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First report of *Thekopsora rubiae* causing rust disease on *Rubia cordifolia* in India

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ABSTRACT

The symptoms of rust disease on *Rubia cordifolia* were investigated during the present study. The diseased symptoms were observed as yellowish-orange powdery mass on the lower surfaces of the leaves. The pathogen causing rust disease on *Rubia cordifolia* in India was identified as *Thekopsora rubiae* based on morphological characteristics and molecular analysis using the internal transcribed spacer region (ITS). This study was further explored to understand the taxonomic placement of this fungus from India. Based on available literature, this is the first report of *Thekopsora rubiae* on *Rubia cordifolia* in India.

Key words: Common Madder, *Pucciniales*, Rust Fungi, Taxonomy, *Thekopsora*

INTRODUCTION

Rubia cordifolia (Common Madder or Manjistha) is a flowering plant belonging to the family *Rubiaceae*. It is a thorny, perennial climber with brown or red roots that can reach up to 12 meters long. The stem is rough, with a lignified base and the branching of stems is 0.3–6 m long, with strong curved prickles on the four ribs, or fully pubescent, or at least hairy below the nodes. The roots are typically woody. It is distributed worldwide and has grown mainly for its red color, obtained from the roots. However, root extract also displays numerous pharmacological properties against various ailments. The roots are used as laxatives, analgesics, rheumatism, dropsy, paralysis and intestinal ulcers. The dried stem is used in blood, skin and urinogenital disorders, dysentery, piles, ulcers, inflammations, erysipelas, skin diseases and rheumatism. A decoction of leaves and stems is used as a vermifuge (Prajapath 2003; Khare 2004; Nyeem & Mannan 2018). *Rubia cordifolia* has been reported to contain more than 100 compounds, mainly including anthraquinones, naphthoquinones, anthraquinone glycosides, naphthoquinone glycosides, bicyclic hexapeptides, triterpenoids and polysaccharides (Natarajan et al. 2019). It was also reported as a source of an important natural dye used for dyeing hair, food and clothing (Ni et al. 2022).

Like other plants, *R. cordifolia* also gets infected with several pathogens, causing various diseases. Durrieu (1987) reported a rust disease on *R. cordifolia* caused by *Phaopsora punctiformis*). Powdery mildew disease on leaves caused by *Leveillua taurica* was reported by Kumar & Pandey (2003). Parashurama & Shivanna (2013) reported a leaf blight on this plant from Bhadra Wildlife Sanctuary, South India, incited by *Cladosporium cladosporioides*. The leaf spot disease of *R. cordifolia* caused by *Colletotrichum gloeosporioides* was reported from China by Tang & Tan (2020), which was later also verified by Talhinhas & Baroncelli (2023). Two diseases, namely, leaf spot and leaf blight, were also reported on this plant, which were caused by *Fusarium oxysporum* and *Colletotrichum* spp. respectively, that lead to defoliation and stem infections (Swamy et al. 2022). The association of *Penicillium polonicum* with *R. cordifolia* was also identified through molecular and morphological features in China/ Nanjing (Almiman 2023). Despite leaf spot, blight, and others, *R. cordifolia* has also been reported to be infected by several rust fungi.

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During the present investigation, fresh samples of rust fungi-infected leaves of *R. coridifolia* were collected and analyzed based on morphological examination and phylogenetic analyses using the internal transcribed spacer region (ITS) to confirm the taxonomic placement of this fungus from India.

MATERIALS AND METHODS

Morphology

The infected leaf specimens of *R. coridifolia* were collected during winter (November 2021) and the disease morphology was studied with the help of a hand lens. Surface scrapings and free-hand cut sections were taken through infected spots and mounted in distilled water for microscopic examination. The microscope slides were examined immediately and photographed.

Molecular characterization

For molecular characterization, the genomic DNA of collected rust fungi was extracted and ITS gene regions were amplified (Padamsee & McKenzie 2014). The obtained sequence was submitted to NCBI GenBank (accession No. PAURH2101). The newly generated sequences were aligned with sequences downloaded from GenBank based on published articles, and molecular phylogeny was inferred following (Hietala et al. 2008; Mostert et al. 2010; Yang et al., 2014; NCBI database 2024). Sequences were aligned using the MAFFT v.7.110 online program (Katoh et al. 2019), with manual adjustments made using BioEdit v.7.0.5.3 (Hall 1999) as needed. Maximum Likelihood (ML) analysis was conducted via the CIPRES Science Gateway (https://www.phylo.org/) employing the GTRGAMMA substitution model with 1,000 bootstrap replicates. Bayesian inference analyses were performed using PAUP* beta 4 and MrBayes v.3.2.1 (Ronquist et al. 2012). The evolutionary model was estimated using MrModeltest 2.2 (Nylander, 2004). Markov Chain Monte Carlo (MCMC) sampling in MrBayes v.3.2.2 (Ronquist et al. 2012) was used to estimate posterior probabilities (PP), with simultaneous Markov chains run for 3,000,000 generations and trees sampled every 1,000 generations. The resulting phylogenetic tree was visualized using FigTree v.1.4.3 (Rambaut 2012).

RESULTS AND DISCUSSION

Morphological identification

The disease specimens analyzed in the present study had the following morphology. Microscopic observations of the fungus revealed hypophyllous, subepidermal, scattered uredinia. Urediniospores were broadly ellipsoid or obovoid-globose, unicellular, echinulate, sessile, 17–23 × 12–17 µm. Telia were not observed (Figure 1).

Molecular characterization

The original collections and several later specimens are examined based on morphological characteristics. There are a total of 22 accessions of *T. rubiae* available on GenBank, depicting analysis of rust samples based on ITS, LSU and SSU gene regions to support the taxonomic placement. The phylogenetic tree generated by the MrBayes analysis of ITS sequences for *Thekopsora* (comprising 38 sequences) was rooted with *Pucciniastrum corni* (TSHR13510). The RAxML analysis produced a tree with a final Maximum Likelihood (ML) optimization value of -3038.256768. The alignment matrix contained 296 distinct patterns and 16.15% undetermined characters or gaps. The estimated base frequencies were: A = 0.296933, C = 0.171416, G = 0.177601, T = 0.354051. The substitution rates were as follows: AC = 1.472717, AG = 2.664480, AT = 1.450466, CG = 0.605120, CT = 2.463308, GT = 1.000000. The proportion of invariable sites was I = 0.428461, and the gamma distribution shape parameter was α = 1.404829. The maximum parsimony analysis involved a dataset of 646 characters, with 393 constant, 208 informative, and 46 uninformative characters. This analysis resulted in 1,000 equally parsimonious trees, each with a length of 466 steps (CI = 0.751, RI = 0.935, RC = 0.702, HI = 0.249). The statistical significance was confirmed with a P value < 0.05. The MrBayes analysis run the 6 million generations with a standard deviation of split frequencies of 0.003280. Bootstrap support values are reported as follows: Maximum Likelihood (ML) support ≥ 80%, Maximum Parsimony (MP) support ≥ 73%, and Bayesian Posterior Probabilities (BPP) ≥ 0.83. These values are indicated above and below the respective branches (ML/MP/BPP) (Fig. 2). However, the recent investigation by Aime & McTaggart (2021) used two gene sequences (KC416007 and KC416010) of the 28S gene region in their investigation of a higher-rank classification for rust fungi and proposed the placement of *Thekopsora rubiae* as *Pucciniastrum rubiae*. However, these genera have prominent morphological variations in the structures of spermogonia and telia (Cummins & Hiratsuka 2003). Recently, Zhao et al. (2023) proposed a new family *Thekopsoraceae*, to accommodate different species of *Thekopsora* based on phylogenetic and morphological distinctions. Their study further revised *Pucciniales* in line with the phylogenetic relationships.

The recent study of Aime & McTaggart (2021) investigated two voucher specimens of the rust *Thekopsora ostryae* (KC416007, KC416010) collected in 2012 from Gansu, northwestern China and considered *T. rubiae* as basionym and proposed it as *Pucciniastrum rubiae*. Their study necessitated further molecular studies to accurately identify other rust samples of the same fungi collected worldwide. Because most of the samples have been determined solely based on morphology, it doesn't seem easy to place all of them under newly established names due to morphological plasticity. As per the phylogenetic

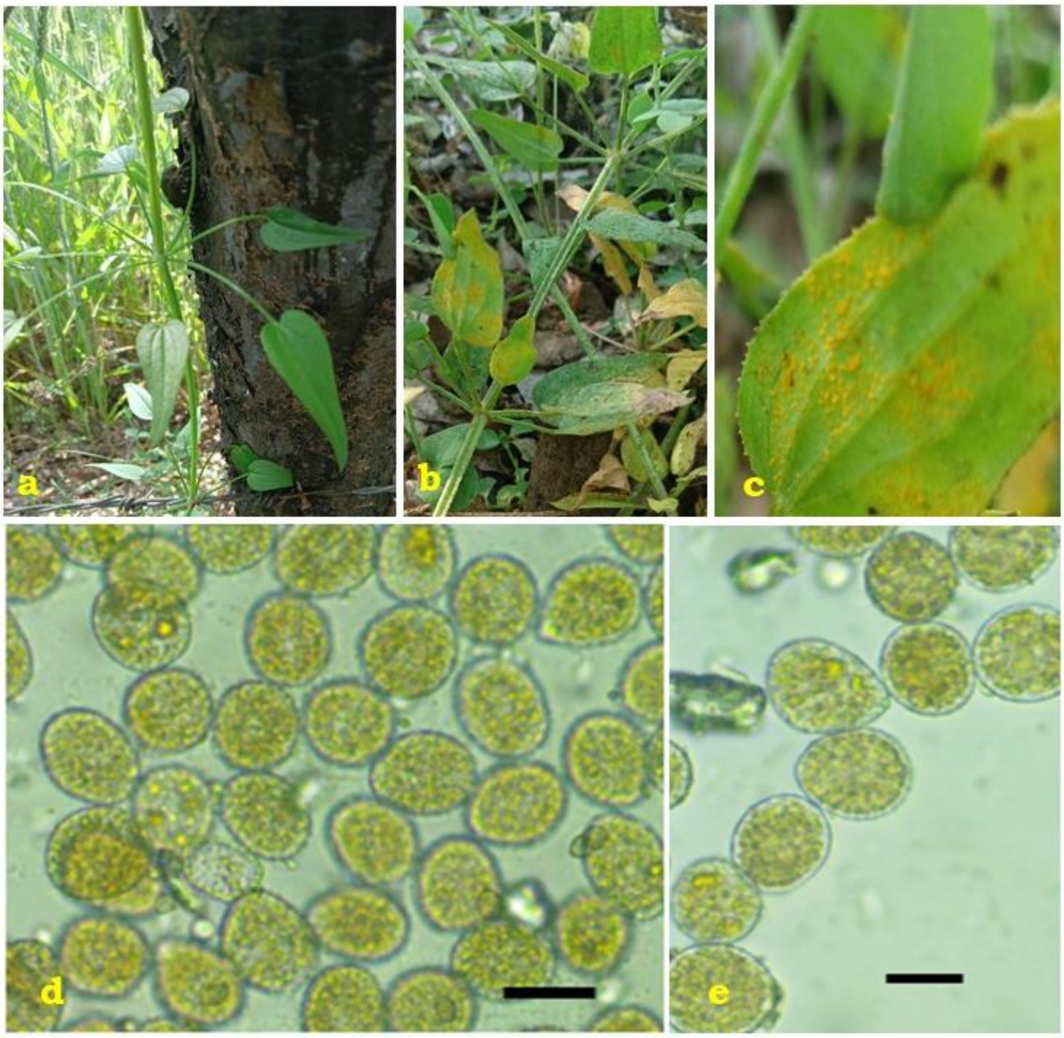

Figure 1. *Thekopsora rubiae* on *Rubia cordifolia*. (a) Healthy plant. (b-c) Abaxial leaf surface with symptoms. (d-e) Urediniospores (Scale=10 µm).

analysis carried out by Zhao et al. (2023), the *Thekopsora* clade, including the type of the genus, *Thekopsora areolata*, was phylogenetically close to *Cronartium* but distinct from *Pucciniastrum* species, which was found in agreement with Aime et al. (2018) (Figure 2). Although *Thekopsora* resembles *Coleopuccinia, Hylospora, Melampsoridium*, and *Pucciniastrum,* it differs from these genera in the aecia, uredinia and telia. In addition, this genus also differs from the phylogenetically allied family *Cronartiaceae* in the structures of spermogonia, uredinia and telia. Thus, a new family *Thekopsoraceae* was proposed to accommodate *Thekopsora* (Zhao et al. 2023). Based on morphology and phylogenetic analysis in the present study, we also proposed the taxonomic placement of rust species isolated in the present study as *Thekopsora rubiae*.

The life history of *Uredineae* is perhaps the earliest record of rust fungi on *R. cordifolia* (Barclay 1890). Likewise, the identification of this rust fungus as *Uredo rubiae* can be considered as another of its earlier records (Dietel 1900). The occurrence of rust disease caused by *Puccinia colletiana* (Barclay 1890; Gautam & Avasthi 2016; Gautam et al. 2021; Avasthi et al. 2025) on *Rubia cordifolia* and *Pucciniastrum rubiae* on *R. argyi* and *R. coridifolia* var. *mungista* was reported by Kakishima et al. (2023) during the analysis of the rust fungi (Pucciniales) collected from 2021 to 2023 at Tsukuba Botanical Garden. This rust fungus was reported as *Thekopsora rubiae* by Hiratsuka et al. (1992) and later by Yamaoka et al. (2009). To our knowledge, this is the first morphological and molecular confirmation of the presence of *Thekopsora rubiae* causing leaf rust on *Rubia cordifolia* in India.

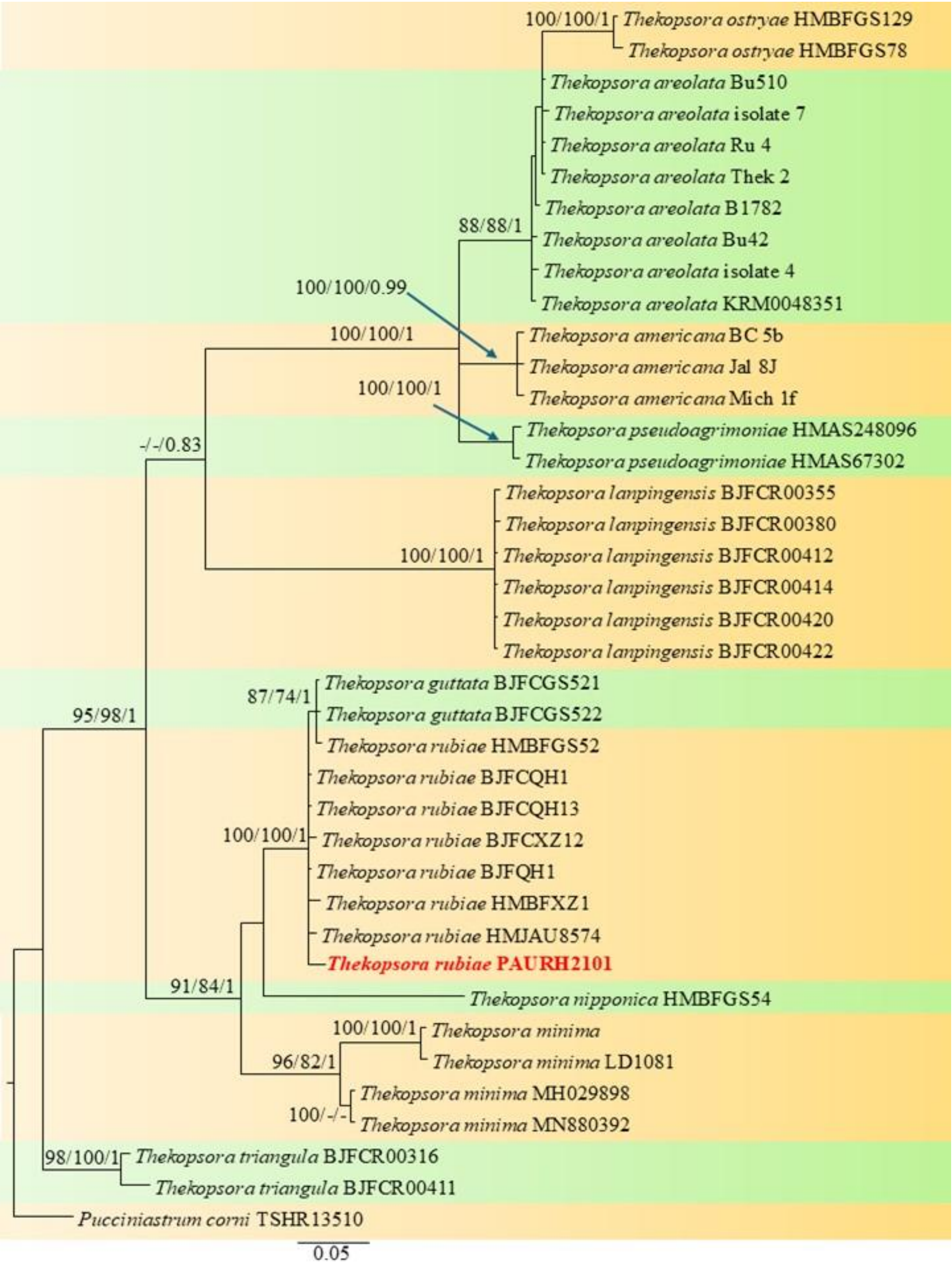

Figure 2. The phylogenetic tree generated by the MrBayes analysis of ITS sequences for *Thekopsora* (comprising 38 sequences) rooted with *Pucciniastrum corni* (TSHR13510).

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