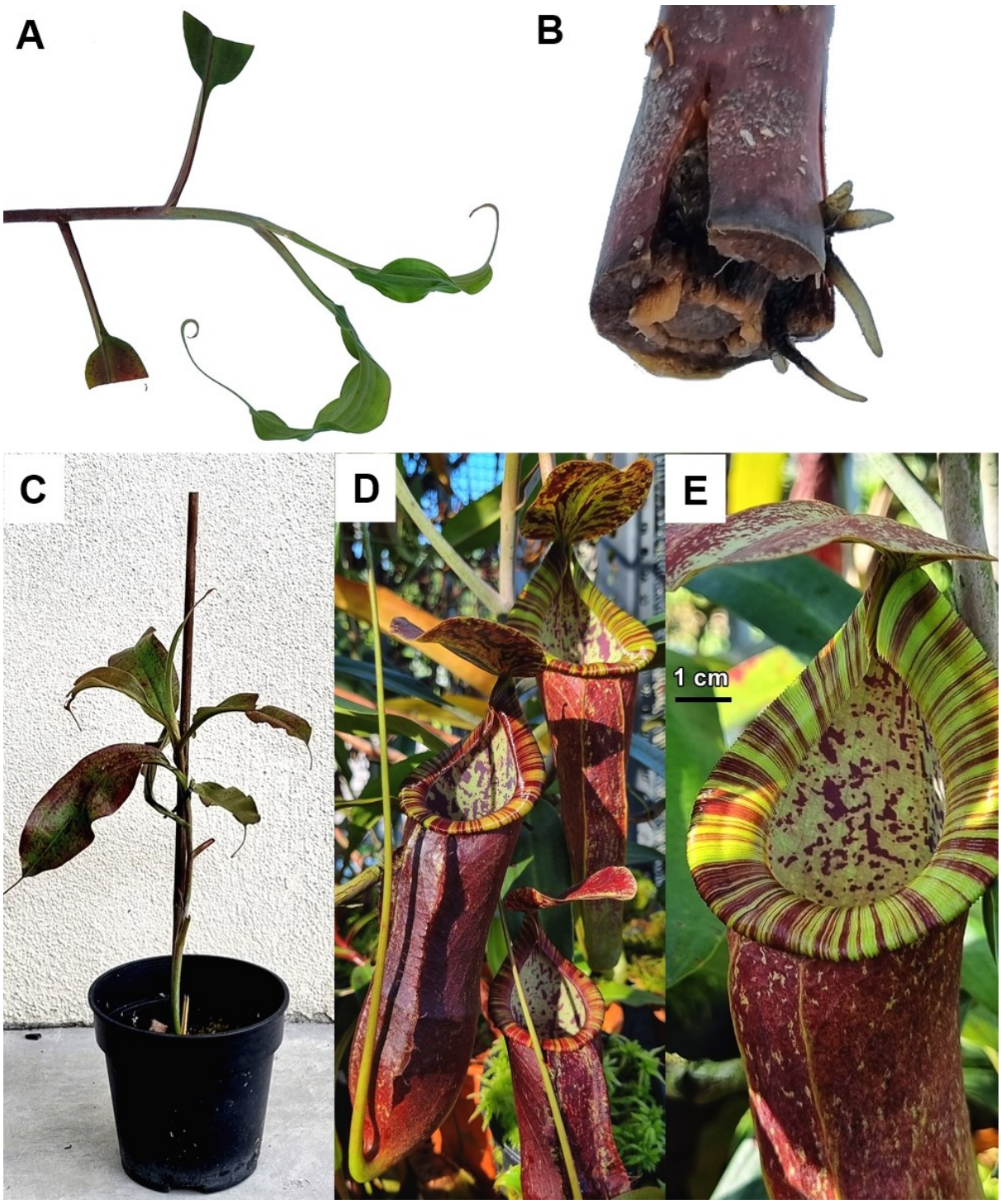

Figure 5. Shoot-based propagation of *N. mirabilis* × *rafflesiana* AHUFR. **A.** A shoot containing three internodes is harvested from the mother plant and prepared for rooting; **B.** Roots start to protrude from the bottom of the cut after a month; **C.** The new plant is transplanted to a bigger pot to encourage root and leaf formations; **D.** Two lower pitchers and one upper pitcher from the primary two-year-old clone; **E.** An upper pitcher exhibiting the flattened peristome typical of *N. mirabilis*, combined with the toothed and striped peristome features of *N. rafflesiana*. Image credits: Amin Asyraf Tamizi.

to that of the original mother plant in AHUFR. Currently, the primary clone, now two years old, has just started to produce a basal stem bearing lower pitchers (Figure 5D).

DISCUSSION

Nepenthes pitcher plants are gaining much interest in the scientific field, recognised for their potential as a source of novel compounds and as an inspiration for biomimetic applications (Miguel et al. 2018); hence, conservation and

documentation of the naturally occurring taxa become priorities for researchers and conservators. Drawing insights from a decade-long *in situ* observation, our study revealed that natural hybridisation and introgression of *Nepenthes* from Peninsular Malaysia can occur in both natural and anthropogenic habitats (Tamizi et al. 2020a, 2020b, 2023a). Currently, there are 19 published species of *Nepenthes*, with seven new species named between 2020 and 2023 (namely *N. berbulu*, *N. domei*, *N. latiffiana*, *N. limiana*, *N. malayensis*, *N. sericea*, and *N. ulukaliana*), recorded from Peninsular Malaysia (Tan et al. 2022; Golos et al. 2023; Lim et al. 2023; Tamizi et al. 2023b). Another taxon, provisionally labelled as *N.* Pesonawangsa223 (Tamizi et al. 2023b), is regarded as a potential new species from the region. Meanwhile, the actual number of *Nepenthes* nothospecies occurring in the peninsula could be underestimated and only a handful of these have been described and assigned with binomial nomenclatures, indicating the lack of study (Masters 1881; Danser 1928; Clarke 2001; Adam & Hamid 2007; McPherson & Robinson 2012; Tamizi et al. 2020b). Clarke (2001) recorded a sum of natural hybrids (and putative hybrids) originating from Peninsular Malaysia; however, scientific names were not designated to these nothospecies. Recently, we encountered three undescribed nothospecies of *N. benstonei*—*N. ampullaria* × *benstonei*, *N. benstonei* × *gracilis*, and *N. benstonei* × *rafflesiana*—bringing the total to 17 nothospecies in Peninsular Malaysia (Tamizi et al. 2023a). Although hybrids have been deliberately produced by the horticultural community and collectors, these are, nonetheless, beyond the taxonomic interest.

Molecular taxonomy of *Nepenthes* remains a fascinating subject, especially when it comes to the phylogenetic inference of closely related taxa and nothospecies, which can be sometimes challenging to resolve solely through morphology (Murphy et al. 2020). Sequence variations in the ITS of fungi and eukaryotes have been widely reported, and researchers have leveraged these variations for taxon delimitation including over 20,000 plant species (Masaoka & Kobayashi 2005; Kaplan & Fehrer 2007; Hodač et al. 2014; Belyakov et al. 2022; Letsiou et al. 2024). Besides, the availability of over 250 ITS sequence accessions in the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov) enhances the dataset's representation, enabling more accurate comparisons between query sequences and reference sequences. In a previous study, we differentiated *N. malayensis* from *N. sanguinea* and *N. domei* from *N. benstonei* using the ITS1-5.8S-ITS2 region, treating each of these species as distinct taxa (Tamizi et al. 2020a; Ghazalli et al. 2020). It is worth noting that the ITS sequence of *N. sanguinea* deposited by Alamsyah and Ito (2013) (GenBank accession no. AB675898) shows complete conservation (100 %) with the ITS sequences of *N. sanguinea* accessions (OR722474 and OR722475) from two distinct populations we sequenced in this study. A similar finding is observed for three distinct *N. gracilis* accessions (KP978789, AB675882, and JX042555) (Table 2), despite their different sources. Bunawan et al. (2017) utilised *trn*L and ITS sequences to deduce the phylogeny of several Peninsular Malaysian *Nepenthes* taxa, and the resulting tree generally supports a cladistic clustering of highland and lowland *Nepenthes*, similar to our finding (Figure 3A). We concluded that the ITS serves as a highly reliable DNA region for *Nepenthes* molecular classification, offering at least a confident species resolution. However, this work demonstrates its utility in deducing the phylogenetic affinity of the nothospecies. The ITS sequence search for *N. mirabilis* × *rafflesiana* AHUFR against the database enlisted *N.* ×*intermedia* and *N. rafflesiana* as the top taxa with the closest hit, followed by *N. gracilis* and *N. mirabilis*. *Nepenthes* ×*intermedia* (syn. *N.* ×*dominii*) is a horticultural hybrid of an ambiguous parentage (Masters 1882; Bednar 1985; Riedel et al. 2007). According to the notes and illustrations provided by Masters (1882) and Bednar (1985), it is evident that *N.* ×*intermedia* is a derivative

of *N. rafflesiana*. Therefore, its high sequence similarity and close positioning with *N. mirabilis* × *rafflesiana* AHUFR on the phylogenetic tree are strongly justified. In brief, the parentage of *N. rafflesiana* in *N. mirabilis* × *rafflesiana* AHUFR is supported through molecular and phylogenetic analysis. Conversely, *N. mirabilis*, the other putative parent, consistently appeared lower than *N. gracilis* in the alignment list (Table 2). This case, however, raises the possibility of introgression from *N. gracilis* due to historical hybridisation events. It is important to note that such inference may not always hold true as it is imperative to complement the molecular data analysis with a thorough morphological examination for a more comprehensive assessment. Additionally, the ITS region being compared was only 512 bp long, and extending the coverage would provide much better resolution. In accordance with the brief morphological analysis of *N. mirabilis* × *rafflesiana* AHUFR, none of the observed characteristics indicated the introgression of any major traits from *N. gracilis* (decurrent leaf attachment, angular stem, reduced peristome, and rounded lid), which would otherwise be noticeable. Such traits, though slightly diluted, were previously observed in many *N. gracilis* nothospecies including *N.* nothosp. Timur (B_aliasiiL421) (*N. gracilis* × *benstonei*), *N.* ×*neglecta*, *N.* ×*trichocarpa*, and *N.* ×*setiuensis*. Henceforth, we strongly accept that *N. mirabilis* × *rafflesiana* AHUFR is strictly an interspecific hybrid of *N. mirabilis* and *N. rafflesiana*. The nothospecies displayed a strong resemblance to *N. mirabilis* especially on the gross shape of the pitchers, position of the hip close to the pitcher midsection, formation of fimbriate leaf margin, and the structure of the stem and leaves (petiolated). Meanwhile, the toothed peristome, relatively large pitcher size, glaucous stem and leathery leaves are the traits attributed to *N. rafflesiana*.

Based on the sequence alignment, the 5.8S and ITS2 regions were found to be highly conserved in *Nepenthes*; therefore, the ITS1 region was selected for secondary structure analysis to better deduce its taxonomic affiliation, due to its higher sequence variability. Nafisi et al. (2023) and Saidon et al. (2023) also reported that ITS1 exhibited a higher degree of accuracy and conveyed more phylogenetic information. Furthermore, the article by Saidon et al. (2023) is the only report on the use of ITS secondary structure to discriminate *Nepenthes* prior to this study. In line with phylogenetic inference, the ITS1 secondary structure of *N. mirabilis* × *rafflesiana* AHUFR highly resembled that of *N. rafflesiana*, with some variations observed in *N. mirabilis*. This observation further supports the close genetic affinity of the nothospecies with *N. rafflesiana*. Beyond plant systematics, the ITS1 sequence and its secondary structure have previously been employed to elucidate low-level phylogenetics in diverse organisms, including ciliates, dinoflagellates, fungi, insects, and animals (Wang et al. 2007; Hoshina 2010; Coleman 2013; Koetschan et al. 2014; Kumar et al. 2018; Thornhill & Lord 2010).

Our study on the pitchers of *N. mirabilis* × *rafflesiana* AHUFR and its parental species has revealed some interesting findings. Generally, *Nepenthes* pitcher acidity typically falls within the pH range of 2 to 6 (Takeuchi et al. 2011; Biteau et al. 2013; Gilbert et al. 2022). Our data from the three studied taxa align with this trend, with one exception: a pitcher from *N. mirabilis* reached an exceptionally acidic level, measuring as low as pH 1.74. The interior glands possess the ability to secrete acid-dependent digestive enzymes (chitinases and proteases) to digest prey and this is further aided by the presence of acidity-specialised bacteria, as well as mutualistic infauna, which break down large prey fragments, particularly insects, into smaller particles (Higashi et al. 1993; Biteau et al. 2013; Bazile et al. 2015). As mentioned earlier, the acidic pitcher fluid is conducive to digestive enzyme reactions, but the shift towards neutrality may be attributed to the depletion of digestive fluid and enzymes during prey digestion. Nevertheless, it is important to

acknowledge that the pH value of the pitcher fluid may also vary depending on the specific taxon, the accumulation of captured prey over time, the mixing with rainwater, and pH modification by pitcher infauna (Takeuchi et al. 2011). We then report the prey and infauna found in the pitchers of *N. mirabilis* × *rafflesiana* AHUFR. In terms of the prey spectrum of the studied nothospecies, the most abundant prey captured were mostly from the class Insecta. This finding is not surprising as insects are known to be the most attracted to pitcher plants due to their nectar secretion and pitcher colouration (Moran 1999). *Nepenthes mirabilis* × *rafflesiana* AHUFR was recorded to have an insatiable appetite for insects belonging to the order Hymenoptera, consistent to studies reported by Marina et al. (2018) and Setiawan et al. (2022). However, the frequency of prey organisms attracted to the pitchers depends on factors such as their abundance in the area, mobility and the presence of pull or push factors influencing their movement towards the pitchers. Research has suggested that the extrafloral nectar secreted by *N. rafflesiana* from the glands around the pitcher's mouth, along with the sweet fragrance produced, might act as attractants for its prey (Moran 1999)—similar may be true for its hybrid in question, *N. mirabilis* × *rafflesiana* AHUFR. In comparison with the other parental species, *N. mirabilis*, we observed the presence of ant prey from the genera *Crematogaster* and *Paratrechina* in the pitchers of *N. mirabilis* × *rafflesiana* AHUFR as well. In contrast, no overlapping insect orders were found between *N. rafflesiana* and the studied nothospecies. This lack of overlap may be attributed to various factors, including differences in pitcher colouration, nectar quality and quantity, as well as spatial considerations. There was no lepidopteran insect found in pitchers observed during this study, while records have shown insects of this order can be found in pitcher plants, although in lower numbers (Marina et al. 2018). This may be caused by their flight ability and habits, as they are more attracted to the flowers as compared to the extrafloral nectars produced by the pitchers. This study provides the first insight into the prey spectrum of *N. mirabilis* × *rafflesiana* growing in AHUFR.

In general, the invertebrates discovered in this study function as prey for *Nepenthes*, with some also serving as symbionts, notably the dipteran larvae (mosquitoes). The genus diversity of mosquito larvae in *N. mirabilis* × *rafflesiana* AHUFR exceeded that in its parental species, possibly due to its average pH being a bit closer to neutral. This heightened diversity is particularly noteworthy, especially considering that the larvae of most mosquito species are typically found in water within the pH range of 3.3 to 10.5, as mentioned by Nikookar et al. (2017). It is worth highlighting that the presence of larvae of an unidentified *Toxorhynchites* mosquito raises intriguing prospects for further investigation. This particular mosquito group is acknowledged as an important natural predator of other mosquitoes and has been observed residing within *Nepenthes* pitchers. (Tsukamoto 1989; Clarke 1998; Lim et al. 2019; Maquart et al. 2023). Although *N. mirabilis* × *rafflesiana* AHUFR exhibits genetic proximity to *N. rafflesiana*, the pitcher architecture and insect-trapping traits of this nothospecies align more closely with those of *N. mirabilis*. The results from this study may still be preliminary, more data can be obtained once more *N. mirabilis* × *rafflesiana* AHUFR individuals are discovered within the forest.

As noted earlier, the sole plant of *N. mirabilis* × *rafflesiana* AHUFR is female and this individual is considered a taxon of high conservation priority. Previously, Siti-Suhaila and Norwati (2021) micropropagated a horticultural hybrid, *Nepenthes* 'Viking' × 'Miranda', using a temporary immersion bioreactor system. The method took more than 16 months, from seed explant preparation to acclimatisation, to regenerate and establish new plantlets with approximately 10 cm long leaves. The reported success rate for this microprop-

agation was 70 %, which is considered high. Alternatively, the vegetative propagation (macropropagation) method that relies on cuttings from mature plants to create new individuals is deemed a much easier way to produce new clones; however, this approach remains underexplored for local *Nepenthes*. Sani et al. (2000) found it inefficient, reporting an overall rooting success rate of less than 50 % even with the use of rooting hormones. Additionally, Bahadur et al. (2007) briefly mentioned that the method is difficult. In a previous study, we conducted a preliminary test in using stem cuttings to vegetatively propagate *N. albomarginata*, *N. ampullaria*, *N. gracilis*, *N. limiana*, *N. mirabilis*, *N. rafflesiana*, *N. sanguinea*, *N.* ×*neglecta* (*gracilis* × *mirabilis*), *N.* ×*trichocarpa* (*ampullaria* × *gracilis*), and *N. benstonei* × *gracilis* (Tamizi et al. 2023a). It was determined that macropropagation using apical shoot cuttings are effective for generating new plants. Building upon this, we utilised shoot cuttings as the starting material to successfully generate multiple clones of *N. mirabilis* × *rafflesiana* AHUFR. Both of our previous and current studies have proven that producing new *Nepenthes* plants from stem cuttings, particularly shoots, is simpler than previously thought.

CONCLUSION

This study aimed to examine the genetic background and further affirming the taxonomic position of the sole living *N. mirabilis* × *rafflesiana* from Johor's last peat swamp forest. The ITS DNA barcode deposited in the GenBank® database will serve as a crucial reference for future population authentication and surveys. We also emphasise the reliability of the ITS region as a robust DNA barcode for determining species limitations in future studies. While there could be more individuals of *N. mirabilis* × *rafflesiana* AHUFR yet to be discovered, the studied specimen has been listed as one of the Johor's iconic plant taxa entitled for conservation priority. The insight into prey preferences gleaned from our data provides a fundamental understanding of the ecological requirements of local *Nepenthes*, while the development of a feasible vegetative propagation method instils optimism for potential future reintroduction programmes aimed at preserving this remarkable nothospecies. Further investigations are needed to discover various aspects of this natural hybrid, encompassing genomic studies, assessments of fecundity, analyses of phytochemical composition, exploration of ecological interactions, and extensive population surveys. These future studies will provide invaluable information and contribute to the conservation and understanding of *N. mirabilis* × *rafflesiana* AHUFR.

AUTHORS' CONTRIBUTIONS

A.A.T., S.M., and A.A.A.R. contributed to the conception and design of the study. All authors (A.A.T., S.M., A.A.A.R., S.N.M., M.S.H., and M.Z.Z.) were involved in material preparations and data collecting process. A.A.T., S.M., and A.A.A.R. conducted the data analyses and prepared the initial draft of the manuscript. Subsequently, all authors provided comments on later versions of the manuscript. The final version was read and approved by all authors.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.

DATA AVAILABILITY

All data generated, accessed, or analysed during this study are included in this published article. The ITS nucleotide sequences have been deposited in GenBank® (www.ncbi.nlm.nih.gov/genbank/).

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES

During the preparation of this work, ChatGPT (a language model developed by OpenAI) was strictly employed to enhance language and readability. Subsequently, the authors thoroughly reviewed and edited the content as necessary, taking full responsibility for the publication's content.

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