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RESEARCH COMMUNICATION

Mycology

First report of *Ramularia coleosporii* associated with rust sori of *Coleosporium plumeriae* on *Plumeria* species in Sri Lanka

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Abstract: In this study, we report the first instance of the fungus *Ramularia coleosporii*, isolated in the sori of *Plumeria* leaf rust caused by *Coleosporium plumeriae* in Sri Lanka. The fungus *R. coleosporii* was cultured on potato dextrose agar (PDA) medium, DNA was isolated, and PCR was conducted targeting the internal transcribed spacer region (ITS) of the genome. Phylogenetic analysis using the obtained sequence data and other homologous sequences retrieved from GenBank revealed a close relationship with *Ramularia coleosporii* strains reported from Eastern and Western regions of Asia. The Sri Lankan isolate showed 99% sequence identity with *R. coleosporii* from South Korea, with strong bootstrap support (99%) for this identification. Although *R. coleosporii* has been reported as a hyperparasite of plumeria rust in other regions, hyperparasitism was not experimentally verified in this study.

Keywords: *Coleosporium plumeriae*, ITS phylogeny, *Plumeria* leaf rust, *Ramularia coleosporii*.

INTRODUCTION

Plumeria, commonly grown as an ornamental tree belongs to the family Apocynaceae and is well adapted to tropical and subtropical climates worldwide. This introduced species in Sri Lanka is locally known as "Araliya" or the temple tree. Two species commonly found on the island are *Plumeria rubra* L. (Figure 1a) and *Plumeria obtusa* L. (Figure 1b) with flowers that are often used in religious ceremonies (Manimohan & Mannethody, 2011). The rust disease was first reported in Sri Lanka in 2006 (Weeraratne & Adikaram, 2006), and can be seen on both *Plumeria* species in Sri Lanka.

In the present case, whitish mycelium-like structures containing sori or rusts were observed on the abaxial surface of leaves of *P. rubra* collected from a tree located on the periphery of the Hanthana conservation forest in Peradeniya, Central Province, Sri Lanka (Figure 2). It was noted that the whitish mycelium appears after 4-5 weeks of the rust's initiation. Previous studies have reported the occurrence of hyperparasites on rust fungi in other regions of the world. Mycoparasites are considered as potential biocontrol agents against plant pathogens (Baiswar et al., 2015). Various species of *Ramularia* have been documented as hyperparasites of rust fungi on different host plants across multiple geographic regions (Pirnia et al., 2021).

MATERIALS AND METHODS

Rust spores from sori were collected using a paintbrush, suspended in sterile distilled water, and transferred to Potato Dextrose Agar (PDA) medium. After three weeks of incubation at 27 °C (relative humidity 78%), a cottony fungal mycelium was observed and single spores were isolated and then incubated under the same conditions (Figure 3).

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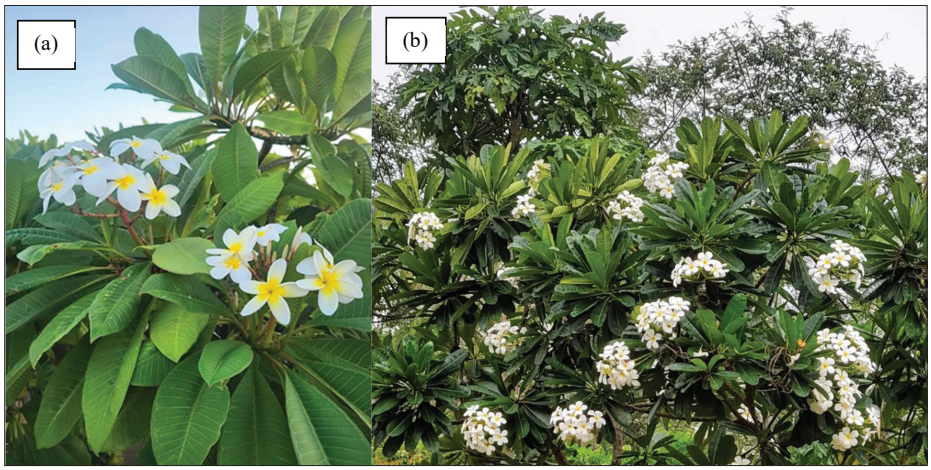

Figure 1: *Plumeria* species (a) *P. rubra* L.; (b) *P. obtusa* L.

Figure 2: The white mycelia of *R. coleosporii*, colonizing the rust sori found on the abaxial surface of the leaves of *P. rubra* L.

Figure 3: Culture characteristics of *R. coleosporii* isolate PQ142333 grown on Potato Dextrose Agar (PDA) medium showing cottony mycelium after single spore isolation; Left half; upper surface; right half; lower surface.

Fresh mycelia (100 mg) were prepared by scraping colonies from a two-week old culture grown on PDA medium at 27 °C in 1.5 mL Eppendorf tubes for the isolation of genomic DNA. Mycelia were homogenized for 20 s with liquid nitrogen using a pestle and mortar. To this homogenate, 500 µL of extraction buffer [200 mmol/L of Tris-HCl (pH 7.5)], 250 mmol/ L of NaCl, 25 mmol/L of EDTA, and 0.5% of SDS (Edwards et al., 1991) were added, and the solutions were mixed and homogenized for five minutes with a Vortex Genie 2 mixer at the maximum speed. Then, 350 µL of phenol was added to each tube and gently inverted four times followed by the addition of 250 µL of chloroform, and the tubes were mixed by inverting them 40 times. The samples were then centrifuged at 2834 x g for 30 min at 4 °C. Subsequently, the aqueous phase of all the samples was transferred to new tubes. The samples were treated with 2 µL RNase (20 µg/mL TE) and allowed to incubate at 37 °C for 10 min, followed by mixing equal volumes of chloroform. The tubes were centrifuged at 4 °C at 2834 x g for 10 min, and the aqueous layer was transferred to new Eppendorf tubes containing 0.5 volume of cold

isopropanol to precipitate the DNA. Tubes with the samples were spun at 4 °C for 2834 x g for 5 min, and the supernatant was discarded by gently pouring it into a sink. Cold 70% ethanol (100 µL) was added to the pellet, and the samples were centrifuged at 4 °C, 2834 x g for 5 min, and the ethanol was discarded. Each pellet was then incubated at 37 °C for 30 min, and suspended in 50 µL of sterile distilled water. The optimum DNA yields and quality of the subsequent DNA were analyzed by the ethidium bromide-stained 1% (w/v) agarose gel method as described by Edwards et al. (1991).

PCR reactions were done using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The amplification reaction for each sample was obtained with 0.20 mM of each dNTP, 0.4 mM each of the forward and reverse primers, 0.5U Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2 µL of genomic DNA solution, and 10 × Easy Taq buffer, bringing the volume up to 50 µL. A typical reaction was as follows: denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 50 s, annealing at 52 °C for 50 s, extension

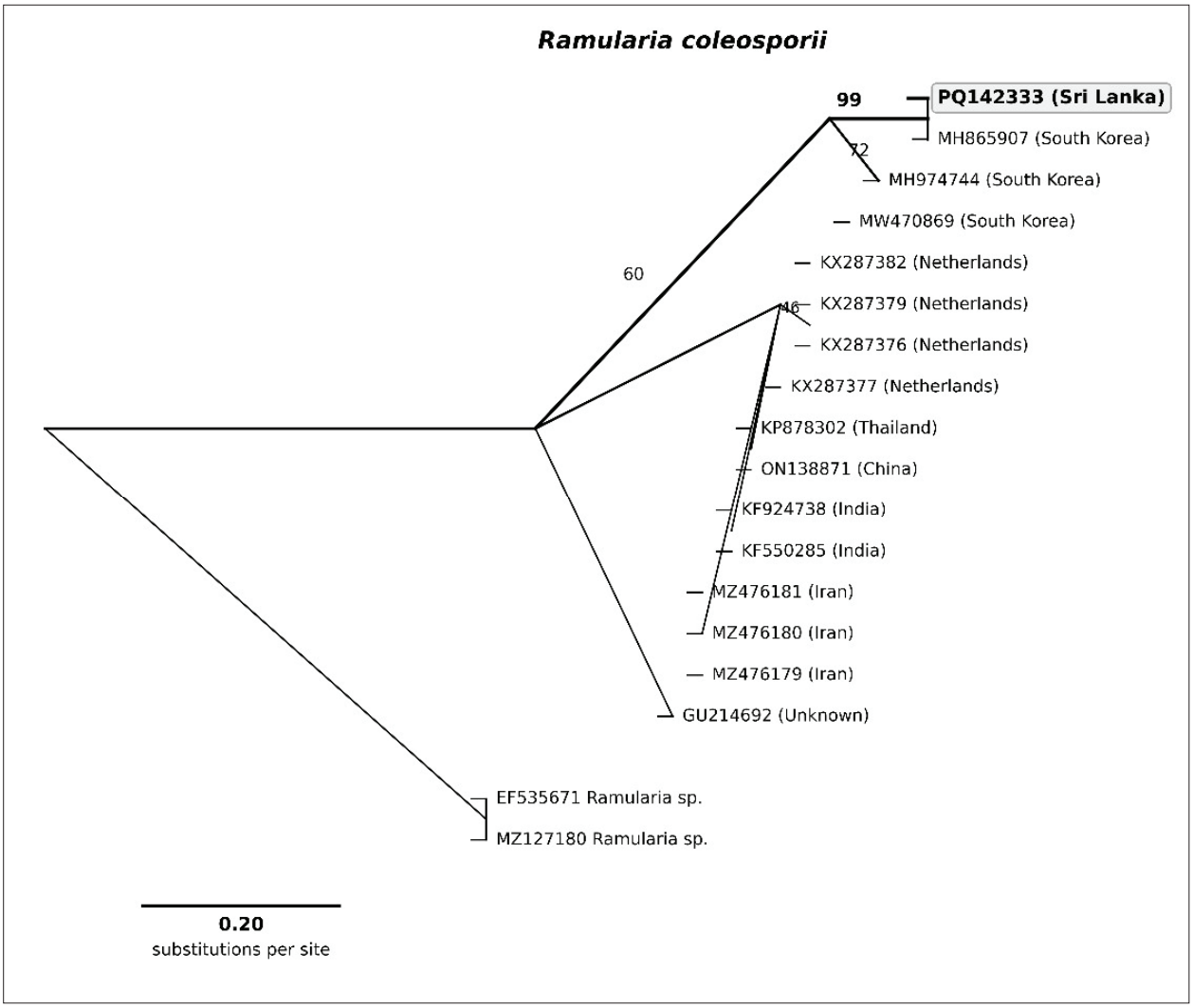

Figure 4: Maximum Likelihood phylogenetic tree of *Ramularia coleosporii* based on ITS sequence data. The tree was constructed using the Tamura-Nei model with 1000 bootstrap replications in MEGA11. Bootstrap support values ≥45% are shown at nodes. The Sri Lankan isolate (PQ142333) is highlighted and clusters with the South Korean isolate MH865907 with 99% bootstrap support. *Ramularia* spp. sequences serve as outgroups. The scale bar indicates 0.20 substitutions per site.

at 72 °C for 60 s, and final extension for another 10 min at 72°C. The PCR products were purified using a PCR purification kit (Qiagen) and sequenced in both directions using the same primers at Macrogen Inc., South Korea.

RESULTS AND DISCUSSION

The sequence was deposited in GenBank under the accession number PQ142333. BLAST analysis showed that the analyzed isolate was 99% homologous to *R. coleosporii* isolate accessioned as MH865907 reported from South Korea. Phylogenetic analysis was conducted in MEGA11 using the maximum likelihood method with the Tamura-Nei model (Tamura & Nei, 1993). Bootstrap replications were set to 1000 to enhance the credibility of the branching patterns. The phylogenetic analysis indicated that the Sri Lankan isolate clusters with *R. coleosporii* sequences from various geographical regions with strong statistical support. The main identification node (Sri Lankan isolate + South Korean isolate MH865907) showed 99% bootstrap support, providing reliable species identification. Internal nodes showed moderate support values (45-72%), which is typical for closely related intraspecific sequences with minimal genetic divergence (Figure 4).

The use of ITS sequences for fungal species identification follows established protocols, as ITS has been validated as the primary DNA barcode for fungi (Schoch et al., 2012). For *Ramularia* species specifically, ITS-based phylogeny has proven effective for species delimitation and has been extensively used in taxonomic studies (Videira et al., 2015; Bakhshi, 2017). While multi-locus phylogenetics could provide additional resolution for population genetic studies, the combination of high sequence similarity and strong bootstrap support observed in this study provides sufficient evidence for species-level identification.

The phylogenetic analysis places the Sri Lankan isolate within a well-supported clade of *R. coleosporii* sequences from multiple geographic origins including South Korea, Netherlands, India, Iran, Thailand, and China. This cosmopolitan distribution suggests that *R. coleosporii* is widely distributed as a fungicolous fungus associated with rust diseases across diverse climatic zones.

It is important to note that while *R. coleospori*i has been reported as a hyperparasite of rust fungi in other studies, the hyperparasitic nature of this interaction was not experimentally verified in the present study through parasitism assays or microscopic examination of host-parasite interactions. Our observations were limited to the morphological association of *R. coleosporii* with rust sori on *Plumeria* leaves. Further studies, including detailed microscopic observations of fungal penetration into rust structures and pathogenicity tests, are needed to conclusively demonstrate hyperparasitism in the Sri Lankan context. This represents the first confirmed report of *R. coleosporii* associated with *Plumeria* rust in Sri Lanka, expanding the known geographic distribution of this fungicolous species. The finding contributes to our understanding of the mycobiota associated with rust diseases in tropical environments and highlights the need for comprehensive studies of fungal interactions.

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