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# *Ramularia coleosporii* (*Mycosphaerella*) on *Plumeria* rust in Thailand

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**Abstract**

A hyperparasitic fungus was found on uredinia of *Coleosporium plumeria* on leaves of *Plumeria rubra* in Thailand. The hyperparasite was identified as *Ramularia coleosporii* following an examination of its morphological characters and a phylogenetic analysis by using ITS sequence data. This is the first record of *R. coleosporii* on *C. plumeriae* in Thailand. *Ramularia coleosporii* has the potential for biocontrol management strategies of the rust.

**Key words** – biocontrol – *Coleosporium plumeriae* – fungicolous fungi – hyperparasite

## Introduction

*Plumeria* is a small tree in the family Apocynaceae. It is commonly known as frangipani, nosegay, or temple tree, and is an ornamental in most tropical and subtropical areas. These trees are commonly found in parks, gardens and landscaped areas in Thailand. They are relatively free from major pests and diseases, however, in the last two decades a rust disease has spread to several parts of the world where *Plumeria* species are grown, often causing extensive defoliation (Kakishima et al. 1995, Manimohan & Mannethody 2011). The rust *Coleosporium plumeriae* Pat. has infected *Plumeria* in many tropical and subtropical regions (Ogata & Gardner 1992, Kakishima et al. 1995, Minter et al. 2001, Yang et al. 2006). This rust fungus was first recorded in Thailand on *Plumeria acuminata* and *P. rubra* by To-anun et al. (2004).

*Coleosporium plumeriae* causes extensive yearly leaf loss, but chemical control of a rust fungus on an ornamental host such as *Plumeria* is not feasible (Moricca & Ragazzi 2008). Fungal biocontrol agents may therefore be an alternative approach. Some species in the genera *Aphanocladium*, *Cladosporium*, *Scytalidium*, *Sphaerellopsis*, *Tuberculina* and *Verticillium* have been found on the sori of rust fungi, and may be potential biocontrol agents (Moricca and Ragazzi 2008). Recently, *Zygosporium gibbum* (Sacc. et al.) S. Hughes was found parasitizing *C. plumeriae* colonies on leaves of *Plumeria* sp. in India, and it was suggested as a potential biocontol agent (Manimohan & Mannethody 2011).

The genus *Ramularia* is the asexual morph of *Mycosphaerella* (Verkley et al. 2004, Hyde et al. 2013). There are 1152 records of *Ramularia* in Index Fungorum (2015), many member of this genus have been reported to cause leaf spots. However, three species, *Ramularia coleosporii* Sacc., *R. uredinearum* Hulea and *R. uredinicola* Khodap. & U. Braun are found associating with rust

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fungi (Braun 1998, Khodaparast & Braun 2005). *Ramularia coleosporii* was original isolated from sori of *Coleosporium* sp. (Saccardo 1880). It has been considered as a mycoparasite on uredinia of several rust taxa including *Coleosporium campanulae* and *C. petasitis* in Austria (Poelt & Fritz-Schroeder 1983, Morgan-Jones et al. 1972), *C. clematidis*, *C. clerodendri* and *Puccinia exhausta* in China (Zhang 2006), *C. tussilaginis* in New Zealand (Braun & Hill 2002) and *C. plumeriae* in India (Baiswar et al. 2015). However, there is no report of *Ramularia coleosporii* being associated with *C. plumeriae* in Thailand.

We collected a *Ramularia*-like mycoparasite on the sori of *C. plumeriae* on the leaves of *Plumeria rubra* in Chiang Rai Province, Thailand. This paper reports its occurrence in Thailand, details its morphological characters and provides a phylogenetic analysis.

## Materials & Methods

### Sampling and morphology

Rust infected leaves of Plumeria rubra were collected on 2nd Feb, 2014 in Chiang Rai Province, Thailand, and were taken to the laboratory in paper bags. Hyphae growing over the uredinia were removed by using a needle, placed in a drop of distilled water on a clean slide and covered by a cover slip. The slides were examined using a Nikon Eclipse 80i compound microscope fitted with a Canon 450D digital camera.

### Isolation

Single spore isolation was carried out following the method described in (Chomnunti et al. 2014). Germinating spores were transferred aseptically to malt extract agar (MEA) plates and grown at 20°C, then were transferred to Potato Dextrose Agar (PDA) and grown at 25 to 20°C. Colony colour and other characters on PDA were observed and measured after one week and one month. The specimens are deposited in the Mae Fah Luang University (MFLU) Herbarium (MFLU 16–0944), Chiang Rai, Thailand. Living cultures are also deposited in the Culture Collection in Mae Fah Luang University (MFLUCC 14–0330).

### DNA extraction, PCR amplification and sequencing

Fresh mycelia (50–100 mg) were harvested from 14-day-old cultures grown on PDA media at 25 °C in 1.5 mL Eppendorf tubes for genomic DNA extraction. DNA was extracted following the protocol of sequence of internal transcribed spacer (ITS) regions was amplified by polymerase chain reaction (PCR) with the primer pairs ITS5 and ITS4 (White et al. 1990). Each amplification reaction included 0.2 mM of each dNTP, 0.4 mM of each primer, 0.5 U of Taq polymerase (Transgen, China), 2 µl of genomic DNA solution, 10 × Easy Taq buffer (Transgen, China) in 50 µl reaction volume. A typical reaction included an initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 50 s, annealing at 52 °C for 50 s, extension at 72 °C for 60 s and a final extension at 72 °C for 10 mins. Reactions were run with positive and negative controls to ensure accuracy and to detect contamination. Automated sequencing was performed by Sino Geno Max Co. (Beijing, China).

### Phylogenetic analysis

The closest taxa to our strain were determined with standard nucleotide blast searches, with ITS sequences against the nucleotide database in GenBank (http://www.ncbi.nlm.nih.gov/). The ITS sequence of *R. cynaraei* closest relatives of *R. coleosporii* was selected as ingroup, and *Acrodontium crateriforme* was employed as outgroup. Multiple sequence alignments were generated with aligned by MAFFT ver. 7.03 using the Q-INS-I strategy (Katoh et al. 2013), Maximum parsimony analyses were conducted with PAUP 4.0b10 (Swofford 2002). Bayesian analysis was performed in a likelihood framework as implemented by MrBayes v3.0b4 software package to reconstruct phylogenetic trees (Huelsenbeck & Ronquist 2001). The best-fit model of evolution was determined by MrModeltest 2.3 (Posada & Crandall 1998, Nylander 2004) through

comparing different nested models of DNA substitution in a hierarchical hypothesis-testing framework. "GTR+G+I" was tested as the best model, and parameters were set as follows: lsetnst = 6, and rates = invgamma; Prset statefreqpr = dirichlet (1, 1, 1, 1). Multiple Bayesian searches using Metropolis-coupled Markov chain Monte Carlo sampling were conducted. One cold and five heated Markov chains were used in the analysis. Bayesian analysis was run for 504000 generations, with trees sampled every 100 generations. The first 1080 trees, which represented the burn-in phase of the analysis, were discarded. To estimate posterior probabilities (PP) of recovered branches (Larget & Simon 1999) 50% majority rule consensus trees were created from the remaining trees using PAUP.

**Table 1** ITS sequence data used in this study

| Species | Collection No[a]. | Origin | Accession No. |
|---|---|---|---|
| *Acrodontium crateriforme* | CBS 144.33 | Contaminant, Netherlands | FN666565 |
| *Mycosphaerella punctiformis* | CBS 115297 | | AY853180 |
| *M. punctiformis* | CBS 115311 | | AY853182 |
| *M. punctiformis* | CBS 115302 | | AY853183 |
| *Ramularia aplospora* | CBS 545.82 | | EU040238 |
| *R. beticola* | 10-RR-35 | *Beta vulgaris*, Denmark | JF330025 |
| *R. beticola* | 09-RR-24 | *B. vulgaris*, Denmark | JF330018 |
| *R. beticola* | 10-RR-22 | *B. vulgaris*, Denmark | JF330013 |
| *R. carthami* | s-527 | USA | DQ466083 |
| *R. coleosporii* | KACC 42485 | rust on *Aster pilosus*, South Korea | EF535674 |
| *R. coleosporii* | Ra_01 | *Coleosporium plumeriae*, India | KF550285 |
| *R. coleosporii* | Voucher KACC 42483 | *C. perillae* on *Perilla frutescens* var. *japonica*, South Korea | EF535672 |
| *R. coleosporii* | voucher KACC 42484 | *C. eupatorii* on *Eupatorium chinense* var. *simplicifolium*, South Korea | EF535673 |
| *R. coleosporii* | Ra 02 | *C. plumeriae*, India | KF924738 |
| *R. coleosporii* | voucher KACC 42485 | rust on *Aster pilosus*, South Korea | EF535674 |
| *R. coleosporii* | MFUCC 14-0330 | *C. plumeriae*, Thailand | KP878302 |
| *R. collo-cygni* | E25 | *Hordeum vulgare*, Denmark | JN003641 |
| *R. collo-cygni* | 18.2 | *H. vulgare*, Denmark | JN003640 |
| *R. collo-cygni* | s43/3 | *H. vulgare*, Switzerland | GU939178 |
| *R. collo-cygni* | s55 | *H. vulgare*, Switzerland | GU939172 |
| *R. collo-cygni* | R7 | *H. vulgare*, New Zealand | AJ536191 |
| *R. collo-cygni* | R10 | *H. vulgare*, New Zealand | AJ536184 |
| *R. collo-cygni* | R4 | *H. vulgare*, New Zealand | AJ536178 |
| *R. collo-cygni* | H12 | *H. vulgare*, Denmark | JN003642 |
| *R. cynarae* | CPC 18426 | *Cynara cardunculu*, USA | HQ728117 |
| *R. cynarae* | CPC 18725 | *C. cardunculus*, USA | HQ728118 |
| *R. endophylla* | CBS 113265 | *Quercus robur*, Netherlands | JQ739802 |
| *R. didyma* | 509 | *Ranunculus asiaticus*, USA | HQ442297 |
| *R. endophylla* | CBS 113265 | *Q. robur*, Netherlands | KF251329 |
| *R. endophylla* | CBS 113265 | *Q. robur*, Netherlands | KF251220 |
| *R. eucalypti* | CPC 13046 | *Corymbia grandifolia*, Italy | EF394861 |
| *R. eucalypti* | CBS 120726 | *C. grandifolia*, Italy | EF394860 |
| *R. eucalypti* | CPC 13304 | *Eucalyptus tereticornis*, Australia | EF394862 |
| *R. lamii* | CPC 11312 | *Leonurus sibiricus*,South Korea | KF251331 |
| *R. lamii* | CPC 11312 | *L. sibiricus*, South Korea | KF251222 |
| *R. lamii* var. *lamii* | KACC 42534 | *L. sibiricus*, South Korea | EF535688 |
| *R. lamii* var. *lamii* | KACC 42523 | *L. sibiricus*, South Korea | EF535683 |

**Table 1 (continued)**

| Species | Collection No[a]. | Origin | Accession No. |
|---|---|---|---|
| *R. lamii* var. *lamii* | KACC 42511 | *L. sibiricus*, South Korea | EF535676 |
| *R. miae* | CBS 120121 | *Wachendorfia thyrsifolia*, South Africa | DQ885902 |
| *R. pratensis* | F0709 | Malus x domestica_Fuji, Japan | AB693924 |
| *R. pratensis* | CPC 11294 | *Rumex crispus*, South Korea | KF251223 |
| *R. pratensis* | s89 | *Rumex* sp., Germany | GU939182 |
| *R. pratensis* var. *pratensis* | CPC 11294 | *R. crispus*, South Korea | EU019284 |
| *R. proteae* | CPC 18294 | *Physalis longifolia*, Australia | JN712499 |
| *R. proteae* | CBS 112161 | *P. longifolia*, Australia | EU707899 |
| *R. rumicis-crispi* | voucher CEO06 | *Rumex japonicus*, Taiwan | JN662315 |
| *R. sphaeroidea* | STE-U 5242 | | AY352584 |
| *R. stellenboschensis* | clone NY218 | *Pinus radiata*, Australia | HQ442297 |
| *R. stellenboschensis* | CBS 130600 | *Protea* sp., South Africa | KJ406791 |
| *R. uredinicola* | IRAN | *Melampsora* sp. on *Salix babylonic*a, Iran | GU939180 |
| *R. uredinicola* | CPC 10813 | *Salix* sp., South Korea | GU214694 |
| *R. vizellae* | CPC 18283 | *Protea* sp., in association with *Vizella interrupta* ,South Africa | JN712500 |

Note: aCBS = Centraalbureauvoor Schimmelcultures, Netherlands; KACC = Korean Agricultural Culture Collection, Korea; MFLUCC = Mae Fah Luang University Culture Collection. The others from the personal Culture Collection.

## Results

### Phylogenetic analyses

Nucleotide sequence blast searches of the NCBI-nrDNA database, showed that isolate MFLUCC 14–0330 had 99% ITS sequence similarity with *Ramularia coleosporii*, and showed high similarity with other species in *Ramularia.*

The combined ITS dataset comprised of 53 taxa (Table 1) including isolate MFLUCC 14–0330 with *A. crateriforme* as the out group taxon. The analysis of Maximum parsimony consisted with total 468 characters, 272 constant characters, 149 parsimony informative and 47 variable characters parsimony uninformative. The Bayesian tree has the similar topology with the best Maximum parsimony tree. The values of the Bayesian posterior probabilities (PP) (equal to or above 95%) from MCMC analyses are shown (Fig. 3). Bootstrap support (BS) values of MP (equal to or above 50% based on 1000 replicates) were shown. According to the phylogenetic analyses, our strain (MFLUCC 14–0330) formed a subclade with other *R. coleosporii* and showed strong high Bootstrap value (85%). These fungicolous *R. coleosporii* could also form a clade with *R. uredinicola* which was another fungicolous fungus of rust fungi and separated from representative species of the genus, however, the value of BS and PP are low.

***Ramularia coleosporii*** Sacc., Michelia 2 (6): 170, t. 983 (1880) Figs 2, 3

MycoBank 163652; Facesoffungi number: FoF 00752

*Parasitic* on colonies of *Coleosporium plumeriae* on leaves of *Plumeria* spp (Fig. 2). *Colonies* effuse whitish, mycelium 1.5–3.0 µm wide, superficial and immersed, sparingly branched, septate smooth, hyphae septate, subhyaline. Stroma, hyphopodia and setae absent. Sexual morph: undetermined. Asexual morph: *Conidiophores* 35–135 × 3–5 µm, macronematous, mononematous, arising from hyphae immersed in uredisporw, or occasionally solitary, arising from superficial hyphae, erect, straight, subcylindrical to geniculate-sinuous, unbranched or branched. *Conidiogenous cells* 10–20 × 2–3 µm, discrete, subulate, gradually tapering to 4 µm wide near the apex, one or two scar on the apex, hyaline. *Conidia* 6–28 × 4–6 µm, catenate, in simple or branched chains, ellipsoid-ovoid, fusiform, old conidia sometimes with 1–4 conspicuous hila.

Culture characteristics – Colonies on PDA slow growing, hyaline when young, light greyish at maturity (Fig 3, a), raised on surface, with the mycelium composed of septa, branched hyphae, formed in abundance, after 30 d, 0.5 cm diameter at 25°C. Colonies effuse whitish, mycelium superficial and immersed, sparingly branched, septate smooth, hyphae septate, subhyaline. *Conidiophores* 35–140 × 3–4 µm, unbranched, straight or flexuous, hyaline. *Conidiogenous cells* 15–26 × 3–4 µm, discrete, subulate, 1 or 3 scar on the apex. *Conidia* 6–26 × 4–6 µm, catenate, in simple or branched chains, ellipsoid-ovoid, fusiform, old conidia sometimes with 1–4 conspicuous hila.

Specimen examined – Thailand, Chiang Rai Province, Chiang Rai city, on colony of *Coleosporium plumeriae* on leaves of *Plumeria rubra***.** MFLU15–033, living culture in MFLUCC10–0064; ROMANIA, Distr. Mures, Sovata Bâl, on *Senecio doria* subsp. *umbrosus* CBS H-17708, CBS H-17709; ROMANIA, Distr, Prahova, Busteni, Valea Jepilor, on leaves of *Petasites kablikianus* CBS H-17710.

Notes – *Ramularia coleosporii* was original isolated from sori of *Coleosporium* sp. (Saccardo 1880). It has been considered as a mycoparasite on uredinia of several rust taxa in Austria (Poelt & Fritz-Schroeder 1983, Morgan-Jones et al. 1972), China (Zhang 2006), New Zealand (Braun & Hill 2002) and India (Baiswar et al. 2015). This specimen was collected from Chiang Rai on colony of *Coleosporium plumeriae* on leaves of *Plumeria rubra*. Compared with the *Ramularia* species associated with rust fungi, our collection has the similar morphological traits of *Ramularia coleosporii.* Meanwhile the phylogenetic analyses also affiliated our isolate to *Ramularia coleosporii.*

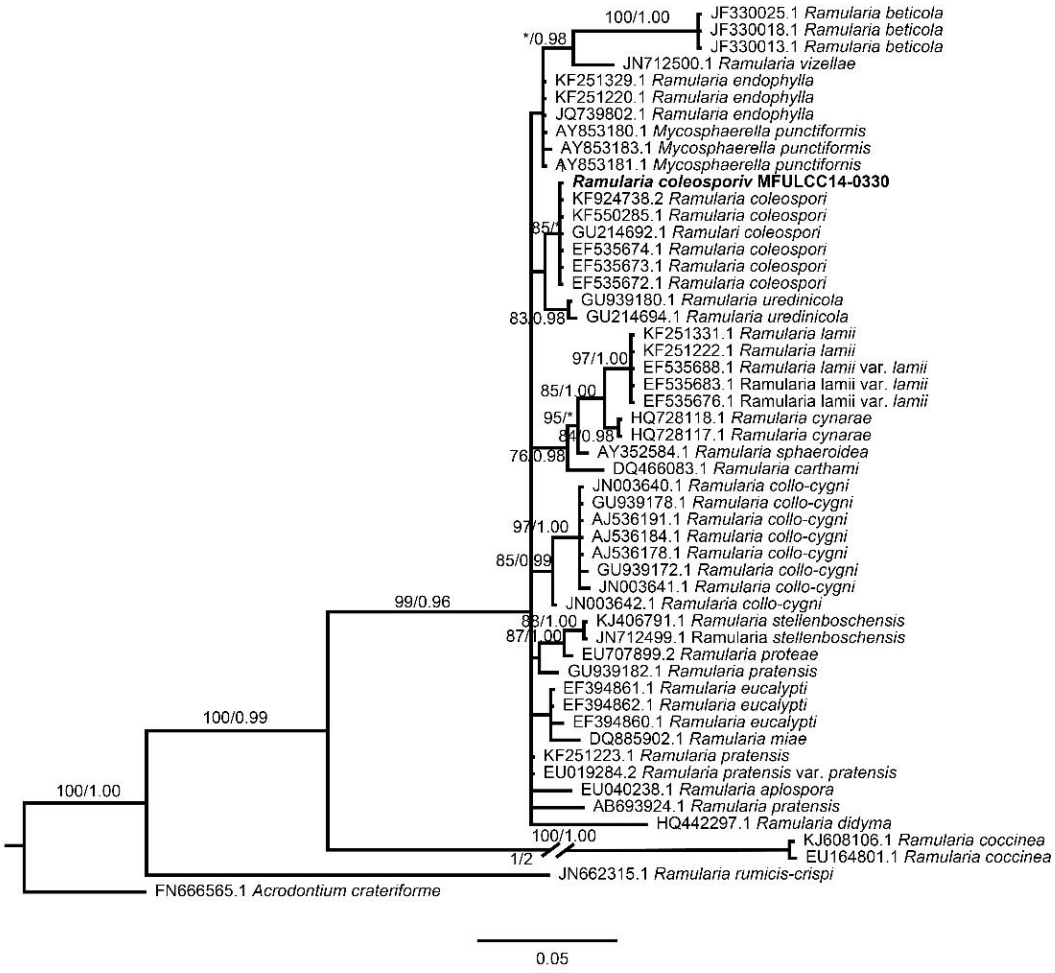


**Fig. 1 –** Phylogram inferred from likelihood analysis using ITS sequences. Bootstrap support values for maximum likelihood (ML) higher than 50% are defined. Bayesian posterior probabilities (BYPP) greater than 0.95 are provided.

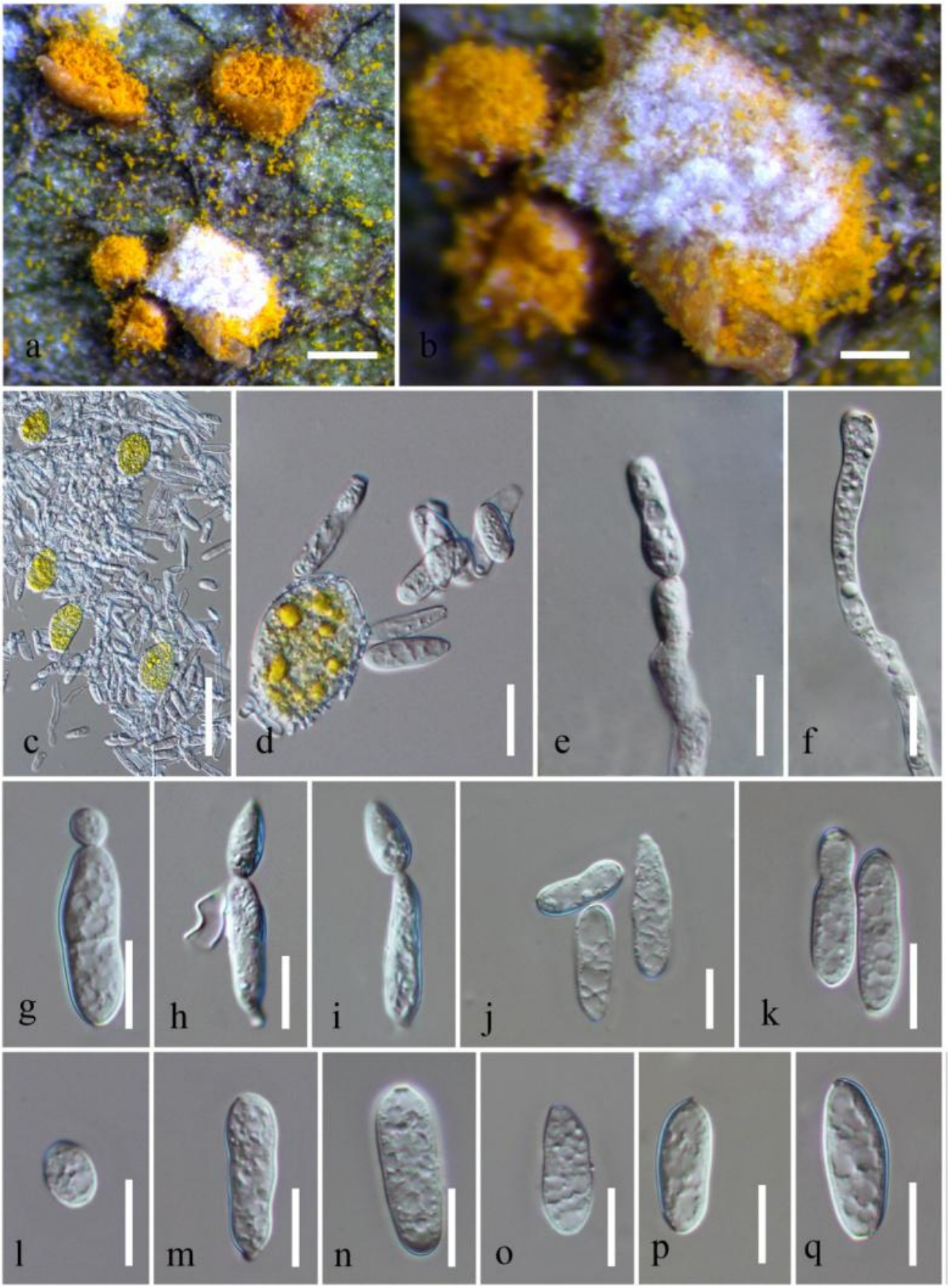


**Fig. 2** – *Ramularia coleosporii.* a, b Colony on uredinia of *C. plumeriae* on *Plumeria rubra* leaf. c d conidia and rust urediniospores. e Conidiophore with conidium. f Conidiophore without conidia; g, q Conidia. Scale bars: a = 500 µm, b = 200 µm, c = 50 µm, d–q = 10 µm.

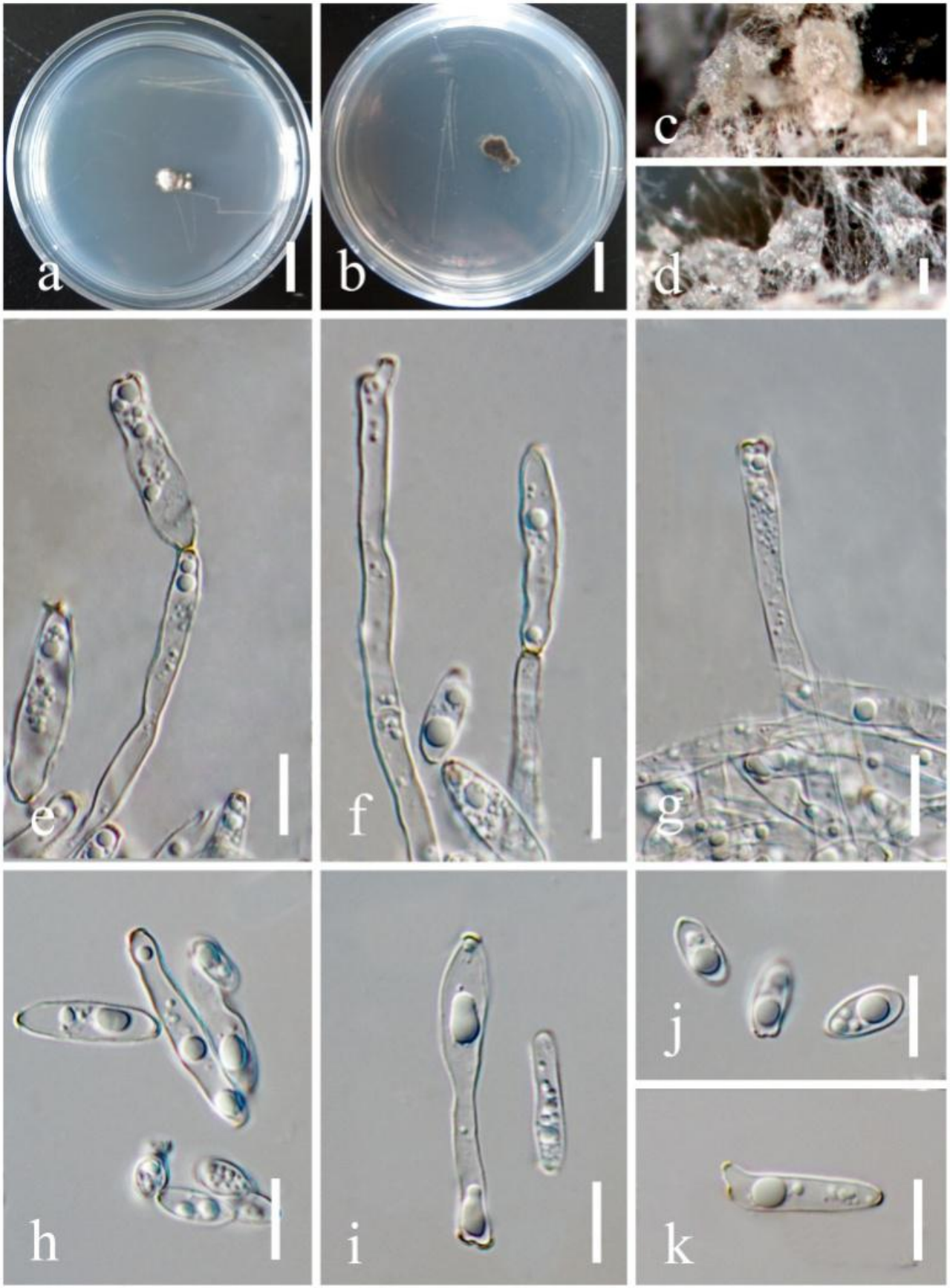


**Fig. 3** – *Ramularia coleosporii* on PDA. a, b Thirty-day-old colony on PDA. c, d Hyphae on PDA, e, f Conidiophores with conidia. g Conidiophore without conidia. h, k Conidia. Scale bars: a, b = 1 cm, c, d = 100 µm, e-k = 10 µm.

## Discussion

*Ramularia* species are mostly leaf pathogens, with only three species found in uredinia of rust fungi. Based on the morphological characters and phylogenetic analyses, our strain was determined to be *R. coleosporii*. *Ramularia coleosporii* showed a close relationship with another fungicolous species, *R. uredinicola.* However, there are differences in the size of conidiophores and conidia. *R. coleosporii* has conidiophores that measure 35–135 × 3–5 µm and conidia that are 6–28 × 4–6 µm in size, whereas the conidiophores of *R. uredinicola* are 20–80 × 2–4 µm and the conidia are 4–15 × 2–4 µm in size (Khodaparast and Braun 2005). The third fungicolous species, *Ramularia uredinearum* produces consistently colorless colonies on its rust host (Braun 1998), but its conidia are, 9–24 × 3–5 µm in size, they are slightly shorter and thinner than *R. coleosporii* (Zhang 2006). However, the molecular data is not available for *R. uredinearum*, the phylogenetic relationships between *R. coleosporii*, *R. uredinicola* and *R. uredinearum* need be further studied.

*Ramularia colesporii* has been reported as a pathogen on leaves of *Campanula rapunculoides* in Armenia (Simonyan 1981), *Clematis gouriana* in China (Poelt & Fritz-Schroeder 1983), *Ipomoea batatas* in Puerto Rico (Stevenson 1975), and *Perilla frutescens* var. *acuta* in China (Yang 2005). It is unclear if the species referred to in these reports is correctly named as substantiating molecular data is lacking. Here, we firstly report *R. colesporii* associated with *C. plumeriae* on the leaves of *Plumeria* sp. in Chiang Rai, Thailand. There are no reports of *R. colesporii* causing leaf spot disease of *Plumeria* and thus this hyperparasite may be a potential agent for biocontrol of *Plumeria* rust.


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