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continued on the back inside cover

Cover: Fish species recorded in the Gowthami-Godavari Estuary, Andhra Pradesh: *Lutjanus johnii* (top left), *Triacanthus biaculeatus* (top right), *Acentrogobius cyanomos*, *Elops machnata*, *Trypauchen vagina*, *Oxyurichthys microlepis*. © Paromita Ray.

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COMMUNICATION

Morphological assessment and partial genome sequencing inferred from matK and rbcL genes of the plant *Tacca chantrieri*

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Abstract: *Tacca chantrieri* is a monotypic perennial plant belonging to the family Taccaceae. It is listed as an endangered species by different authors. The plant was found in Thorangtlang Wildlife Sanctuary, a protected area in Lunglei District, Mizoram. Although there is a record of its existence from the forests of Mizoram, there are no detailed studies based on morphology, partial or whole genome sequencing. Plant samples collected from Thorangtlang Wildlife Sanctuary were used for morphological assessment and partial genome sequencing of *mat*K and *rbc*L genes. This study provides information useful in making conservation decisions.

Keywords: Black Bat Flower, Endangered, genetics, genomics, herb, morphology, northeastern India.

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Author contributons: PCL and EVN conducted field studies, collected data, and wrote the manuscript. FL, VR, and JLP all gave valuable inputs. PCV analysed the data.

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INTRODUCTION

Tacca chantrieri Andre, or Black Bat Flower, belongs to the family Taccaceae (Fu & Jin 1992). The bractea of this particular species is very similar to that of bats, hence the common name Bat Flower. In local language, Mizo, it is called 'Thialkhasuak'. It is a perennial herb with underground rhizomes distributed mainly in tropical regions of Asia (Drenth 1972, 1976; Ding & Larsen 2000). The family Taccaceae tends to be divergent in the number of genera and species. According to Linn & Kuntz 2010, it is represented by two genera and about 13 species. Taccaceae comprises 10 species of pan-tropical distribution (Zhang & Li 2008) and comprised of one genus and 11 species (Ding et al. 2000). They are commonly found in the forest understorey, and a majority species are rare in the wild.

Black Bat Flower has a unique shape which mimics bats, with broad wings and numerous long dangling filaments with rich maroon black or deep purple color (Charoensub et al. 2008). It exhibits a low germination rate and can survive only under specific environmental conditions. As a result of its rapidly disappearing natural habitats and low germination rate, the species has become an endangered plant (Fu & Jin 1992).

Globally there are 10 species representing this genus, with nine confined to the Indo-Malaysian region. Beyond this region, only two species are found; an inclusive species: *T. leontopetaloides* distributed mainly from the Indo-Malaysian region to tropical Africa and the other species *T. parkeri*, the only native to South America. There are five species presently occurring in Malaysia, viz., *T. leontopetaloides, T. integrifolia, T. palmata, T. chantrieri*, and *T. bibracteata*. In both peninsular and eastern Malaysia *T. leontopetaloides, T. integrifolia,* and *T. palmata* are found whereas *T. chantrieri* is found only in the northern parts of peninsular Malaysia while *T. bibracteata* , a very rare plant is only found in Sarawak (Saw 1993).

Tacca chantrieri was first reported from Assam in 2015 as a new record from India (Baruah et al. 2015). Morphologically, *Tacca chantrieri* resembles *Tacca khanhhoaensis* which is assessed as Critically Endangered (CR) on the IUCN Red List Categories (IUCN 2012). To date, a study on reproductive biological observation of *Tacca* is still lacking (Faegri & van der Pijl 1971; Drenth 1972; Saw 1993). Mizoram is situated in the northeastern part of India along with its sister states of Manipur, Nagaland, Tripura, Arunachal Pradesh, Assam, and Meghalaya. It is abundantly furnished with dense forests and diverse species of flora and fauna but many areas of several regions are unexplored. Although a preliminary record of the plant's existence is recorded, there are no detailed studies based on its morphology, anatomy, and partial genomic sequencing. Due to exploitation and destruction of forests, the habitat of this species has diminished. *Tacca chantrieri* exhibits improvident floral arrangement and a high reproductive structure investment, which leads to highly suitable of it for out crossing thus possessing sapromyophilous (pollination by flies where the flower mimic rotting meat) syndrome of pollination (Drenth 1972; Saw 1993).

DNA bar coding based techniques such as DNA sequencing are the most relevant and innovative techniques which can analyze the genetic linkage and evolution of plants and species identification. CBOL (Consortium for the Barcode of Life) plant-working researchers suggest that rbcL and matK (the 2-locus) combination is the standard plant barcode based on the sequence attribute or trait, levels of species differentiation, and evaluation of resiliency. A brief reflection of Maturase K Gene in plant DNA barcoding and phylogenetics (Kar et al. 2015).

MATERIALS AND METHODS

The plant sample was collected from Thorangtlang Wildlife Sanctuary at an elevation of 500–550 m, where necessary investigation of *Tacca chantrieri* was done by field observation and measurement of observable morphological features and the state of its efflorescence within the natural habitat. The research analysis was conducted between September 2017 and December 2020. *Tacca chantrieri* prefers moist, shaded brushwood habitats (Image 1A,B). Plants are 2–4 feet tall with rhizomes imperfectly cylindrical, leaves are oblong or elliptic shape with caudate apex and attenuate base in various sizes and are green in color. Petiole slightly dark brown to black. Our study reveals that *T. chantrieri* bears inflorescence from late April to September and by October to November berries are ripened. T.S. and L.S. of both stems and leaves were observed under fluorescence microscope. The exposure of the anatomical studies for exceedingly large organs or tissues require to be dissected into tiny segments for microscopic observations. Section cutting or sectioning is the most stereotypic technique of studying microscopic anatomy or histology of large specimens (Karuppaiyan & Nandini 2016). Sections were stained using the double staining method, a technique involving a mixture of two contrasting dyes (safranin and methylene blue). These procedures can be used on

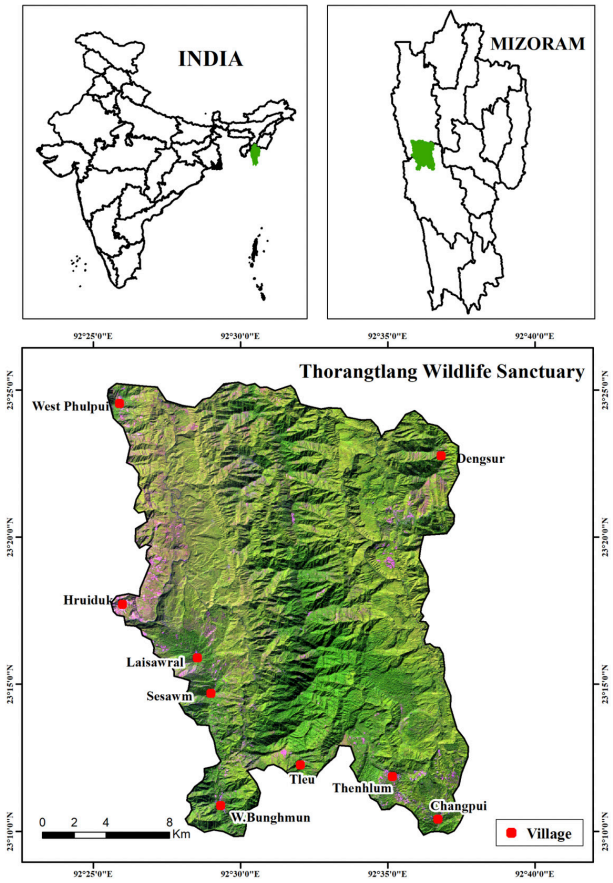

Figure 1. Study area: Thorangtlang Wildlife Sanctuary.

paraffin, paraplast, or historesin-embedded free hand and microtome sections. A section of young leaves was preserved in 70% alcohol which needs to undergo further partial genome sequencing process. Partial DNA sequencing was inferred from matK and rbcL genes. The length of DNA bands acquired from matK and rbcL genes are 635 and 675 respectively and are deposited in GenBank, NCBI with accession no MW289205 (matK) and MW289206 (rbcL).

DNA extraction, PCR amplification and sequencing

DNA isolation of the specimen was obtained from leaves and stems of *Tacca chantrieri* following the protocol recommended by White et al. (1990). For polymerase chain reaction (PCR) analysis, each DNA sample was diluted to the appropriate concentrations. A total reaction volume of 25 µl consisted of 12.5 µl Tag Master Mix (Takara), 9.5 µl of nucleus free water, 1 µl each of primers, and 1 µl of DNA sample. Maturase-K region was amplified using: Forward primer matK390F: 5'-CGATCTATTCATTCAATATTTC-3' and Reverse primer matK1326R: 5'-TCTAGCACACGAAAGTCGAAGT-3' with the following parameters; initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 45 sec, followed by the final elongation step at 72 °C for 7 min. RbcL region was amplified using: forward primer rbcL 1F: 5'-ATGTCACCACAAACAGAAAC-3' and reverse primer rbcL 724R: 5'-TCGCATGTACCCTGCAGTAGC-3' with the following parameters; initial denaturation at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min, followed by the final elongation step at 72 °C for 7 min (Bafeel et al. 2012).

The PCR products were electrophoresed on 0.8% (w/v) agarose gel in 1.0 x TAE buffer [containing 1 µl Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific) per 20 ml of 10 gel] at 150 V for 20 minutes. The amplified PCR products were sequenced by Sanger's dideoxy method (Sanger et al. 1997) on ABI 3730XL automated sequencer (AgriGenome Labs Pvt. Ltd., Smart City Kochi, Kerala, India). Consensus sequences for contigs were trimmed and aligned using Bioedit sequence alignment editor (Hall 1999). Sequences were then compared to those in GenBank database using the BLASTn (Altschul et al. 1990) search tool for similarities. DNA sequence of matK and rbcL data of the studied species have been submitted to GenBank. The sequences were then aligned with Clustal W (Larkin et al. 2007) and the phylogenic tree was established using maximum likelihood in MEGAX. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighhbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 12 nucleotide sequences.

Study Area

Thorangtlang Wildlife Sanctuary is situated about 245 km south of Aizawl, the state capital of Mizoram between 23.28°–23.19° North & 92.50°–92.62° East and 1,396 m at highest altitude falling in Lunglei District (Fig 1). The Sanctuary lies close to the Indo-Bangladesh border. It possesses both evergreen and semi-evergreen forests and its richness in wildlife is the most distinctive feature compared to other wildlife sanctuaries in the forests of Mizoram. Disastrous practices of events

like shifting cultivation and hunting from nine fringing villages leads to biotic pressure on flora and fauna.

RESULTS

Morphological and anatomical observations

Morphological evaluation was conducted primarily in its natural habitat. The morphological patterns of *Tacca chantrieri* plant was investigated intensively from September 2017 to December 2020. According to our observations, *Tacca* bears inflorescence from late April to September, and berries ripen from October to November. Plants are 2–4 feet tall, rhizomes imperfectly cylindrical, leaves oblong or elliptic shape having arcuate, reticulate, palmate, camptodromous and brochidodromous venation which measure 35–50cm x 14–20cm (Image 1E–G) and are green in color. Petiole 45–60 cm by 3–6 mm slightly dark brown to black (Image 1C). Inflorescence 2, up to 20–30 flowers comprising of involucral bracts (Image 1D).

Figure 2 depicts the schematic diagram of *Tacca chantrieri* inflorescence bearing numerous flowers along with its trailing-like filaments and leaves which resemble bats consequently giving the plant the common name Black Bat Flower. The inflorescence arrangements exhibit numerous flower stalks which spread from a common point, thus referred to as cymose umbellate inflorescence demarcated by the dark colored bracts and also consist of long trailing filamentous bracteoles. The flowers are nearly black, deep maroon or purple-red in color. The number of inflorescence per plant was two and in each of the two inflorescences 20–30 florets with around 25–30 long trailing like filaments were present. The inflorescence lasted for two to three weeks. The root of the plant is extensive and rhizomatous which is imperfectly cylindrical.

T.S. of the stem shows conductive collateral vascular bundles arranged in circular motion in which xylem protrudes towards the inner side and phloem projects outwards (Image 1K,L). The inner core mainly consists of the ground tissue. L.S. of stems of *Tacca* shows sieve tubes and sieve plate (Image 1I) T.S. of leaf shows a single layer of upper cuticle followed by epidermis which is transparent. Next to the epidermis are tightly packed rod-shaped cells known as mesophyll cells. Beneath the mesophyll cells, loosely bound spongy mesophyll cells are present. Stomatal pores (tiny pores) are present in some regions (Image 1J). The stomata present are anomocytic (Image 1M).

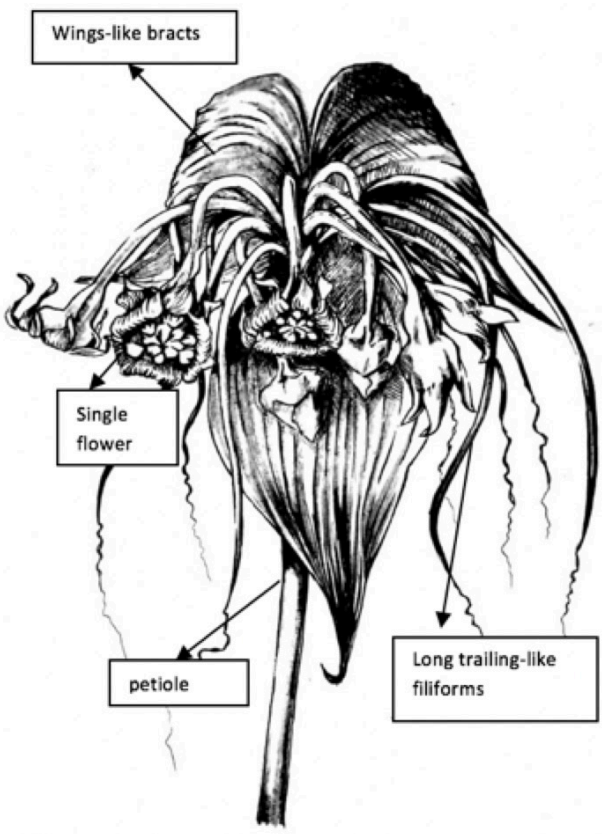

Figure 2. Schematic diagram of *Tacca chantrieri*. Illustrated by Vesper Lalrinawma.

Table 1. matK sequences.

Name of species	Accession Number
Tacca chantrieri	JQ733736
Tacca chantrieri	MH748936
Tacca reducta	MK153216
Tacca reducta	MK153205
Tacca palmata	MK153192
Tacca palmate	MK153200
Tacca bibracteata	MK153225
Trichopus sempervirens	KP083035
Tacca plantaginea	AY973842
Tacca maculate	MK153197
Tacca leontopetaloides	MK153196
Tacca leontopetaloides	MK153193
Tacca sumatrana	MK153224
Tacca havilandii	MK153210

Nucleotide analysis and Phylogeny

To construct phylogeny of major lineages, representative taxa of members from the major species

were chosen. Table 1 comprises all the taxa analyzed herein and their accession numbers.

The matK sequences of our specimen (MW289205) had 3 nucleotide differences with zero gap, from the two species of *Tacca chantieri* (JQ733736 and MH748926). The rbcL sequences (MW289206) of our specimen had 13 nucleotide differences with zero gap, from the species of *Tacca chantieri* (KX171420 and JN850578).

The evolutionary history was inferred using the maximum likelihood method and Tamura-Nei model base on the matK region (Figure 3). The final positioning for the merged sequences for the two regions (matK and rbcL) comprised of 897 base pairs.

In the phylogenetic tree (Figure 3), as expected, a close relationship between the specimens examined (MW289205 Voucher BMZU) and the two species of *Tacca chantieri* (JQ733736 and MH748926) was observed. The two species of *Tacca chantieri* (along with the specimen examined MW289205), form a distinct clade with a high support bootstrap value of 96 (Figure 3). Assessments of the two selected loci culminated in a well-supported phylogenetic tree. *T. leontopetaloides* and *T. maculata* formed the sister clade to all other Tacca species. *T. palmata, T. plantaginea* and *T. bracteata* form a clade with low support values (Figure 3). Section *Tacca* has been well supported based on the phylogeny shown by Zhang et al. (2001). This section is distinguished by its geophytic behavior, perennial leaves with decompound foliar blades, a long ascending peduncle, substantially more inflorescences, more than two inner segments, many threadlike floral bracteoles, and a low number of ovules per fruit. According to Tanaka (1954) and Li & Li (1997), the contemporary genetic diversity dispersal patterns of *Tacca chantrieri* populations are believed to be the result of a hypothetical evolutionary event involving vicariance from a single common ancestor and fragmentation of the species' historic geographic range. Genetic drift affects the genetic structure and increases differentiation among populations when populations are small and geographically and genetically distant from one another (Barrett & Kohn 1991; Ellstrand & Elam 1993). This highlights a shortage of gene flow between groups, which may be inadequate to combat genetic drift. Both morphological and phylogenetic analysis confirm that the specimen analysed (MW289205 Voucher BMZU) is identical to *Tacca chantrieri.*

DISCUSSION

The species *T. chantrieri*, though not included in the IUCN Red List, is still described by many authors as an endangered species as they are rare even in their wild habitats. *T. chantrieri* consists of several dark colored

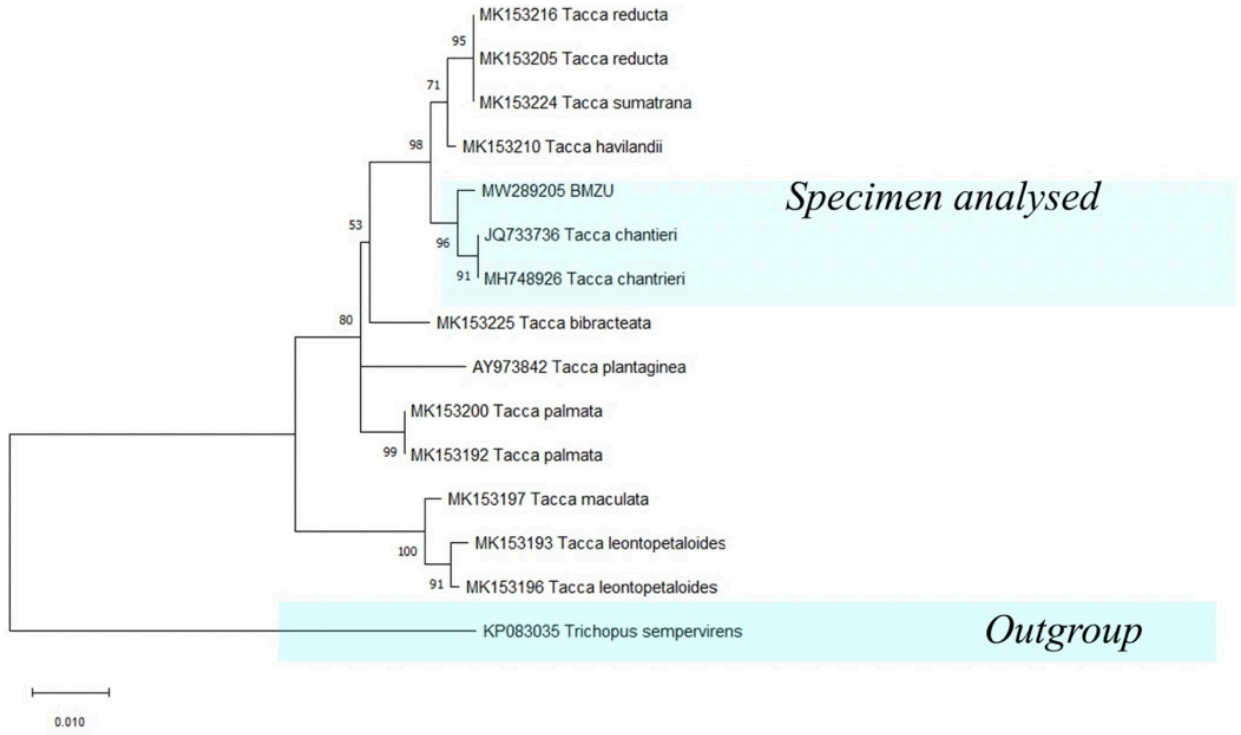

Figure 3. Phylogenetic tree.

Image 1. A,B—Habitat | C,D—Inflorescence | E–G—Leaves of *Tacca chantrieri*. © P.C. Lalbiaknii

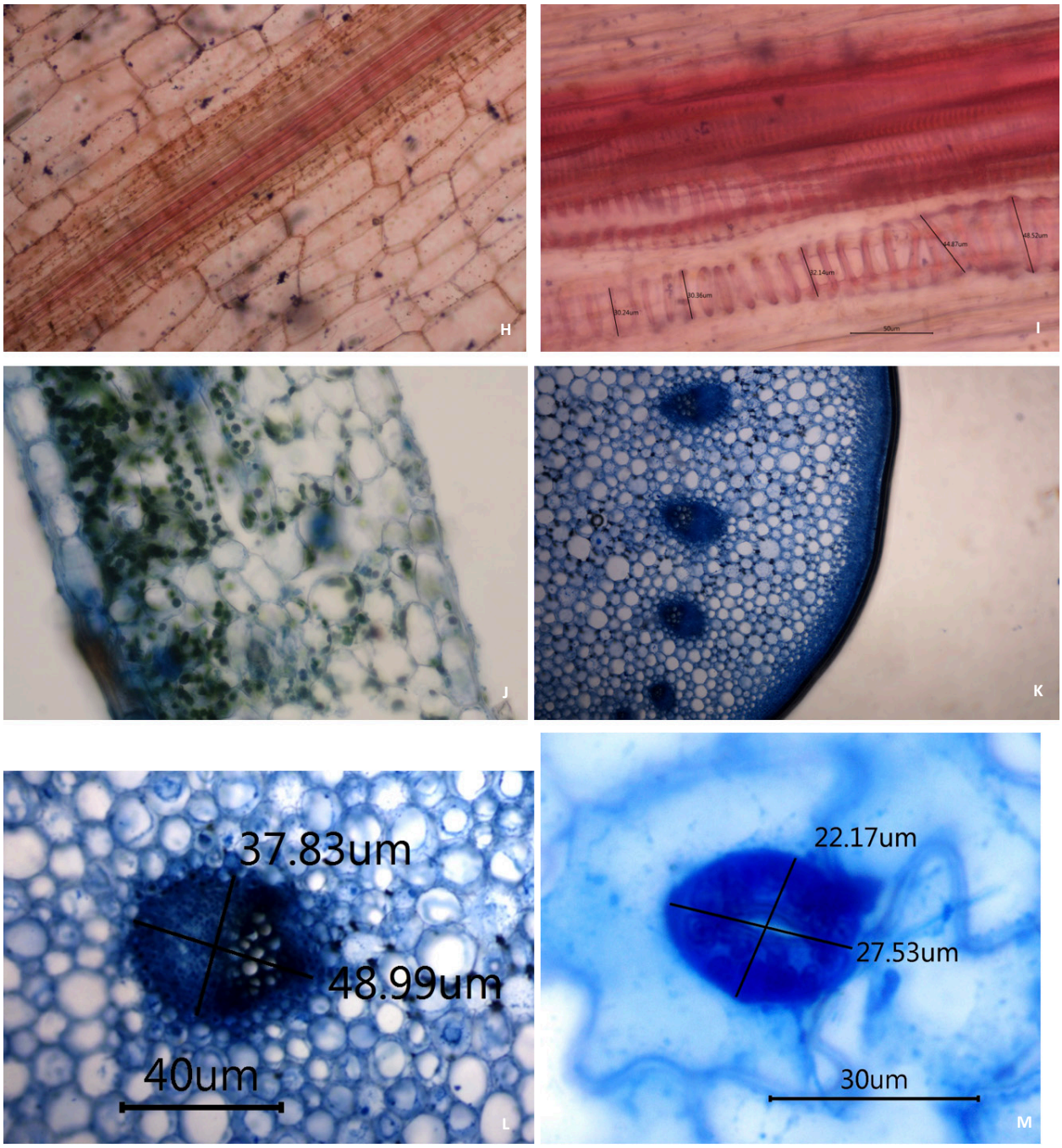

Image 1. H—L.S. of stem (40X) | I—L.S. of stem (40X) | J—T.S. of leaf (40X) | K—T.S. of stem (4X) | L—vascular bundle (40X) | M—single stomata. © P.C. Lalbiaknii

or maroon inflorescence with bracts and whisker like filiforms that makes it captivating. According to Zhang et al. (2005), it is a shade loving plant in its own natural habitat and florets are primarily self-pollinated and have several characteristics that encourage autonomous self-pollination. A potential explanation for its unusual inflorescence structure is that it aids in photosynthesis in the shady understory while also protecting the plant from herbivores. And due to the changes in the climatic conditions and landscape morphology of its native habitats it can be considered a rare, endangered or threatened species. The plant is very difficult to grow in an artificial or controlled environment, requiring specific temperature, moisture, and shade, and can take up to 11 to 12 months to germinate when cultivated by agriculturists. Hence, there is a significantly larger

potential for it to be developed as ornamental plants so as to conserve it from extinction. Apart from the species detailed, there might be many more species that are yet to be discovered in the unexplored terrains. So, it is imperative that we protect and conserve whatever species have been found regardless of their abundance and scarcity. Considering that habitat loss and overharvesting have been the primary cause of species endangerment, a central component of species recovery has been to establish a network of conservation areas and reserves that represent all the pertinent terrestrial and riparian natural communities. Species delineation provided by DNA-based techniques would provide important insights into the evolutionary biology and species diversity, but their versatility is limited in the apparent lack of multigene phylogenetic analysis. Future research in phylogenetic analysis will be critical in determining relevant perception to organise and better understand the basic similarities and differences between organisms, as well as other emergent properties of early life.

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