

***rbcL* nucleotide sequence-based phylogenetic analysis of *Nepenthes distillatoria* L.**

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Abstract:

Among carnivorous plant families, Nepenthaceae is a very peculiar family which has only one genus, *Nepenthes*. In Sri Lanka, this genus is represented by a single endemic species; *Nepenthes distillatoria* L. (1753). In that aspect, this plant has a key taxonomic value in Sri Lankan flora. This study was intended to identify the phylogenetic position and to establish a relationship between *N. distillatoria* and other selected carnivorous plant species of Nepenthaceae using *rbcL* sequences. DNA was extracted from leaves of *N. distillatoria* and subsequent PCR amplification of the *rbcL* region was performed. The amplified PCR products were subjected to DNA sequencing. The *rbcL* sequence alignments were constructed using MEGA v.7.0 and phylogenetic analyses were carried out under the Maximum Likelihood method. This study mainly introduces the partial coding sequence of the *rbcL* gene of *N. distillatoria*. The reconstructed phylogenetic tree based on the sequence results revealed that *N. ventricosa*, *N. merrilliana*, *N. khasiana*, *N. bellii*, and *N. fusca* are closely related species to *N. distillatoria*.

Keywords: Carnivorous plants, Endemic species of Sri Lanka, *Nepenthes*, Phylogenetic relationships, *rbcL*

1. Introduction

Carnivorous plants have gained the interest of the scientific community for their outstanding performance in insect prey digestion-based modes of living. The plants are equipped with chemical and morphological features specifically evolved to attract, retain, trap, and digest the insects to satisfy the requirement of nutrients such as Nitrogen, Phosphorus, Potassium, Calcium, and Magnesium [1]. Botanical carnivory has evolved independently in five angiosperm orders: Poales, Caryophyllales, Oxalidales, Ericales, and Lamiales [2]. Ten different carnivorous families belong to five separate botanical orders. Among them, Nepenthaceae is a very peculiar family which is represented by only one genus *Nepenthes* where it further includes nearly 150 species [3]. They are found around the Indian Ocean from

Madagascar to Southeast Asia, New Guinea, and northern Australia. The genus *Nepenthes* in Sri Lanka is restricted to only one species: *Nepenthes distillatoria* which is a tropical pitcher plant endemic to Sri Lanka [4]. Danish physician Thomas Bartholin first mentioned *Nepenthes distillatoria*, a type of carnivorous plant, in 1677 and named it *Miranda herba*. In 1753, it became the second *Nepenthes* species to be described in print and the first to receive a scientific name under the Linnaean system of taxonomy, *Nepenthes distillatoria*, by Swedish naturalist Carl Linnaeus [5]. In that aspect, this plant has a key taxonomic value in Sri Lankan flora. This study was conducted to find the phylogenetic relationship of *N. distillatoria* and other selected carnivorous species by using sequences of the *rbcL* gene. First, we introduced the partial coding sequence of *rbcL* gene of *N. distillatoria*. Then, we constructed the maximum likelihood phylogenetic tree to identify the taxonomic location of *N. distillatoria* with other species of *Nepenthes*.

2. Materials and Methods

The leaves of *N. distillatoria* were collected from the Department of Botany, University of Peradeniya, Sri Lanka. Collected plant leaves were stored at -80 °C for the DNA extractions. In addition, GenBank data for molecular phylogenetic analyses from representative species in carnivorous plants were collected [6]. The total DNA extraction from leaves of *Nepenthes distillatoria* was carried out by a modified Cetyl trimethylammonium bromide (CTAB) method [7]. The quality and the concentration of the extracted genomic DNA were determined by agarose gel electrophoresis and spectrophotometry (Analytic Jena Spec200 - Germany). The PCR was performed using a Takara TP600 (Japan) thermal Cycler using primers for *rbcL* F (5' ATGTCACCAACAGAAAC 3' [8]) and *rbcL* R (5' CTTTCCAAATTCACAAG CAGCA 3' [9]) for the amplification of the *rbcL* region of *N. distillatoria*, at the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka. The total PCR reaction mixture was 25 µL. The PCR conditions used were, 94 °C for 2 minutes followed by 35 cycles at 94 °C for 30 seconds and 55 °C for 30 seconds. The final extension was performed at 72 °C for 2 minutes. This final PCR product was subjected to DNA sequencing (Macrogen, South Korea).

2.1 Data Analysis

The partial coding sequence of the *rbcL* gene of *N. distillatoria* was submitted to GenBank using BankIt tool [10]. For the gene alignments, 16 *rbcL* sequences of other *Nepenthes* species were downloaded from the National Center for Biotechnology Information (NCBI) [11]. The *rbcL* sequence of *N. distillatoria* was aligned with them using the ClustalW [12] in MEGA v.7.0 [13] software. The maximum likelihood method (ML) [14] was used in the substitution model by PhyML 3.0 online software [15] to construct the phylogenetic trees using the *rbcL* sequences.

3. Results and Discussion

We provided the newly sequenced *rbcL* gene of *N. distillatoria* in our study to GenBank and it is now registered with the accession number, MK059425. According to sequence alignment results, the length of the partial coding sequence of *rbcL* gene in *N. distillatoria* was 1264 base pairs. The *rbcL* sequence of *N. distillatoria* was aligned with the available 16 *rbcL* sequences of other *Nepenthes* species and randomly selected two species from Sarracenaceae as outgroups. The total length of aligned *rbcL* sequences was 453 base pairs where 438 (97%) of which were invariant, 6 (1%) were parsimony informative, and 9 (2%) were parsimony uninformative. According to AIC [16] and BIC criteria [17] in the phylogenetic tree of Maximum Likelihood, the best model was given as K80+G model with a -828.05668-log likelihood value. Using the aligned sequence, the pairwise genetic distances among the given taxa ranged from 0 to 7%. *N. distillatoria* shows a pairwise genetic distance range of 0.5% - 1.1% with other *Nepenthes* species. The two major clusters of Nepenthaceae were formed and all these clusters are well supported (posterior probability 100%, bootstrap 100%).

Tree topology within group II indicates species *N. ventricosa*, *N. merrilliana*, *N. khasiana*, *N. bellii*, and *N. fusca* are closely related to *N. distillatoria*. When considering their geographical distribution, group I is restricted to *Nepenthes* species found in Philippines. But group II represents the species from both Philippines (*N. ventricosa*, *N. merrilliana* and *N. belli*) and several other countries such as Sri Lanka, (*N. distillatoria*) India (*N. khasiana*) and the island of Borneo (*N. fusca*) (Figure 1). When considering the evolutionary and geographical origins of *Nepenthes* species, it is difficult to get a clear idea due to the lack of fossil records. [18]. One of the phenomena currently being used to explain the diversification of the genus *Nepenthes* is associated with the continental drift theory. According to Danser (1928) [19], the genus *Nepenthes* was evolved at least 80 million years ago, at a time when the Indian subcontinent was connected to Australia. According to this hypothesis, *Nepenthes* which was present in Indian continent was carried northwards after the breakup of Gondwana. It is believed that subcontinent's collision with Asia led to the spread of the genus eastwards via several migrations into Indochina and the Malay Archipelago, where it underwent rapid diversification [20].

Considering the hypothesis of Meimberg and Heubl (2006) [20], combined with the gene tree, the following propositions can be made. As Sri Lanka and India were believed to be located in the the Indian subcontinent while Borneo and Philippines were located in the Southeast region of the Asian continent (which is situated at the boundaries of three major plates, Eurasia, India–Australia, and Pacific–Philippine Sea) of the old world. The origin of *Nepenthes* is suspected to be from the mainland Indian subcontinent [20]. Furthermore, it can be assumed that all the examined species from the genus *Nepenthes* had the same ancestor at a time [2], [21]. Further migrations to different geographical places can be through the effects of continental drift, land bridges, seafloor spreading, and long-distance dispersal. After colonization in different geographical locations, the ancestral *Nepenthes* species would have evolved to withstand different local environmental conditions such as soil pH, soil nutrients, availability of water, and altitudinal range. This may have caused the progression into differentiated new species

through speciation processes [22], [23]. This could be the reason for the occurrence of closely related *Nepenthes* species from different regions of the world which belong to the same cluster. Moreover, *ITS* nuclear ribosomal DNA-based phylogenetic analysis also revealed that *N. distillatoria* is a sister species to *N. khasiana* [24].

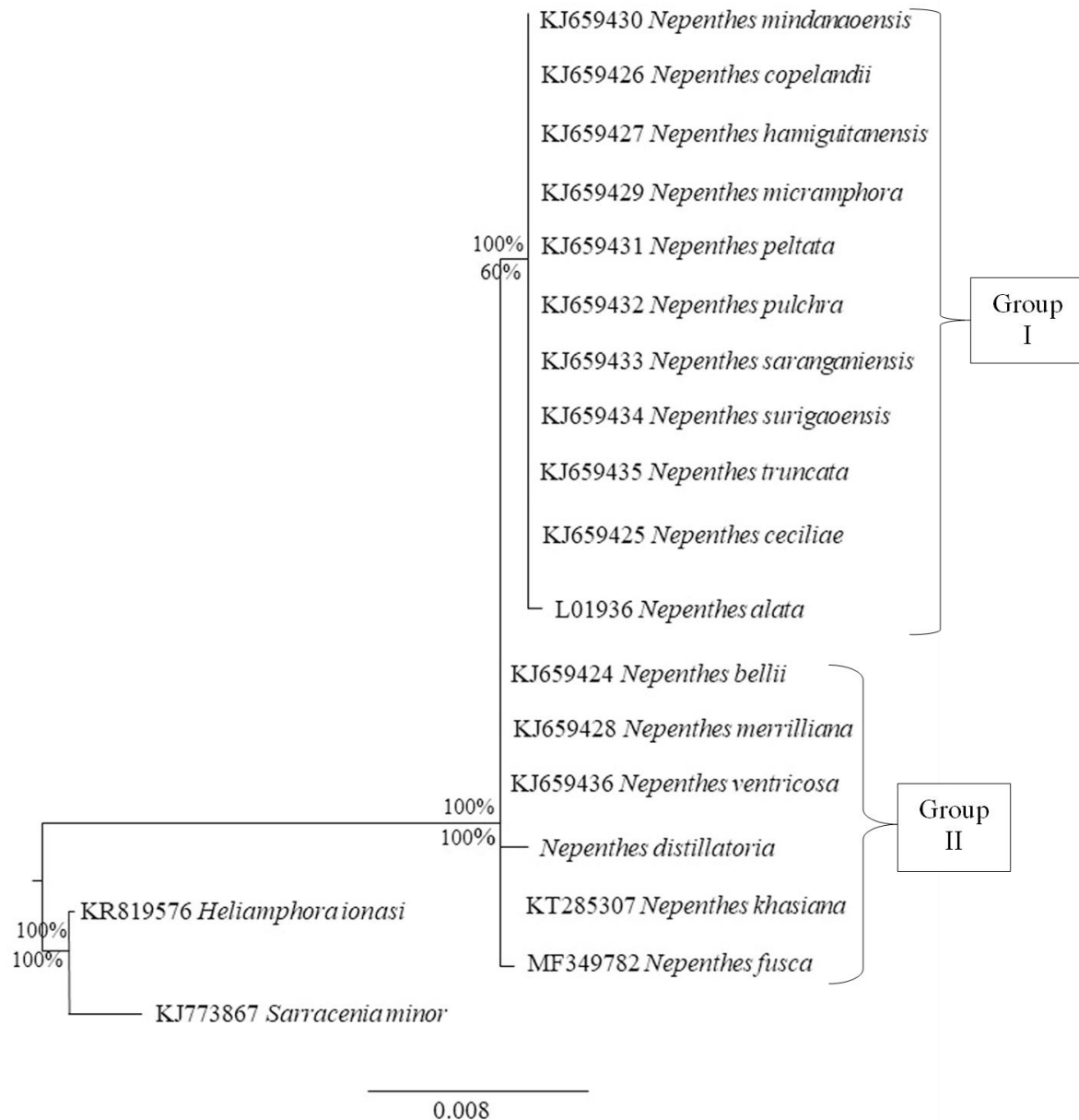


Figure 1. Maximum Likelihood tree of the *rbcL* gene for *N. distillatoria* with other *Nepenthes* species, constructed using PhyML 3.0. The numbers above and below the nodes represent bootstrap values (>70%) and Bayesin posterior probabilities (>70%), respectively. *H. ionasi* and *S. minor* are used as the outgroup. The scale bar represents the number of substitutions/sites.

4. Conclusions

This study introduced the partial coding sequence of *rbcL* gene of *N. distillatoria* and elucidated its location in the phylogenetic tree of Nepenthaceae species. The phylogenetic analysis based on *rbcL* gene reveals that *N. ventricosa*, *N. merrilliana*, *N. khasiana*, *N. bellii* and *N. fusca* are closely related to *N. distillatoria*. Thus, our phylogenetic tree suggests that *N. distillatoria* may share the closest common ancestor with *N. khasiana* and each species may have adapted to the local environment after the geographical separation of the species.

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