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Cover: Common Silverline *Spindasis vulcanus vulcanus* in poster colours adapted from photograph by Kalpesh Tayade. © Pooja R. Patil.

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COMMUNICATION

New distribution record and DNA barcoding of *Sapria himalayana* Griff. (Rafflesiaceae), a rare and endangered holoparasitic plant from Mizoram, India

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Abstract: *Sapria himalayana* Griff. is a rare and endangered holoparasitic plant that prefers a specific host (*Tetrastigma* sp.). It is one of the lesser-known and poorly understood plants facing threats of extinction owing to human interference in the evergreen forests of Mizoram. The flower is the only visible part of this endophyte and blooms from November to December. The plant was encountered for the first time in the evergreen forest near Rullam village in the Serchhip District of Mizoram, India. In the present study, DNA barcoding was used to identify the plants, and the internal transcribed spacer 2 (*ITS2*) region of *S. himalayana* was amplified and sequenced. The *ITS2* sequence could accurately identify up to the species level for this endangered species. The absence of the ribulose-biphosphate carboxylase gene (*rbcL*) region in the genome supports its holoparasitic nature. Hence, DNA barcoding can help in taxonomic and biodiversity research and aid in selecting taxa for various molecular ecology and population genetics studies. The phylogenetic tree was analyzed using the maximum-likelihood method, and our findings showed that species from different families were clearly discriminated in a phylogenetic tree. To the best of our knowledge, this is the first report of DNA barcoding using *ITS2* region of *S. himalayana* from Mizoram, India.

Keywords: DNA barcoding, endangered species, endophyte, holoparasitic, ITS2, Mizoram, *Sapria himalayana*.

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Author contributions: LR—data collection, data handling, data curation, writing, review and editing of manuscript; HS—writing, review, and editing of manuscript; SPS—review, and editing of manuscript; LK—statistical analysis; YTS—supervision, review, and editing of the manuscript.

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INTRODUCTION

Angiosperms, commonly known as flowering plants, are the most diverse group of land plants, and this group also includes parasitic plants. Parasitic plants lack chlorophyll and depend on the host plants for water and nutrition (Osathanunkul 2019). Rafflesiaceae comprises holoparasitic plants (Rubiales et al. 2011) and includes three genera, namely *Rafflesia* (28 species), *Rhizanthes* (four species) and *Sapria* (three species) (Trần et al. 2018). *Sapria himalayana* Griff. (Rafflesiaceae) is also a holoparasite with a preference for specific hosts-*Tetratstigma sp*ecies (Elliott 1990).

Sapria himalayana consists of endophytic vegetative tissues with microscopic strands called haustoria, ramifying through the root cambium of the host plant. The flowers (Image 1A) are about 20 cm across, bright red and mottled with yellow spots. They appear above the ground, emitting a putrid odour. The flowers usually remain in bloom for two-three days and eventually decompose. The flowering stalks are short, erect and unbranched. The flower buds (Image 1B) are globose and covered basally by light pink bracts. The fruit is swollen, blackish-brown, and crowned with perianth remnants. The flowering and fruiting of *S. himalayana* occur during winter, usually during December-February. The seeds have been reported to be the size of grapes and blackish-brown in colour (Borah & Ghosh 2018).

Sapria himalayana has been reported to have a preference for specific hosts, so the removal of the host plants might eventually result in the death of this parasitic plant (Osathanunkul 2019). In addition, fragmentation and loss of habitat, intensive agriculture to meet human needs and other anthropogenic activities threaten the existence of this holoparasitic plant (Osathanunkul 2019). Apart from biodiversity conservation, accurate taxonomic assignment is important for this rare species as it may be accidentally collected, adding to the threat of its existence.

Traditionally, the taxonomic assignment has mainly been the responsibility of taxonomic experts (Yang et al. 2018). Above that, population genetic studies are also restricted because of their limited distribution (Elliott 1990). DNA barcoding using nucleotide comparisons of approved gene regions allows simple, rapid and reliable identification of species (Cosaic et al. 2016; Saddhe & Kumar 2018). The internal transcribed spacer Two (*ITS2*) region of nuclear ribosomal DNA is considered one of the candidate DNA barcodes since it has several desirable characteristics, including conserved regions for designing universal primers, ease of amplification, and adequate variability, to distinguish even closely related species (Kang et al. 2017).

Global distributions of *S. himalayana* have been reported from Myanmar, northeastern India, southeastern Tibet, Thailand and Vietnam (Elliott 1990; Hajra 1996). In India, William Griffith first reported *S. himalayana* in 1847 from the tropical wet evergreen forests of Mishmi Hills of Lohit District in Arunachal Pradesh. Since then, *S. himalayana* has also been reported from Assam, Manipur, and Meghalaya (Borah & Ghosh 2018; Ahmad et al. 2020). In Mizoram, *S. himalayana* was first reported by Lakshminarasimhan et al. (2013) from Tawi Wildlife Sanctuary in Aizawl, Mizoram. However, no molecular analysis has been undertaken thus far on *S. himalayana* plants found in Mizoram.

Recently, *S. himalayana* was spotted in an evergreen forest near Rullam village in Serchhip District of Mizoram, India. The plant is locally called 'lei pangpar,' meaning flower without a stalk. This study aimed, for the first time, to identify *S. himalayana* using DNA barcoding combined with morphological characterization. The genome of *S. himalayana* collected from Thailand has recently been published by Cai et al. (2021). However, to our knowledge, DNA barcoding of Indian materials of this rare species has not been conducted so far.

MATERIALS AND METHODS

Study area

Flowering buds of *Sapria himalayana* were collected from an evergreen forest near Rullam village in Serchhip District, Mizoram, India (Figure 1). The locality has an average elevation of 888 m and is situated at 23.44°N & 92.99 °E. The annual daily average temperature ranges 15–27 °C with moderate rainfall.

Collection of samples and Isolation of DNA

The samples were found attached to the roots of *Tetrastigma* species (*T. obovatum* Gagnep*, T. pachyphyllum* (Hemsl.) Chun*, T. cruciatum* Craib & Gagnep). The collected samples were brought to the Department of Botany, Mizoram University, for further investigation. Isolation of genomic DNA was done using the standard CTAB method (Doyle & Doyle 1990) with some modifications. Briefly, 200 mg of two flower buds was ground and analysed separately using a sterile mortar and pestle with 500 µl of extraction buffer (100 mM TrisHCl, 1.4M NaCl, 2 mmM EDTA, 2% CTAB, 1% PVP at pH 8), and incubated at 60^{0}C for 30 mins followed by

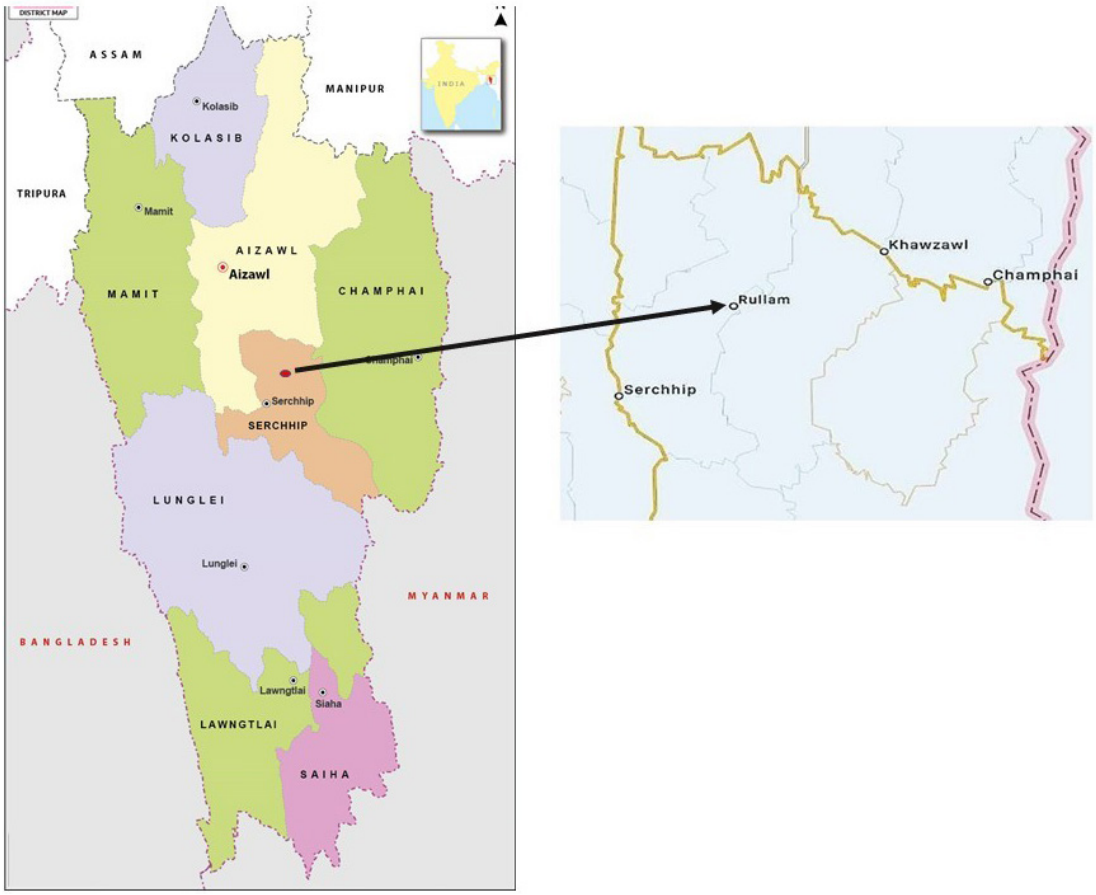

Figure 1. Political map of Mizoram, India, showing the collection site, Rullam. Source: www.mapsofindia.com.

centrifugation at 11,000 rpm for 15 mins. After RNase A treatment, the sample was incubated at 30^{0}C for 30 mins. Then, 500 µL Chloroform Isoamyl was added to the sample and centrifuged at 11,000 rpm for 1 min. A 0.7 volume of ice-cold isopropanol was added to precipitate the genomic DNA at -20^{0}C. The DNA was washed with 70% ethanol and dissolved in 30 µL TE buffer (10 mM TrisHCl, 1 mM EDTA).

Amplification of DNA, sequencing and analysis

The isolated DNA was amplified using *ITS2* primers: F – GAAGGAGAAGTCGTAACAAGG, R – TCCTCCGCTTATTGATATGC and *rbcL* primers: F- CTGTATGGACCGATGGACTTAC, R-CGGTGGATGTGAAGAAGTAGAC (Zahra et al. 2016) in a Veriti 96-Well Thermal Cycler (ABI, Thermo Fisher Scientific).

The amplified DNA products were cleaned and sent for commercial sequencing to AgriGenome (Cochin, India). The resultant sequence was analyzed using NCBI BLAST (ncbi.nlm.gov), and the similarity indices with the reference sequences from GenBank database were used for the species identification of the samples.

Phylogenetic analysis

A phylogenetic tree was constructed in MEGA X (Kumar et al. 2018) using the maximum likelihood (ML) method. The model suggested by Bayesian information Criterion (BIC) was T92 + G, with the lowest BIC score. The models with the lowest BIC scores were considered to describe the best substitution pattern (Posada & Crandall 2001). The phylogenetic tree was constructed using similar sequences identified from BLASTn analysis from Genbank. Species of closely related families from the same order (Malpighiales)- *Euphorbia canariensis* (Euphorbiaceae), *Chaetocarpus echinocarpus* (Peraceae) were also used, and a non-photosynthetic plant *Conopholis americana* (Orobanchaceae) was taken as an outgroup. Only when conspecific and congeneric species in the study formed a single clade with bootstrap P >50, the ML tree was considered successful.

RESULT AND DISCUSSION

Morphological characters of *S. himalayana* and the host plant

Sapria himalayana flowers and flower-buds were found growing on the roots of *T. cruciatum*. (Vitaceae). The host plant had leaves with tendrils arising from the bases of the petioles.

The collected flowers of *S. himalayana* (Image 1) were dark-red, mottled with yellowish-white dots, and had a bowl-shaped disk. Leaves were absent. The flowering occurs during winter, from November to February.

Table 1. Flower description of *Sapria himalayana.*

Floral parts	Size
Flower	8 cm high; 14.5–15.5 cm in diameter
Outer Perigone Lobes	3.5–4.5 cm long; 3–4 cm wide
Inner Perigone Lobes	2.5–3 cm long; 2–2.5 cm wide
Disk	3.5–4 cm in diameter
Host Plant's Root	1–2 cm in diameter

DNA - Isolation, Amplification, Sequencing and Analysis

The genomic DNA from *S. himalayana* was successfully isolated and amplified using *ITS2* primer (Image 2). However, the *rbcL* primer failed to amplify the DNA.

The amplified DNA of *S. himalayana* was subjected to sequencing and the sequence was submitted to the GenBank database (MW788913). The amplicon (731 bp) also showed a high percentage similarity (97.44%) with the reference sequence (EU882286) from GenBank database.

Phylogenetic analysis

The ML-based phylogenetic tree of *ITS2* showed high bootstrap values, and species of each genus were clustered on different branches and nodes as monophyletic taxon and clustered with the genus of other clades. The taxonomic units were statistically branched from their nodes with bootstrap P >70 for most of the sub-trees. Thus, the present study revealed that *ITS2* had a high-resolution potential for the molecular taxonomy of *S. himalayana*. The collected sample was clustered together with other *Sapria* species. Here, *S. himalayana* formed a monophyletic group (bootstrap value = 100), and *S. himalayana* individuals showed coalescent stochasticity with high branch support value (bootstrap value = 100) (Figure 2) while other species were grouped into a different clade. Our study showed that the species from different families were discriminated clearly in a phylogenetic tree. Therefore, *ITS2* locus-based ML phylogenetic tree can be used to identify unknown samples for molecular taxonomy and identification of rare and endangered species.

The identification and classification of plants based on their morphological characteristics are an integral part of taxonomy; however, identifying plants based on their morphology alone may sometimes be inaccurate (Feng et al. 2017). DNA barcoding is a valuable taxonomic tool for the identification of species. A study was conducted to identify *S. himalayana* from Thailand using *ITS2* employing environmental DNA (eDNA) (Osathanunkul 2019). In our study, DNA barcoding of *S. himalayana* gDNA was successfully done using *ITS2* primers,

Image 1. *Sapria himalayana*: A—Flower | B—Flower bud with the root of its host (*Tetrastigma* sp.) collected from Rullam forest, Serchhip District. © Hmingremhlua Sailo.

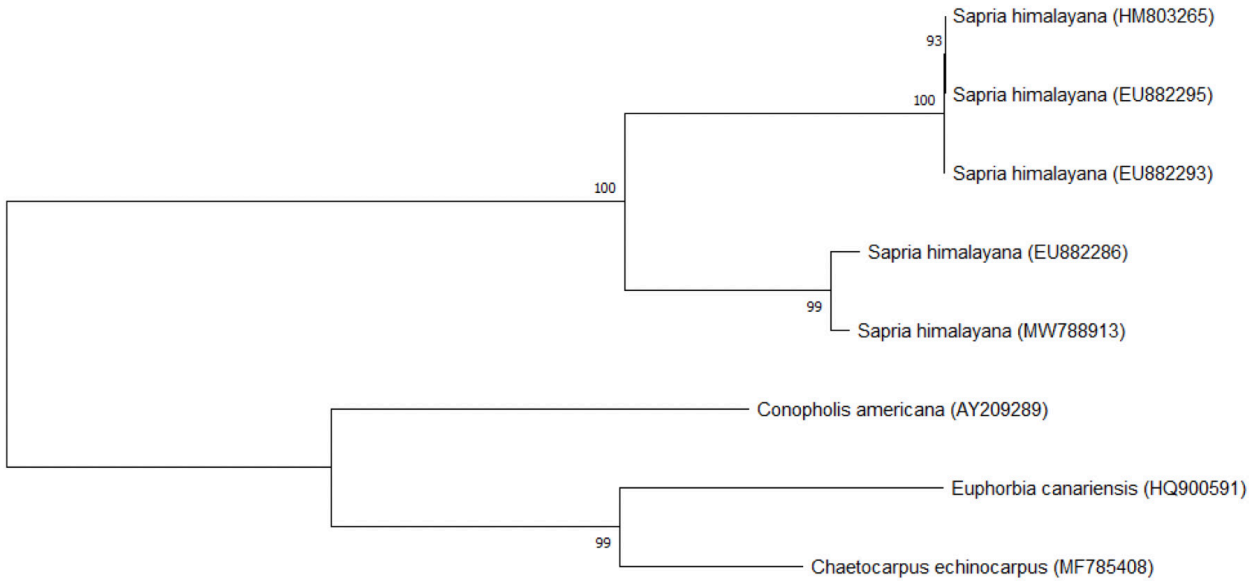

Figure 2. A maximum likelihood phylogenetic tree of *Sapria himalayana* using ITS2 region.

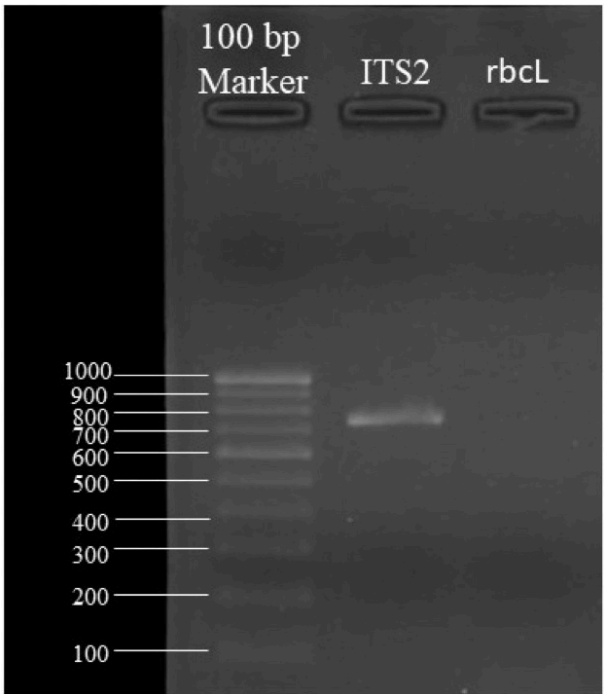

Image 2. A 1.5% agarose gel electrophoresis of amplified products of *S. himalayana* using ITS2 and rbcL.

resulting in a 97.44% similarity with the reference sequences from the GenBank database; this confirms the identification of the studied plant sample. Another interesting observation was the failure to amplify the *rbcL* region of the species; this could be primarily due to heavy gene loss, including the plastid genome, as already reported for the genus *Sapria* (Cai et al. 2021) and hence loss of its photosynthetic activity. Thus, DNA barcoding can help derive an accurate phylogenetic classification. Therefore, the identification and classification of plants based on their morphology and DNA complement each other to attain accurate species identification.

CONCLUSION

Sapria himalayana, a rare and endangered holoparasitic plant, was collected from Mizoram. The results of DNA barcoding confirms the identification of this species. However, the distribution of this little known taxon is highly restricted in the region. This study suggests that focused explorations must be conducted in similar habitats to assess the population size. Suitable conservation measures are needed to protect this rare and interesting species from threat of extinction from the region.

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